# Bioaccessibility and antioxidant activity of *Calendula officinalis* supercritical extract as affected by *in vitro* co-digestion with olive oil

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**ABSTRACT**

Supercritical extracts of marigold (ME) were produced and characterized. The bioaccessibility of terpenes, especially that of pentacyclic triterpenes (PT), the particle-size distribution and antioxidant activity after the *in vitro* co-digestion of ME with olive oil (OO), were determined. ME produced without co-solvent was richer in taraxasterol, lupeol, α-amyrin and β-amyrin than extracts with co-solvent. All terpenes showed high bioaccessibility without OO (>75%). Significant correlations were found between the molecular properties of compounds (logP and number of rotatable bonds) and their bioaccessibility. Co-digestion with OO enhanced the bioaccessibility (around 100% for PT), which could be related to a higher abundance of low-size particles of the digestion medium. The antioxidant activity of the digested ME increased around 50%, regardless of OO. PT-rich extracts from marigold display high bioaccessibility and improved antioxidant activity after *in vitro* digestion, although complete bioaccessibility of PT can be reached by co-digestion with oil, without affecting antioxidant activity.

**Keywords:** *Calendula officinalis*, pentacyclic triterpenes, bioaccessibility, lipid digestion, excipient foods

## INTRODUCTION

*Calendula officinalis* L. (common name marigold) belongs to the order of Asterales and is a member of the family Asteraceae. This herbaceous plant, native of the Mediterranean climate areas, is traditionally cultivated in several countries for ornamental, medical and cosmetic purposes. Although it is not extensively known, some edible uses have been described for flowers and leaves. The fresh petals can be chopped and added to salads, curry or custard. The dried petals have a more intense flavor and are used as seasoning in soups, cakes, drinks and baked products. A tea can be prepared from the flowers and petals, and the leaves can be also eaten raw in salads.[1–3]

The traditional medicinal use of the marigold is related to its great variety of phytochemicals of bioactive interest, such as terpenoids, sterols, saponins, carotenoids and phenolic compounds, mainly in flower extracts. Due to this complex composition, the extracts of marigold have been related to activities such as antioxidant, antiinflammatory, immunostimulant, anticancer, hepatoprotective, antimicrobial and wound healing.[1–4] The triterpenoids as pentacyclic triterpenes (PT) have been described as one of the main responsible for the biological activities of marigold, especially as anti-inflammatory.[4] The typical PT of the marigold are monohydroxy alcohols (α-amyrin, β-amyrin, taraxasterol and lupeol), and dihydroxyalcohols (faradiol, arnidiol, brein or calenduladiol).

Taking into account the biological interest of all these compounds, the production of extracts of marigold rich in these bioactive compounds is of current interest. Within the most popular methods for production of plant extracts, the green technology of supercritical fluid extraction (SFE) is quite popular nowadays, with special use in the extraction of compounds with low polarity that are soluble in supercritical $CO_2$, such as the PT. The supercritical $CO_2$ extraction assisted by co-solvents such as ethanol may also enhance the yield of extraction. In the specific case of marigold, diverse studies have reported the use of SFE,[5–7] but the use of co-solvents has been scarcely explored.[7] Furthermore, many of them have been focused in the extraction and characterization of the essential oil rich in sesquiterpenes,[8–10] although the content in PT is not always reported.

It is important to remark that most of the described bioactive compounds of marigold are compounds of a typical low polarity and high hydrophobicity, which leads to a limitation in their potential use and bioactivity. However, it seems that the available information on the behavior of PT during the gastrointestinal process, bioaccessibility and bioavalability is still scarce and contradictory. It has been suggested that the bioavailability of PT is poor due to a difficult solubilization in the aqueous media of the gastrointestinal tract, necessary for a proper absorption.[11] However, other studies reported that some PT of fruits and plants were effectively absorbed and deposited in their intact forms in diverse tissues in mice.[12] In the specific case of the typical PT of marigold, some studies have described that lupeol is bioavailable,[13] whereas other studies have suggested the opposite.[14,15] Ching, Lin, Tan & Koh[16] also described a low bioavailability of the amyrin in mice, whereas diverse studies have shown that this compound is orally effective, which would not be in agreement with a poor bioavailability.[11,17] Concerning other PT from the marigold, such as taraxasterol or faradiol, a lack of information on bioavalability has been found.

One of the factors that might be related to these inconclusive results could be the variability on the composition of the digestion medium. In this respect, for most lipophilic compounds, it has been demonstrated that their bioaccessibility can be improved by the coexistence of other lipids in the intestinal tract.[18] This is because the lipid digestion leads to the release of fatty acids and monoglycerides that enhance the formation of micellar structures with bile salts and phospholipids. These micelles will include other hydrophobic compounds present in the medium and in turn, the dispersion and absorption of the compounds vehiculized by these micellar structures. In fact, this is one of the fundamentals of current interest in the development of lipid-based delivery systems or the recent term “excipient foods” (a food that increases the bioavailability of bioactive agents that are co-ingested with it), for the improvement in the bioactivity of compounds for food and nutraceuticals.[19,20] On the other hand, the association of bioactive compounds with lipid components has been suggested as a strategy with other advantages of interest, such as the protection of labile compounds against the conditions of the gastrointestinal tract.[21,22]

The aim of the present study was the production of a supercritical extract of *Calendula officinalis*, in absence and presence of co-solvent, and the characterization on their terpene composition. The subsequent *in vitro* gastrointestinal digestion of the extract was performed, both in absence and in co-existence with olive oil, in order to evaluate the bioaccessibility and the distribution of particle sizes of the digestion medium. Furthermore, the impact of the gastrointestinal digestion process on the antioxidant activity of the extract was also evaluated.

## MATERIALS AND METHODS

### Reagents and Materials

Dry *Calendula officinalis* flowers were purchased from a local herbalist supplier (Murcia, Spain). The flowers were ground (particle size smaller than 500 μm) in a knife mill (Grindomix GM200 RETSCH). Extra virgin olive oil was purchased from a local supermarket.

Thymol, β-caryophillene, valencene, cedrol, β-sitosterol, tocopherol and alkane mixture (C7-C30) were from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). The standard α-amyrin was from Extrasynthese (Genay, France).

Trizma, maleic acid, Amano lipase A from *Aspergillus niger*, pepsin, pancreatin from porcine pancreas, bile salts, phosphatidyl choline from egg yolk and 2,2-diphenyl-1-picryl-hydrazyl (DPPH) were from Sigma-Aldrich Chemie GmbH (Steinheim, Germany).

### Supercritical extraction method

Extractions were carried out in a pilot-scale supercritical fluid extractor (Thar Technology, Pittsburgh, PA, USA, model SF2000) comprising a 2 L cylindrical extraction cell and two different separators (S1 and S2), each of 0.5 L capacity, with control of temperature and pressure. The extraction cell was loaded with 400 g of the milled marigold and the $CO_2$ flow rate was set to 70 g/min. The extractions were carried out using pure $CO_2$ or $CO_2$ with ethanol as cosolvent (10% p/p). The extraction pressure and temperature were selected at 140 bar and 40 ºC and were kept constant

for all experimental assays. The separator conditions were 40 bar and 40 ºC. The total time extraction was 180 min. Samples were stored at -20 ºC until analysis.

