



## The role of pristine carbon nanotubes as nanocarriers of 7,8-dihydroxyflavone

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### ABSTRACT

Flavonoids are secondary plant metabolites with a wide range of pharmacological effects. Among others, their antioxidant, anti-inflammatory and vasoprotective actions are noteworthy. However, the low bioavailability of flavonoids limits their direct clinical use. Nanoencapsulation of flavonoids is an effective tool to improve their biopharmaceutical characteristics, as the drug is protected inside the nanocarrier and specifically released into the therapeutic target. Bearing this in mind, pristine single- and multi-walled carbon nanotubes have been studied in this work as nanocarriers of 7,8-dihydroxyflavone, 7,8-DHF. Flavone encapsulation is optimized according to the influence of pH, the type of CNTs and their concentration on the association process. The equilibrium binding constants of 7,8-DHF to the CNTs are determined by measuring the variation observed in the flavone absorbance when the concentration of carbon nanotubes increases. CNTs/flavone complexes are characterized by particle size distribution and Z-potential measurements, as well as Transmission Electron Microscopy. The antioxidant capacity of free 7,8-DHF and the CNTs/7,8-DHF complexes are estimated by using the DPPH·, 2,2-diphenyl-1-picrylhydrazyl radical scavenging method. The results show that the encapsulation of the flavone in the CNTs results in a preservation of its pharmacological properties and provides stability to the encapsulated drug.

### 1. Introduction

Flavonoids are the most abundant polyphenols in higher plants. They are secondary metabolites derived from the propanoic acid biosynthetic pathway [1]. Flavonoids are involved in the formation of pigments. They are found in flowers and fruits, where they serve to attract pollinating vectors, and in leaves, where they act as photo-screens, protecting sensitive photosynthetic organelles from solar UVB radiation. These molecules are also involved in the plant's response to pathogens and

insects, and in the activity of several plant hormones, such as auxins and cytokinins [2–4]. Chemically, flavonoids have the diphenylpropane structure, C6–C3–C6, consisting of two aromatic rings linked by a three-carbon bridge. Their basic structure consists of a benzene ring condensed to a  $\gamma$ -pyrone, or its derivatives, substituted at the position 2 by a phenyl radical (Fig. 1A), with the exception of isoflavones, a type of flavonoid where the phenyl is at the position 3. The 3, 5, 7, 3' and 4' positions are usually substituted by hydroxyl groups. These can be found free, methylated or attached to a sugar, the latter being the most

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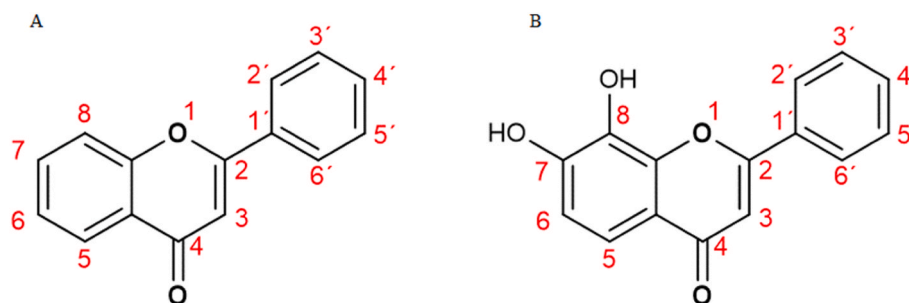


Fig. 1. (A) General structure of flavonoids. (B) Structure of 7,8-dihydroxyflavone, 7,8-DHF.

common form in the plant kingdom. Depending on the chemical structure and the degree of unsaturation and oxidation of the three central carbons, flavonoids can be divided into six main groups: isoflavonoids, flavanones, flavanols, flavonols, flavones and anthocyanins [5].

As is well known, flavonoids have potential health-promoting effects for humans due to their therapeutic activities: (i) anti-inflammatory [6], (ii) anticancer [7,8], (iii) anti-allergic [9], (iv) antimicrobial [10], (v) antiviral [11], (vi) antiplatelet [12], (vii) diuretic [13], (viii) anti-hypercholesterolemic [14,15] and (ix) spasmolytic [16]. Flavones is one of main subsets of flavonoids more extensively studied due to the variety of pharmacological properties they exhibit [17,18]. Their administration produces positive results in the treatment of metabolic diseases originated from oxidative stress such as diabetes, cancer, neurodegenerative diseases, atherosclerosis etc. [18]; and in the treatment of microbial and viral diseases, such as periodontal diseases, influenza, COVID 19, etc. [19] However, the efficacy of flavones and other flavonoids as therapeutic agents is reduced due to their limited bioavailability, which results from their poor solubility in aqueous solutions, low permeability and instability in physiological media [19,20].