**Analysis of terpene compounds of the extracts**

Samples were prepared at 15 mg/mL in chloroform:methanol (2:1, v/v) and were analyzed in an Agilent 7890A system (Agilent Technologies, Santa Clara, CA, USA) comprising a split/splitless injector, an electronic pressure control, a G4513A auto-injector and a 5975C triple-axis mass spectrometer detector. The column used was an Agilent HP-5MS capillary column (30 m × 0.25 mm i.d., 0.25 μm phase thickness). Helium was used as carrier gas at 2 mL/min. The injector temperature was 260 °C and the mass spectrometer ion source and interface temperatures were 230 and 280 °C, respectively. The sample injections (1 μL) were performed in splitless mode. The separation method of Crabas et al.[8] was used with slight modifications. The oven temperature was initially at 60 °C and increased to 250 ºC at 4 ºC/min, followed by an increase to 310 °C at 3 °C/min, and held for 5 min. The mass spectra were obtained by electronic impact at 70 eV. The scan rate was 1.6 scan/s at a mass range of 30-700 amu. Identification of compounds was performed by the NIST MS Data library, the retention indexes of the compounds, the mass spectra according to literature, or according to those of pure commercial compounds whenever possible. Quantitation of compounds was performed by calibration curves obtained from commercial standards whenever possible: thymol was used for monoterpenes, caryophillene and valencene were used for hydrogenated sesquiterpenes, cedrol was used for oxygenated sesquiterpenes, sitosterol was used for phytosterols, α-amyrin was used for PT, and tocopherol was quantitated by its own commercial standard. Other compounds such as alkanes were also quantitated by their own commercial compounds.

***In vitro* gastrointestinal digestion**

The *in vitro* digestion model was based on Martin, Moran-Valero, Vázquez, Reglero & Torres[23] with brief modifications and the inclusion of a gastric phase. For gastric digestion, 30 mg of marigold extract (ME) and 10 mg of lecithin were mixed with 14 mL of a gastric solution (150 mM NaCl, 6 mM $CaCl_2$, pH 4.5). In case of the coexistence of dietary lipids, olive oil (OO) was added at a ratio of ME to oil of 1:2 (w/w). The mixture was placed in an orbital incubator at 200 rpm and 37 ºC. After 2 min of agitation to allow the dispersion of the components, the gastric digestion was initiated by the addition of a fresh extract of gastric enzymes (170 mg of gastric lipase and 15 mg of pepsin in 3 mL of gastric solution and stirred for 10 min). Reaction was continued during 45 min.

For intestinal digestion, a solution to simulate biliary secretion was prepared by mixing 0.1 g of lecithin, 0.25 g of bile salts, 0.5 mL of 325 mM $CaCl_2$ solution, 1.5 mL of 3.25 mM NaCl solution, and 10 mL of Trizma-maleate buffer 100 mM pH 7.5. This mixture was homogenized for 1 min at 3500 rpm (Ultra-Turrax IKA T18). Then, the biliary secretion was added to the gastric digestion and shaken in the orbital incubator for 2 min at 200 rpm and 37 ºC. The simulation of intestinal digestion was started by the addition of fresh pancreatin extract (0.5 g of pancreatin in 3 mL of Trizma-maleate buffer, stirred for 10 min and centrifuged at 1600 x *g* for 15 min). Reaction was continued during 60 min. The *in vitro* digestion of each sample was performed at least in triplicate.

**Determination of bioaccessibility**

At the end of digestion the medium was submitted to centrifugation at 4000 rpm for 40 min (5810R Eppendorf Iberica, Madrid, Spain). After centrifugation, an upper aqueous phase and a minor precipitated phase were obtained. The aqueous phase was filtered in order to isolate the aqueous solution containing the micellar structures (micellar phase, MP) from visible and non-solubilized particles of the ME. The components of the ME included in the MP were extracted and analyzed by GC-MS following the same procedure previously described.

The bioaccessibility of each compound was determined as the fraction of each compound that was considered available for intestinal absorption, that is, included within the aqueous MP, as:

146 % bioaccessibility = (mg of compound in MP/mg of compound in digestion media) x 100

147

148 **Extraction of compounds from the digestion media**

149 At the end of digestion or after isolation of the MP, the components of the ME were extracted with
150 hexane:methyl tert-butyl ether (50:50, v/v) at a ratio of 3:1 (v/v) of solvent to sample. The mixture
151 was stirred for 1 min and centrifuged for 10 min at 4000 rpm (ScanSpeed mini, Micro Centrifuge).
152 A second extraction was performed whith chloroform:methanol (2:1, v/v) at a ratio 3:1 (v/v) of
153 solvent to sample. The two organic phases obtained were mixed and the solvent was removed by
154 rotary evaporator. The obtained extract was solubilized in chloroform:methanol (2:1, v/v) at 15
155 mg/mL and analyzed by GC-MS following the same procedure previously described.

156

157 **Particle size distribution after *in vitro* digestion**

158 The particles size distribution of the isolated MP from the *in vitro* digestion of ME in absence and
159 presence of OO was measured. Furthermore, the particles size distribution was also determined for
160 two control samples: 1) the MP isolated after *in vitro* digestion in absence of ME and in absence of
161 OO, and 2) the MP isolated after *in vitro* digestion in absence of ME and in presence of OO. By this
162 procedure, it was possible to determine the particles size distribution of the digestion medium itself,
163 in order to distinguish the differences due to the presence of the experimental components.
164 The particle size distributions were analyzed by mean of laser diffraction technique. A Mastersizer
165 2000 instrument (Malvern Instruments Ltd., Malvern, Worcestershire, UK), equipped with a
166 dispersion unit of solid particles in liquids (Hydro 2000MU) working at 2000 rpm, was used.
167 Trizma-maleate buffer 100 mM pH 7.5 was used as dispersant. Assuming a volume distribution, the
168 largest particle size $D_{90}$ and the volume mean diameter $D_{43}$ were the reported parameters. Analyses
169 were performed in quintuplicate.

170

171 **Antioxidant activity of compounds by DPPH assay during *in vitro* digestion**

The antioxidant activity of the digestion media was measured before *in vitro* digestion and after gastric and intestinal digestion by the DPPH test. The procedure of Martin, Moran-Valero, Casado, Reglero & Torres[24] was used with brief modifications. The digestion medium was diluted in methanol:chloroform (5:1 v/v) up to 0.8 mg of ME/mL. An aliquot (500 µL) was added to 1500 µL of DPPH in methanol (0.06 mM). Samples were centrifuged at 12000 rpm for 5 min (miniSpin plus, Eppendorf). Then, reaction was completed after 60 min at room temperature and darkness, and absorbance was measured at 517 nm. Control experiments of the digestion medium in absence of the tested compounds (ME and OO) were also performed following the same procedure. The remaining DPPH concentration in the reaction medium was estimated by proper calibration curves of DPPH.

Antioxidant activity was expressed as percentage of inhibition of DPPH as:

$$\% \text{ inhibition DPPH} = 100 - [(\mu g \text{ DPPH/mL}_{sample} / \mu g \text{ DPPH/mL}_{control}) \times 100]$$

**Statistical analysis**

Statistical analyses were performed by means of the general linear model procedure of the SPSS 17.0 statistical package (SPSS Inc., Chicago, IL, USA) by one-way analysis of variance. Differences were considered significant at $p \leq 0.05$. Post-hoc Tukey's tests were performed in order to establish significant differences. Pearson's correlation tests were used to study the bioaccessibility of compounds as related to their molecular properties.

## RESULTS AND DISCUSSION

### Characterization of supercritical extracts of *Calendula officinalis*

The presence or absence of co-solvent in the supercritical extraction of *Calendula officinalis* led to some differences regarding yield and composition of the extract. The extraction yield of ME by $CO_2$ in absence of co-solvent was 2.1%, whereas that value was up to 7.5% in case of ethanol-$CO_2$ extraction. The identified compounds of both extracts are detailed in Table 1. More than 94% of the

198 volatile compounds were identified for both extracts. According to the area percentage, the major
199 abundance corresponded to alkanes (HC), followed by sesquiterpenes (S) and oxygenated
200 triterpenes (OT), in case of extraction without co-solvent. In case of ethanol-$CO_2$ extraction, a slight
201 qualitative difference was found, mainly due to a lower proportion of HC and a higher proportion of
202 OT. However, the major difference due to co-solvent was that the chromatographic areas for all the
203 compounds were lower than the $CO_2$ extraction without co-solvent (Table 1). Taking into account
204 the higher yield of the ethanol-$CO_2$ extraction, this result would suggest that the detected
205 compounds could be diluted with other extracted compounds but non-detected by GC-MS under the
206 used conditions.
207 In any case, the general composition of both ME was in agreement with previous similar studies
208 about supercritical extracts from this plant. Thus, α-cadinol, τ-cadinol, ɣ-cadinene and δ-cadinene
209 are typical volatile compounds described for marigold.[8,10,25] Furthermore, in the current study, the
210 main detected PT were taraxasterol, lupeol, α-amyrin and β-amyrin, as well as diverse non-
211 identified sterols. The identification of PT from supercritical extracts of marigold has not been
212 usually described in most previous studies, but most of them were focused on the typical terpenes of
213 the essential oil of the extracts.
214 It is important to remark that the relative abundances of the compounds in Table 1 cannot be
215 considered to determine the major compounds of the extracts, due to their differences on
216 chromatographic responses. Therefore, taking into account the importance of characterizing the
217 extract due to its bioactive compounds of interest, especially PT, a proper quantitation as possible
218 was performed for some of the compounds with the available commercial standards. As shown in
219 Table 2, in absence of co-solvent, close to 15% of the chemical composition of the extract was
220 quantitated, being the OT the most abundant family of compounds. In the specific case of bioactive
221 PT (taraxasterol, lupeol, α-amyrin and β-amyrin), these compounds were close to 3% of the extract.
222 Furthermore, up to 5% of the extract was also quantitated as non-identified sterols.

Regarding the ethanol-$CO_2$ extract, due to the dilution effect with other extracted but non-identified compounds, the total amount of quantitated compounds by GC-MS was lower than 5%, but similarly to the $CO_2$ extract, the major compounds were the OT (4%) and the bioactive PT were around 1.5% (Table 2).

As summary, both supercritical ME might be considered of interest due to their content on bioactive PT, although the extraction of marigold in absence of co-solvent resulted in the preferred procedure. Therefore, taking into account the interest of the bioactive PT, the following studies were performed with the ME produced in absence of co-solvent.

**Bioaccessibility of supercritical extract of *Calendula officinalis***

After *in vitro* digestion of the ME, the bioaccessibility of the major quantitated compounds of the extract was determined. Due to the complex composition of the extract, as well as the increased complexity of the chromatographic analyses due to the co-elution of other compounds from the digestion medium itself, a selection of compounds for the study of bioaccessibility was performed. Such selection was based on the preferential characterization of the bioactive compounds of interest (PT), as well as the consideration of other representative compounds of each chemical family of the extract (SH, OS and HC) that were present in the extract at concentration $\geq$ 0.1% at least. The selected compounds and their values of bioaccessibility are shown in Table 3.

According to Table 3, the bioaccessible fraction of the compounds was quite variable, since percentages higher than 80% were found for some compounds, whereas other compounds showed values lower than 50%. In general, it seemed that the bioaccessibility of the family of compounds decreased in the following order: SH (84%) > OS (81%) > OT (77%) > HC (42%). Thus, it could be considered that most terpenes, included the PT, showed a high bioaccessibility, since more than 75% of the amount of terpenes were found within the bioaccessible MP, including the bioactive PT (Table 3). The available information about the bioaccessibility of terpenes in general is scarce, and bioaccessibility values of PT in particular has not been found in the scientific literature, whereas

249 contradictory results have been reported about the bioavalability of compounds such as lupeol or
250 amyrins.[11, 13–17] According to the obtained results, the present study showed that the studied PT of a
251 supercritical extract of ME might have high bioaccessibility.
252 Diverse reasons might be considered to explain the observed results. In general, the bioaccessibility
253 of compounds in the aqueous medium of the intestinal lumen is determined by their solubility. [26,27]
254 This solubility is not a problem for those compounds with a good hydrosolubility or
255 hydrophilic/lipophilic balance that ensure its dispersion in the medium, either directly, or indirectly,
256 by inclusion in vesicles, emulsion droplets, lamellar or micellar structures of bile salts and
257 phospholipids naturally present in the intestinal tract. In fact, absorption of lipophilic products
258 takes place supported by these structures of the MP, which enhances the transport of such products
259 to enterocytes through the unstirred water layer close to the microvillous membrane, where they are
260 absorbed.[26,27] According to this theory, most of terpenes might show a proper dispersion in the
261 aqueous media after *in vitro* digestion. On the contrary, alkanes might not be so effectively
262 dispersed within the medium and hence their lower bioaccessibility.
263 In order to understand whether the obtained results were related to the lipophilicity of the
264 compounds, the logP value of each individual substance listed in Figure 1 was considered (Food
265 Database FooDB, www.foodb.ca). As it is illustrated in Figure 1, a negative correlation was found
266 between the logP value of the compounds and their bioaccessibility ($r$ = -0.771, $P < 0.001$).
267 Therefore, the higher lipophilicity of the compounds, the worse the bioaccessibility is. Thus, logP
268 values lower than 8 might be preferred for a high bioaccessibility (closer to 80%). All PT identified
269 in ME showed logP values around 6.
270 Another molecular property that has been popularly related to the bioavailability of drugs is the
271 molecular flexibility, due to the number of rotatable bonds (NRB) described by Veber, Johnson,
272 Cheng, Smith, Ward & Kopple[28]. A low NRB value has been suggested as one strong criterion for
273 drug candidates with proper bioavailability, although the exact reason for such relation has not been
274 established. According to Figure 2, a strong negative correlation was found between the NRB value

of the studied compounds (Molinspiration Cheminformatics, Bratislava, Slovak Republic) and their bioaccessibility ($r$ = -0.860, $P < 0.001$). Therefore, the higher the molecular flexibility, the worse the bioaccessibility is. Thus, NRB values of 0 or 1 might be desirable for a high bioaccessibility of the studied compounds. This was considered an interesting result, since previous information on the relation between the molecular flexibility and bioaccessibility of compounds has not been described, but only the relation of NRB with bioavailability.