7,8-Dihydroxyflavone, 7,8-DHF, is a type of natural flavonoid belonging to the subset flavones, which is widely distributed in plants. Its basic molecular structure consists of a 2-phenyl-1-benzopyran-4-one and two hydroxyl groups at the positions 7 and 8 (Fig. 1B). This substance is able to mimic the function of brain-derived neurotrophic factor (BDNF) by activating tropomyosin-related kinase B (TrkB) receptors, cross the haematoencephalic membrane and enter the central nervous system [21]. In addition, 7,8-DHF stimulates the synthesis of antioxidant and DNA repair enzymes by increasing the Nrf2 protein [22,23]. Some studies suggest that 7,8-DHF has antioxidant, anti-inflammatory and antiapoptotic effects which are beneficial in the treatment of neurodegenerative disorders, such as Alzheimer's disease, Parkinson's disease, etc. [24] Also, this flavone improves depression [25], reduces the addictive effects of some drugs [26], enhances the obesity reducing fat production [27], decreases blood pressure [28], improves the effects of ageing on the skin by increasing collagen synthesis and decreasing oxidative stress [29] and shows anticancer activity by inhibition of cell proliferation and enhancement of apoptosis [30].

As other flavonoids, 7,8-DHF is poorly bioavailable (low aqueous solubility and permeability) and unstable in physiological media, which hampers its potential application in medicine.<sup>31</sup> Nanotechnology is an important tool to effectively improve the oral bioavailability of 7,8-DHF, and the flavonoids in general. Nanoencapsulation of 7,8-DHF increases its solubility, dissolution rate and permeability. Besides, the use of nanocarriers prevents the flavone degradation in the gastrointestinal tract and it delivers the drug directly to its physiological targets [20,31]. It also decreases the dose administered of 7,8-DHF to obtain the desired therapeutic effect [32].

Carbon nanotubes, CNTs, can be defined as a graphene sheet rolled up on itself to form a hollow cylinder. They were synthesized for the first time in the 1970s by Oberlin et al. [33] and in the 1990s by Ijima et al. [34] CNTs have a diameter between 0.4 nm and 5 nm, and a length ranging from a few nanometers to several microns and, based on the

number of layers, they can be classified in single-walled carbon nanotubes, SWCNTs, and multi-walled carbon nanotubes, MWCNTs. They show exceptional mechanical, thermal, electronic, optical and chemical properties and, therefore, they have a wide range of applications such as electronics, medicine, environmental remediation and other industrial applications [35]. In the field of medicine, the use of CNTs in drug delivery is of interest due to their high internal volume, ability to immobilize many species, excellent functionalization capacity, adsorption capacity, biocompatibility and ability to cross biological barriers [36–39]. In fact, these carbon nanostructures have been used as nanocarriers of anticancer drugs [40–44], nucleic acids [45], proteins [46], drugs used in mental disorders [47] and antibiotics [48].

Nanoencapsulation can be carried out by covalent functionalization of the CNTs [48] or by adsorption of the drug at the CNT walls [41–43]. Covalent functionalization, which is more stable, is not always possible because many drugs or biomolecules cannot withstand the necessary reaction conditions. The encapsulation by adsorption is generated by  $\pi$ - $\pi$  stacking, hydrophobic or electrostatic interactions. In this case, the release may occur prematurely if the affinity between the drug and the CNTs is not strong enough. On the contrary, the adsorption of planar molecules, containing aromatic rings, at the CNTs walls via  $\pi$ - $\pi$  interactions (or by electrostatic ones when both the drug and the carbon nanotubes are charged) can provoke a more adequate release [42]. Bearing this in mind, and in order to enhance the bioavailability of 7,8-DHF, non-functionalized single- and multi-walled carbon nanotubes, SWCNTs and MWCNTs, have been studied as nanocarriers of the flavone in this work. 7,8-DHF encapsulation was optimized studying the influence that factors such as pH, the type of CNTs and their concentration exert on the association process. The equilibrium binding constants of 7,8-DHF to the CNTs have been determined by measuring the flavone absorbance at different carbon nanotubes concentrations. Since a good nanocarrier should not affect the pharmacological properties of the encapsulated drug, the antioxidant capacity of the 7,8-DHF free and completely associated to the CNTs has been determined by using the DPPH $\cdot$ , 2,2-diphenyl-1-picrylhydrazyl radical scavenging method.

## 2. Materials and methods

### 2.1. Materials

7,8-Dihydroxyflavone (purity > 98%) was purchased in TCI (Zwijndrecht, Belgium). SWCNTs were obtained from CD Bioparticles (Shirley, U.S.A.), with 1–3  $\mu$ m length and 1–2 nm diameter. MWCNTs were supplied by Dropsens S.L. (Oviedo, Spain), with 1.5  $\mu$ m length and 10 nm diameter. DPPH $\cdot$ , as free radical form (95 % purity) was obtained from Alfa Aesar (Haverhill, U.S.A.). L-Ascorbic acid (purity > 99.0%), all the organic solvents (purity > 99.9%) used as well as all the components of the buffer solutions prepared (with the highest purity available) were provided by Sigma (Darmstadt, Germany).