Therefore, according to the obtained results, the compounds of bioactive interest of the ME showed high bioaccessibilities that might be related to their favorable molecular properties. At any case, further studies at this respect would be necessary in order to understand whether such proper bioaccessibility might lead to a positive bioavailability and bioactivity.

**Bioaccessibility of *Calendula officinalis* supercritical extract co-digested with olive oil**

Despite that the studied compounds showed a proper bioaccessibility, it was considered interesting the study of the role of the coexistence of lipids (olive oil, OO) during the digestion process in order to evaluate whether it would be possible to reach a complete bioaccessibility of the bioactive compounds of interest. Preliminary studies were performed in order to find the best ratio ME to oil that allowed the best bioaccessibility for most compounds (data not shown). This ratio was established as 1:2 (w/w) and the corresponding results are shown in Figure 3. In general, a higher bioaccessibility due to the OO factor was found ($P<0.001$). The different chemical families increased around 20% their bioaccessibility, and most compounds of interest reached values of bioaccessibility closer to 100%.

During lipid digestion, the major hydrolysis products as fatty acids and monoglycerides are released. These compounds lead to the formation of micellar structures with bile salts and phospholipids, which is necessary for the proper absorption of fats by enterocytes. [27] This increase in the micellar surface compared to the absence of oil increases the available structures for inclusion of other hydrophobic compounds present in the aqueous media and hence, their bioaccessibility is

301 enhanced.[18-20] This mechanism would be related to the results obtained in the present study.
302 Therefore, the current study showed that despite the bioaccessibility of bioactive compounds such
303 as PT from a supercritical extract of marigold was high; the co-digestion with particularly low
304 levels of a typical dietary fat would be enough to reach a complete bioaccessibility of such
305 compounds. In this respect, according to the term of "excipient food" recently described by
306 McClements et al.,[19] as a food that increases the bioavailability of bioactive agents that are co-
307 ingested with it, olive oil might be a potential candidate as "excipient food" to enhance the
308 bioaccessibility of compounds of ME in general, and of bioactive PT in particular. As far as we
309 know, previous studies about the effect of coexistence of oils on the gastrointestinal digestion
310 behavior and bioaccessibility of PT have not been described in the scientific literature.
311
312 **Particle size distribution after *in vitro* digestion**
313 The particle size distribution of the isolated MP was characterized in order to deepen the
314 understanding of the hypothesis that a better bioaccessibility of the compounds was due to an
315 enhanced dispersion by digested lipids. Previously, we considered necessary to understand the
316 typical particles size distribution of the own MP in absence of any of the experimental compounds,
317 that is, in absence of ME and OO. As shown in Figure 4.a, there were two major peaks, one in the
318 range of 0.2 μm, and a second one in the range of 1 μm. Furthermore, another abundant volume of
319 particles was found within a wide range of sizes between 4 and 240 μm. This distribution led to a
320 MP characterized by particles most of them lower than 25 μm ($D_{90}$) and a volume mean diameter
321 around 11 μm ($D_{43}$). It is complicated to determine the precise components of the medium
322 responsible of such distribution, but it might probably be related to the particles formed by
323 phospholipids, bile salts, either individually or in combination as micelles or vesicles.[29]
324 When OO was digested, the particle size distribution of the MP changed (Figure 4.b). The typical
325 modes at 0.2 and 1 μm were also present, but the area of the mode at 0.2 μm was higher, whereas
326 the modes at 1 and 4-240 μm were much lower. Thus, the MP was characterized by particles most

of them lower than 2 μm ($D_{90}$) and a volume mean diameter around 7 μm ($D_{43}$). The obtained results would suggest that the hydrolysis products of OO, mainly fatty acids and monoglycerides, contributed to an increase in the number of particles of lower size within the MP. These observed results were quite useful since they could confirm that the *in vitro* digestion model led to a physiological and favorable situation to enhance the dispersion of other lipophilic compounds in the aqueous media.

When the ME was digested in absence of OO, the typical modes of the medium at 0.2 and 1 μm were present again; however, a relevant decrease was produced at the expense of an increase in the abundance of bigger particles (Figure 4.c). In fact, three new modes of particles appeared in the range of 6 μm, 65 μm and 500 μm. Thus, in presence of ME, the MP was characterized by bigger particles, because the 10% of particles were even higher than 190 μm ($D_{90}$) and the volume mean diameter was around 47 μm ($D_{43}$). This distribution was quite different compared to the control samples (Figure 4.a and 4.b). Therefore, it could be thought that the obtained results might be mainly related to particles of the ME dispersed in the aqueous media, either in isolation or by interacting with components of the medium.

When the ME was digested in co-existence with OO, the basic modes of the medium at 0.2 and 1 μm increased again and the big particle sizes previously observed for the ME sample decreased at the expense of the formation of a wide mode in the range of 15-200 μm (Figure 4.d). Thus, in presence of OO, the MP from the digestion of ME was characterized by lower particles than in absence of OO, being most of them lower than 28 μm ($D_{90}$) and a volume mean diameter around 9 μm ($D_{43}$). Those values were closer to those obtained for the control sample of MP in absence of ME and OO (Figure 4.a). This might confirm our proposed theory that hydrolysis products of lipids could enhance the dispersion of hydrophobic compounds of ME, by increasing the number of lower size particles.

Therefore, the study of the distribution of particle sizes after *in vitro* digestion of ME showed that the co-digestion of the extract with low levels of a typical dietary fat might enhance the dispersion

353 of the marigold components within the aqueous phase of the intestinal medium and, in turn, would
354 enhance its bioaccessibility (Table 3). As far to our knowledge, previous studies on the particle size
355 distribution of the aqueous medium after digestion of ME, either with or without oils, have not been
356 described in the scientific literature. In any case, further studies would be necessary in order to
357 confirm the observed evidences, taking into account that the diluting and stirring conditions
358 commonly used for particle size measurement might lead to the formation of artefacts.
359

360 **Antioxidant activity of *Calendula officinalis* supercritical extract during *in vitro* digestion**

361 The modification of the antioxidant activity of diverse compounds, either negatively or positively,
362 after the process and conditions of gastrointestinal digestion has been previously described. In case
363 of a detrimental effect after digestion, the association of bioactive compounds with lipid
364 components has been reported as a strategy to protect those labile compounds from the conditions
365 of the gastrointestinal tract.[21,22] Therefore, taking into account that some components of marigold
366 have been described as antioxidants, it was considered interesting the study of the impact of the
367 digestion process on such activity, either in absence or in presence of olive oil.
368 As shown in Figure 5, the ability of the ME to inhibit the DPPH radical significantly increased with
369 the course of the gastrointestinal digestion (P=0.006). Such increase was especially significant after
370 intestinal digestion for both treatments. Furthermore, the final inhibitory activity that was observed
371 after intestinal digestion was quite similar between treatments, regardless of the presence or absence
372 of OO. Thus, the inhibitory activity of ME increased after gastrointestinal digestion around 50%
373 and 40% for the treatments in absence and presence of OO, respectively. The improvement of the
374 antioxidant activity of compounds during the gastrointestinal process has been previously described,
375 especially for polyphenols, due to their release during the hydrolysis processes from other complex
376 molecules.[30] As far as we know, previous information about the antioxidant activity of compounds
377 from ME as affected by gastrointestinal digestion has not been described.

Therefore, the observed results showed that the antioxidant effect of ME was not negatively affected, but rather enhanced by the *in vitro* gastrointestinal process. Additionally, it seemed that the better dispersion of the extract that was found in presence of OO was not related to these results, at least in the case of intestinal digestion, since during gastric digestion the antioxidant activity was significantly higher in presence of OO. Other *in vitro* and *in vivo* studies would be necessary in order to evaluate whether these preliminary results would be related to enhanced antioxidant activities of a digested ME.

As a summary, the present study showed that supercritical extraction in absence of co-solvent is the preferred procedure for producing a marigold extract rich in bioactive compounds such as pentacyclic triterpenes. Although that such bioactive compounds show a good bioaccessibility, it can be even improved by the co-digestion with particularly low levels of a typical dietary fat such as olive oil, thanks to a better dispersion of the extract in the aqueous media during gastrointestinal digestion. Additionally, the gastrointestinal process enhances the antioxidant activity of the extract, regardless of the co-digestion with olive oil. The obtained results are of interest either to obtaining a deeper knowledge on the potential of the marigold plant as a possible bioactive ingredient of foods, as well as to contributing to the general knowledge on the gastrointestinal digestion of bioactive compounds such as pentacyclic triterpenes, together with other typical compounds of supercritical extracts of plants in general, such as sesquiterpenes or alkanes.

**ABBREVIATIONS USED**

| | |
|---|---|
| ME | Marigold extract |
| PT | Pentacyclic triterpenes |
| OO | Olive oil |
| SFE | Supercritical fluid extraction |
| MP | Micellar phase |
| HC | Alkanes |

| | |
|---|---|
| S | Sesquiterpenes |
| SH | Sesquiterpenes hydrocarbons |
| OT | Oxygenated triterpenes |
| OS | Oxygenated sesquiterpenes |
| NRB | Number of rotatable bonds |

398

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402

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492

493 **Notes**

494 The authors declare no competing financial interest.
495

**Figure Captions**

**Figure 1.** Correlation between lipophilicity (logP) of compounds from supercritical marigold extract and their bioaccessibility (%)

**Figure 2.** Correlation between molecular flexibility (NRB) of compounds from supercritical marigold extract and their bioaccessibility (%)

**Figure 3.** Bioaccessibility (%) of compounds from supercritical marigold extract as affected by olive oil during *in vitro* digestion. Bars within the same compound are significantly different if $p \leq 0.05$ (*), $p \leq 0.01$ (**) or $p \leq 0.001$ (***).

**Figure 4.** Volume particle size distribution of the digestion media after *in vitro* digestion. Isolated aqueous micellar phase after *in vitro* digestion of a) without marigold and without olive oil, b) without marigold and with olive oil, c) with marigold and without olive oil, and d) with marigold and with olive oil.

**Figure 5.** Evolution of the antioxidant activity of supercritical marigold extract throughout *in vitro* digestion. Different letters within the same treatment are significantly different. Bars within the same color are significantly higher if $p \leq 0.05$ (*) or $p \leq 0.001$ (***).

Table 1. GC-MS Characterization of Supercritical Extracts of *Calendula Officinalis*

| RI | Compound | $CO_2$ | | Ethanol-$CO_2$ | |
|---|---|---|---|---|---|
| | | Area | % | Area | % |
| 1295 | Thymol | 1535567 | 0.74 | 414731 | 0.91 |
| 1304 | Carvacrol | 613051 | 0.30 | 185772 | 0.41 |
| 1351 | α-Cubebene | 416655 | 0.20 | 105666 | 0.23 |
| 1378 | α-Copaene | 968862 | 0.47 | 272124 | 0.60 |
| 1391 | β-Cubebene | 625195 | 0.30 | 113123 | 0.25 |
| 1430 | β-Gurjunene | 481604 | 0.23 | 69838 | 0.15 |
| 1442 | β-Humulene | 95765 | 0.05 | 96099 | 0.21 |
| 1464 | Alloaromadendrene | 898623 | 0.43 | 163108 | 0.36 |
| 1478 | ɣ-Muurolene | 901727 | 0.43 | 248135 | 0.55 |
| 1487 | β-Ionone | 617759 | 0.30 | 119421 | 0.26 |
| 1490 | β-Selinene | 363937 | 0.18 | 33297 | 0.07 |
| 1497 | (+)-Ledene | 2385902 | 1.15 | 495858 | 1.09 |
| 1502 | α-Muurolene | 1756737 | 0.85 | 464359 | 1.02 |
| 1516 | ɣ-Cadinene | 5778816 | 2.78 | 2006429 | 4.42 |
| 1525 | δ-Cadinene | 6024229 | 2.90 | 1726232 | 3.80 |
| 1528 | Dihydroactinidiolide | 2694113 | 1.30 | 795656 | 1.75 |
| 1535 | Cadina-1(2),4-diene | 1330919 | 0.64 | 115862 | 0.25 |
| 1539 | α-Cadinene | 1086998 | 0.52 | 249607 | 0.55 |
| 1544 | α-Calacorene | 408864 | 0.20 | 61864 | 0.14 |
| 1593 | Viridifloror | 3118592 | 1.50 | 389411 | 0.86 |
| 1610 | 1-10-di-epi-cubenol | 805223 | 0.39 | 212891 | 0.47 |
| 1616 | δ-Cadinol | 749509 | 0.36 | 151504 | 0.33 |
| 1629 | Cubenol | 824483 | 0.40 | 190310 | 0.42 |
| 1645 | τ-Cadinol | 10030609 | 4.83 | 2369705 | 5.22 |
| 1648 | n.i. oxygenated sesquiterpene | 1062037 | 0.51 | 264170 | 0.58 |
| 1652 | β-Eudesmol | 2101899 | 1.01 | 430441 | 0.95 |
| 1658 | α-Cadinol | 12114169 | 5.83 | 2456127 | 5.41 |
| 1670 | n.i. [a] | 1095520 | 0.53 | 218458 | 0.48 |
| 1711 | 3-Hydroxy-5,6-epoxy-β-ionone | 1793930 | 0.86 | n.d. [b] | n.d. |
| 1740 | 1-cyclohexanone, 2-methyl-2-(3-methyl-2-oxobutyl) | 10426982 | 5.02 | 2808684 | 6.18 |
| 1779 | 9,10- | 16481274 | 7.93 | 5541526 | 12.20 |