The buffered aqueous media used in the study were: HCl/NaCl pH= 2.0 ([HCl]=  $8.1 \cdot 10^{-3}$  mol dm $^{-3}$ /[NaCl]=  $5.48 \cdot 10^{-3}$  mol dm $^{-3}$ ), Tris/HCl pH= 7.4 ([HOCH $_2$ ) $_3$ CNH $_2$ ]=  $8.9 \cdot 10^{-2}$  mol dm $^{-3}$ /[HCl]=

$2.83 \cdot 10^{-4} \text{ mol dm}^{-3}$ ) and  $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$  pH= 9.2 ( $[\text{Na}_2\text{HPO}_4]=0.12 \text{ mol dm}^{-3}/[\text{NaH}_2\text{PO}_4]=4.25 \cdot 10^{-3} \text{ mol dm}^{-3}$ ).

All solutions were prepared with deionized water (from a Millipore Milli-Q system, Burlington, U.S.A.) with a conductivity  $< 10^{-6} \text{ S m}^{-1}$ .

## 2.2. Encapsulation of 7,8-DHF in the CNTs

The encapsulation of 7,8-DHF in the pristine CNTs investigated was performed by using spectrophotometric measurements. The encapsulation process was carried out at the three different pH conditions, keeping constant the flavone concentration at  $3 \cdot 10^{-5} \text{ mol dm}^{-3}$  and varying the CNTs concentration within the range 0–1.33 g/L. First, an adequate aliquot of the stock flavone solution was added to an Eppendorf tube of 2 mL. After evaporating the alcohol with an  $\text{N}_2$  flow, 1.8 mL of a CNTs solution of a known concentration, in buffered media, was added to the tube. Subsequently, the Eppendorf tube was sonicated for 20 min in the darkness. Finally, the mixture was filtered and, once the supernatant was separated, the absorbance was measured, at  $298.1 \pm 0.1 \text{ K}$ , under the working conditions indicated in Supplementary Material. Since the absorption of the CNTs can contribute to the absorbance measured for the 7,8-DHF, a correction for the CNTs contribution should be done. Therefore, the procedure described is repeated without adding flavone, i.e. only the carbon nanostructures are added into the Eppendorf tube. In this way, the amount of 7,8-DHF not bound to the CNTs can be known by using the corrected absorbance and the calibration curve at the desired pH. Finally, the encapsulation efficiency was determined. Each experiment was done by triplicate.

## 2.3. Stability of the CNTs/7,8-DHF complexes

For the purpose of studying the stability of the SWCNTs/7,8-DHF and MWCNTs/7,8-DHF complexes, the following procedure was used. The flavone and CNTs concentrations were maintained constant at  $1 \cdot 10^{-4} \text{ mol dm}^{-3}$  and 1.33 g/L, respectively, and ethanol was used as solvent to increase the amount of solubilized 7,8-DHF. The resulting solution was centrifuged at 13,500 rpm during 15 min. Once the solid and supernatant are separated by filtration, the ethanol from an aliquot of the supernatant (1.8 mL) is evaporated. Afterwards, 1.8 mL of buffer pH= 2.0 is added. After 15 min of ultrasonication, the resulting solution is used to accurately quantify the amount of encapsulated 7,8-DHF by absorbance measurements as described above.

The solid is collected and dried under vacuum by using an Eppendorf Concentrator Plus from Eppendorf Ibérica (Madrid, Spain) for 45 min at room temperature. Afterwards, 1.8 mL of the desired buffer solution was added to 2.4 mg of dried CNT/7,8-DHF complex within an Eppendorf tube, which was maintained under continuous magnetic stirring (200 rpm) for 24 and 48 h in the darkness. Finally, the complex was filtered and the absorbance of a supernatant sample was measured for calculating the released flavone concentration, using the corresponding calibration curve as is indicated in Supplementary Material.

The stability was studied at pHs 2.0, 7.4, and 9.2. Each experiment was done by triplicate.

## 2.4. Antioxidant activity of free 7,8-DHF

The determination of the antioxidant activity of 7,8-DHF was performed by UV-vis absorption spectrophotometry following the DPPH· radical scavenging method described by Villaño et al., with some modifications [49]. The concentration of the flavone solution was varied in the range  $[\text{7,8-DHF}]=0\text{--}5 \text{ mg L}^{-1}$  and the DPPH· concentration was kept constant at  $6.3 \cdot 10^{-5} \text{ mol dm}^{-3}$ . Methanol was used as solvent for both compounds.