| | | | | | |
|---|---|---|---|---|---|
| | dimethyltricyclo[4.2.1.1.(2,5)]decane-9,10-diol | | | | |
| 1840 | n.i. | 857747 | 0.41 | n.d. | n.d. |
| 1846 | Hexahydrofarnesyl acetone | 4377593 | 2.11 | n.d. | n.d. |
| 1901 | Nonadecane | 4110435 | 1.98 | 862927 | 1.90 |
| 1970 | Verticiol | 2312239 | 1.11 | 547645 | 1.21 |
| 1996 | Palmitic acid, ethyl ester | 315697 | 0.15 | 111172 | 0.24 |
| 2001 | Eicosane | 980904 | 0.47 | 163966 | 0.36 |
| 2101 | Heneicosane | 6802339 | 3.27 | 1116421 | 2.46 |
| 2201 | Docosane | 630054 | 0.30 | 104863 | 0.23 |
| 2302 | Tricosane | 8930569 | 4.30 | 1385382 | 3.05 |
| 2402 | Tetracosane | 1111476 | 0.54 | 148787 | 0.33 |
| 2503 | Pentacosane | 13651831 | 6.57 | 1894387 | 4.17 |
| 2601 | Hexacosane | 980678 | 0.47 | 89782 | 0.20 |
| 2708 | Heptacosane | 18232039 | 8.78 | 1943518 | 4.28 |
| 2797 | Octacosane | 2050351 | 0.99 | 242332 | 0.53 |
| 2892 | Nonacosane | 19427953 | 9.35 | 2277964 | 5.01 |
| 2978 | Triacontane | 2191196 | 1.05 | n.d. | n.d. |
| - | Hentriacontane | 11165954 | 5.38 | 1739227 | 3.83 |
| - | α-Tocopherol | 827910 | 0.40 | 442105 | 0.97 |
| - | Dotriacontane | 180722 | 0.09 | n.d. | n.d. |
| - | n.i. sterol | 175177 | 0.08 | n.d. | n.d. |
| - | n.i. sterol | 728069 | 0.35 | 540417 | 1.19 |
| - | n.i. sterol | 2017797 | 0.97 | 710705 | 1.56 |
| - | β-Amyrenone | 578061 | 0.28 | 262928 | 0.58 |
| - | β-Amyrin | 2940308 | 1.42 | 1433928 | 3.16 |
| - | n.i. sterol | 595111 | 0.29 | 287959 | 0.63 |
| - | α-Amyrin + Lupeol | 4327976 | 2.08 | 2095993 | 4.61 |
| - | n.i. oxygenated triterpene | 643359 | 0.31 | 226066 | 0.50 |
| - | Taraxasterol | 6422306 | 3.09 | 3910398 | 8.61 |
| - | n.i. | 565285 | 0.27 | 128466 | 0.28 |
| | Oxygenated monoterpenes | | 2.3 | | 3.1 |
| | Sesquiterpenes hydrocarbons | | 11.6 | | 14.0 |
| | Oxygenated sesquiterpenes | | 14.3 | | 13.7 |

| | | |
|---|---|---|
| Oxygenated diterpenes | 1.1 | 1.2 |
| Oxygenated triterpenes | 7.3 | 17.9 |
| Alkanes | 43.5 | 26.3 |
| Other compounds | 16.1 | 18.6 |
| Total identified compounds | 96.3 | 94.8 |

[a] n.i. = non identified; [b] n.d. = non detected

Table 2. Quantitative Composition (mg/g) of Supercritical Extract of *Calendula Officinalis*

| Compound | $CO_2$ | Ethanol-$CO_2$ |
|---|---|---|
| Thymol | 0.60 | 0.16 |
| Carvacrol | 0.24 | 0.07 |
| α-Cubebene | 0.18 | 0.05 |
| α-Copaene | 0.42 | 0.12 |
| β-Cubebene | 0.27 | 0.05 |
| β-Gurjunene | 0.21 | 0.03 |
| β-Humulene | 0.04 | 0.04 |
| Alloaromadendrene | 0.39 | 0.07 |
| ɣ-Muurolene | 0.39 | 0.11 |
| β-Selinene | 0.18 | 0.02 |
| (+)-Ledene | 1.19 | 0.25 |
| α-Muurolene | 0.88 | 0.23 |
| ɣ-Cadinene | 2.89 | 1.00 |
| δ-Cadinene | 3.02 | 0.86 |
| Cadina-1(2),4-diene | 0.67 | 0.06 |
| α-Cadinene | 0.54 | 0.13 |
| α-Calacorene | 0.20 | 0.03 |
| Viridifloror | 1.11 | 0.14 |
| 1-10-di-epi-cubenol | 0.29 | 0.08 |
| δ-Cadinol | 0.27 | 0.05 |
| Cubenol | 0.29 | 0.07 |
| τ-Cadinol | 3.56 | 0.84 |
| n.i. [a] Oxygenated sesquiterpene | 0.38 | 0.09 |
| β-Eudesmol | 0.75 | 0.15 |
| α-Cadinol | 4.30 | 0.87 |
| Nonadecane | 1.02 | 0.21 |
| Eicosane | 0.25 | 0.04 |
| Heneicosane | 1.81 | 0.30 |
| Docosane | 0.18 | 0.03 |
| Tricosane | 2.62 | 0.41 |
| Tetracosane | 0.34 | 0.05 |

| | | |
|---|---|---|
| Pentacosane | 4.64 | 0.64 |
| Hexacosane | 0.38 | 0.03 |
| Heptacosane | 8.55 | 0.91 |
| Octacosane | 1.19 | 0.14 |
| Nonacosane | 14.27 | 1.67 |
| Triacontane | 2.13 | n.d. [b] |
| Hentriacontane | 10.83 | 1.69 |
| α-Tocopherol | 1.29 | 0.69 |
| Dotriacontane | 0.18 | n.d. |
| n.i. sterol | 2.65 | n.d. |
| n.i. sterol | 11.00 | 8.16 |
| n.i. sterol | 30.48 | 10.74 |
| β-Amyrenone | 1.20 | 0.54 |
| β-Amyrin | 6.08 | 2.97 |
| n.i. sterol | 8.99 | 4.35 |
| α-Amyrin + Lupeol | 8.96 | 4.34 |
| n.i. oxygenated triterpene | 1.33 | 0.47 |
| Taraxasterol | 13.29 | 8.09 |
| | | |
| Oxygenated monoterpenes | 0.8 | 0.2 |
| Sesquiterpenes hydrocarbons | 11.5 | 3.1 |
| Oxygenated sesquiterpenes | 10.9 | 2.3 |
| Oxygenated triterpenes | 85.5 | 40.4 |
| Alkanes | 48.6 | 6.1 |
| Total quantitated compounds | 156.9 | 52.1 |

[a] n.i. = non identified; [b] n.d. = non detected

Table 3. Bioaccessibility (%) of Supercritical Extract of *Calendula Officinalis*

| Compound | Chemical group [a] | Bioaccessibility |
|---|---|---|
| ɣ-Cadinene | SH | 79.3 ± 9.7 |
| δ-Cadinene | SH | 87.9 ± 11.8 |
| τ-Cadinol | OS | 75.1 ± 3.4 |
| α-Cadinol | OS | 87.1 ± 3.7 |
| Nonadecane | HC | 48.1 ± 2.0 |
| Tricosane | HC | 57.2 ± 11.0 |
| Pentacosane | HC | 48.3 ± 2.8 |
| Heptacosane | HC | 13.8 ± 2.4 |
| α-Tocopherol | OT | 71.1 ± 6.5 |
| β-Amyrin | OT | 79.8 ± 4.8 |
| α-Amyrin + Lupeol | OT | 82.1 ± 8.8 |
| Taraxasterol | OT | 75.5 ± 2.1 |
| | | |
| Sesquiterpenes hydrocarbons | | 83.6 ± 10.8 |
| Oxygenated sesquiterpenes | | 81.1 ± 3.6 |
| Oxygenated triterpenes | | 77.1 ± 2.9 |
| Alkanes | | 41.8 ± 3.5 |

[a] SH = sesquiterpene hydrocarbon, OS = oxygenated sesquiterpene, HC = alkanes, OT = oxygenated triterpene

Figure 1.

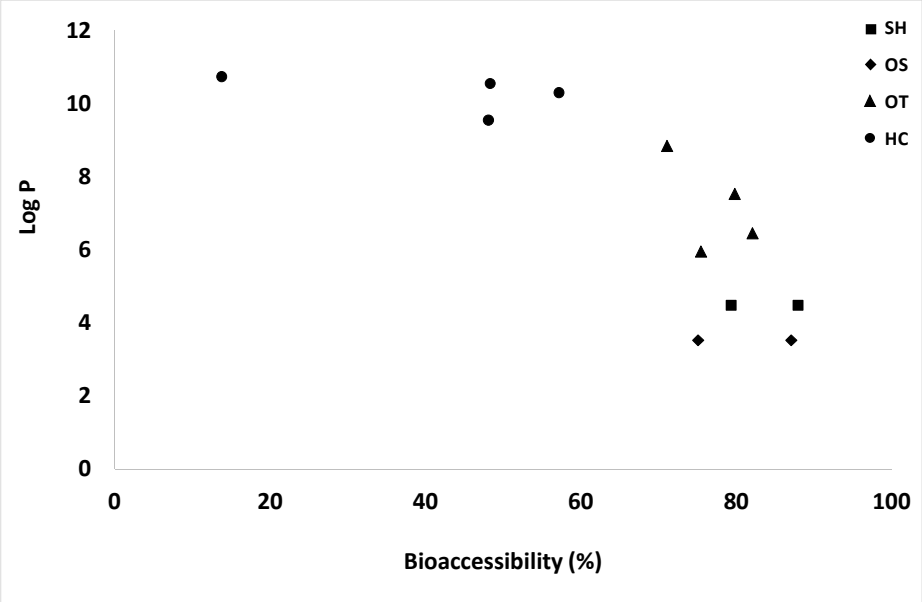
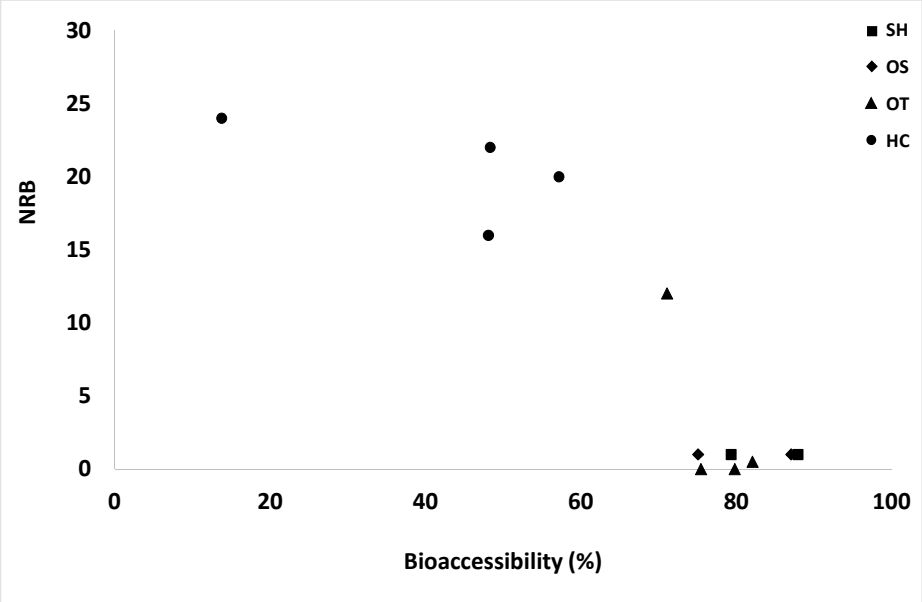


Figure 2.


30
25
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15
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NRB
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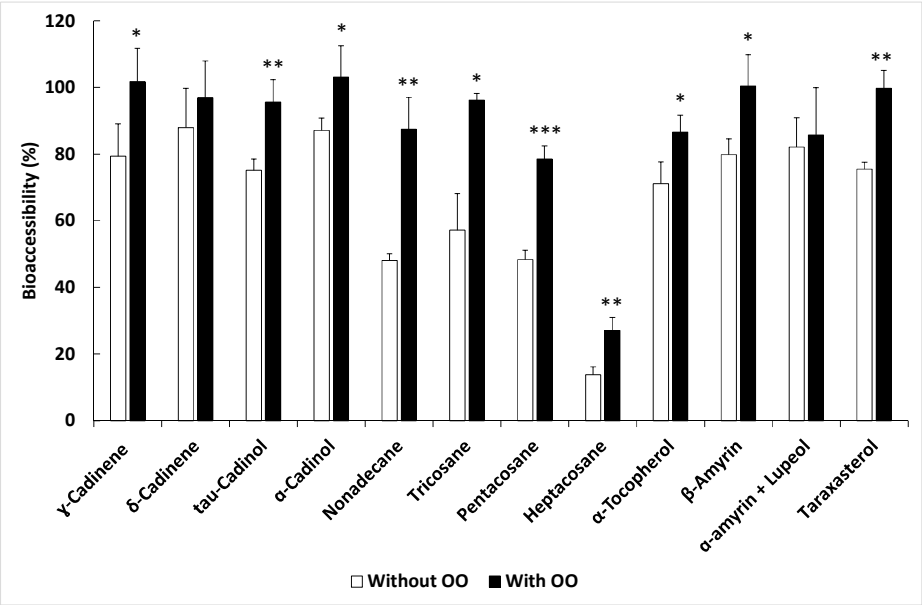
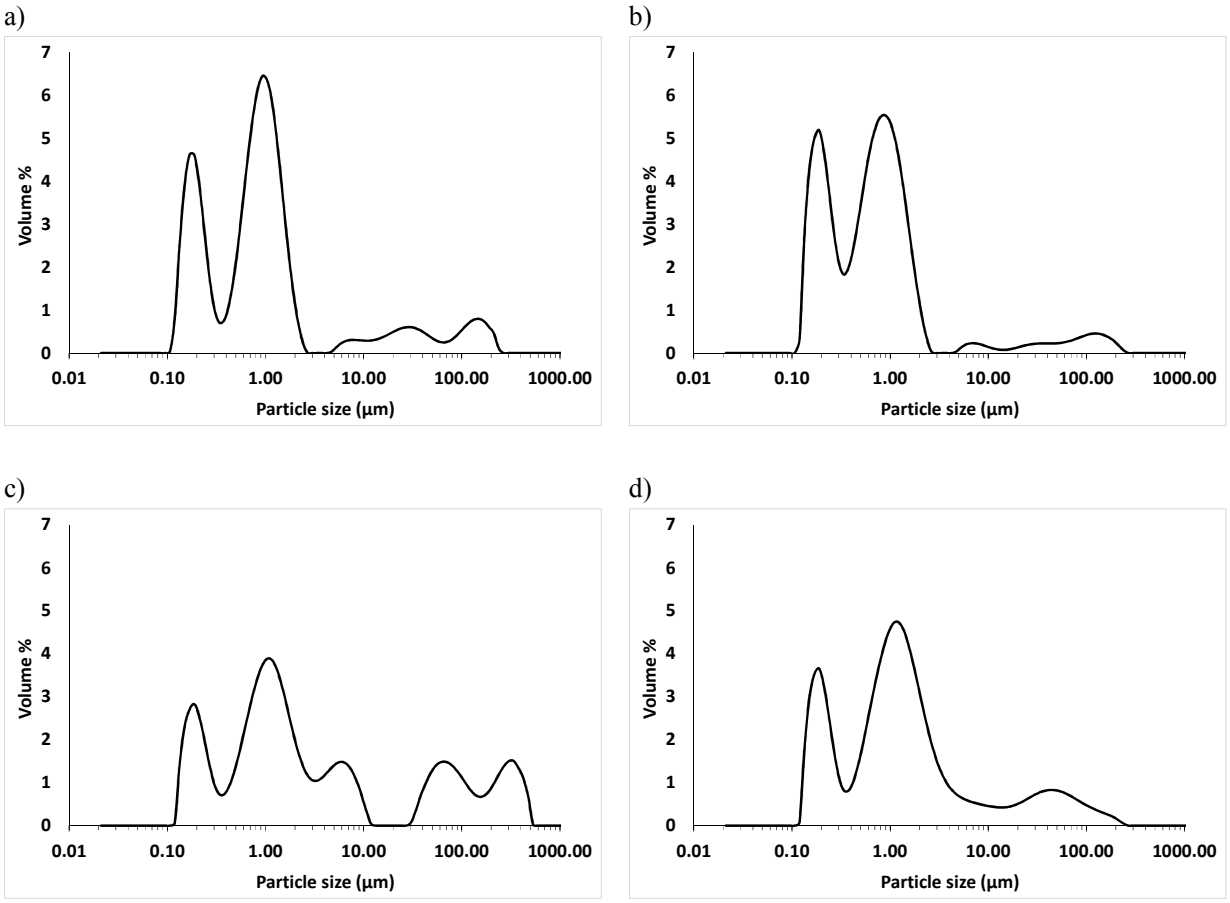
60
80
100
Bioaccessibility (%)
SH
OS
OT
HC

Figure 3.



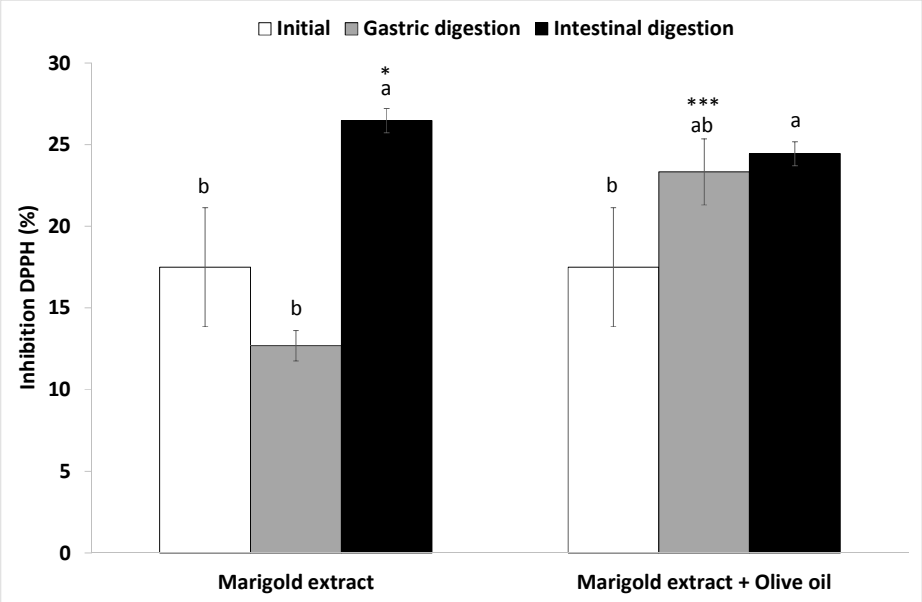

Bars within the same compound are significantly different if $p \leq 0.05$ (*), $p \leq 0.01$ (**) or $p \leq 0.001$ (***).

Figure 4.




Isolated aqueous micellar phase after *in vitro* digestion of a) without marigold and without olive oil, b) without marigold and with olive oil, c) with marigold and without olive oil, and d) with marigold and with olive oil

Figure 5.




Different letters within the same treatment are significantly different. Bars within the same color are significantly higher if $p \leq 0.05$ (*) or $p \leq 0.001$ (***).

## GRAPHIC FOR TABLE OF CONTENTS