Stock solutions were prepared in methanol as solvent, ( $[\text{7,8-DHF}]=3.9 \cdot 10^{-3} \text{ mol dm}^{-3}$  and  $[\text{DPPH}\cdot]=1.2 \cdot 10^{-4} \text{ mol dm}^{-3}$ ). From the flavone stock solution, methanolic solutions with concentrations varying within the range 0–200  $\text{mg L}^{-1}$  were prepared. Then, 60  $\mu\text{L}$  of each

solution was mixed with 157.5  $\mu\text{L}$  of the DPPH· stock solution. Afterwards, methanol was added up to a final volume of 3 mL. The mixture was homogenized and the absorption spectrum of DPPH· was recorded after incubation at 25 °C for 15 min in the dark. This time was long enough for the reaction to be complete. The wavelength selected for the absorbance measurements was  $\lambda=515 \text{ nm}$ , corresponding to the absorption maximum of DPPH·.

The radical scavenging activity of flavone, expressed as percentage of inhibition, was determined by the following equation:

$$\% \text{ Inhibition} = \frac{A_0 - A}{A_0} \times 100 \quad (1)$$

where  $A_0$  and  $A$  are the absorbance at  $\lambda=515 \text{ nm}$  of the blank and sample, respectively. The alcoholic solution of  $[\text{DPPH}\cdot]=6.3 \cdot 10^{-5} \text{ mol dm}^{-3}$  was used as a blank.

Ascorbic acid was used as a reference antioxidant and its antioxidant activity was determined by the same procedure described above.

## 2.5. Antioxidant activity of encapsulated 7,8-DHF

The antioxidant activity of 7,8-DHF encapsulated in CNTs was also performed by using the DPPH· radical scavenging assay. Stock solutions of CNTs/7,8-DHF complex, obtained as indicated in section 2.3, and free radical were prepared in methanol as solvent,  $[\text{CNT}]=1.33 \text{ mg mL}^{-1}/[\text{7,8-DHF}]=1 \cdot 10^{-4} \text{ mol dm}^{-3}$  and  $[\text{DPPH}\cdot]=1.2 \cdot 10^{-4} \text{ mol dm}^{-3}$ . The complex solution was sonicated for 20 min in the darkness until total dispersion. Afterwards, the necessary aliquots of both solutions and methanol were mixed to obtain a final mixture where the concentration of CNT/7,8-DHF varied within the range of  $[\text{CNT}]=0\text{--}0.296 \text{ mg mL}^{-1}/[\text{7,8-DHF}]=0\text{--}5.64 \text{ mg L}^{-1}$  and the DPPH· concentration was kept constant at  $6.3 \cdot 10^{-5} \text{ mol dm}^{-3}$ . After incubation at 25 °C for 15 min in the dark, the mixture was filtered and the absorption spectrum of the DPPH· present in the supernatant is recorded under the working conditions indicated in Supplementary Material. The absorbance value is measured at  $\lambda=515 \text{ nm}$ , as was indicated above.

Since DPPH· shows a planar structure with aromatic rings and can be adsorbed on CNTs, the absorbance values measured in the previous step must be corrected from the contribution of the radical adsorption on the nanotubes wall. Therefore, the procedure described above was repeated without adding flavone.

Finally, the percentage of DPPH· scavenged by the encapsulated 7,8-DHF, %Inhibition, is determined by equation (1), using the absorbance of the samples corrected for the contribution of DPPH· adsorption.

## 2.6. Dynamic light-scattering measurements

DLS technique was used in order to determine the size and the polydispersity index, PDI, of CNTs/7,8-DHF complexes. The measurements were carried out using a Zetasizer Nano ZS Malvern Instrument Ltd. (Malvern, Worcestershire, UK) at  $298.1 \pm 0.1 \text{ K}$ . Samples were illuminated by a laser with a fixed detection arrangement of 90° to the center of the cell area, and the intensity fluctuation in the scattered light was analyzed. The hydrodynamic diameter,  $d_H$ , was calculated by using the Stokes–Einstein equation. Each result is the average of at least ten measurements. The samples were a dispersion of CNTs/7,8-DHF complexes,  $[\text{CNT}]=0.05 \text{ mg mL}^{-1}/[\text{7,8-DHF}]=3 \cdot 10^{-5} \text{ mol dm}^{-3}$ , in aqueous buffer solution at pH= 7.4.

## 2.7. Zeta-potential measurements

Zeta-potential ( $\xi$ ) experiments were performed with the same instrument and samples used in DLS experiments by measuring the electrophoretic mobility of the samples from the velocity of the particles at  $298.1 \pm 0.1 \text{ K}$ , using a Laser Doppler velocimeter, LDV, (Malvern, Worcestershire, UK). DTS1060 polycarbonate capillary cells were used. Each

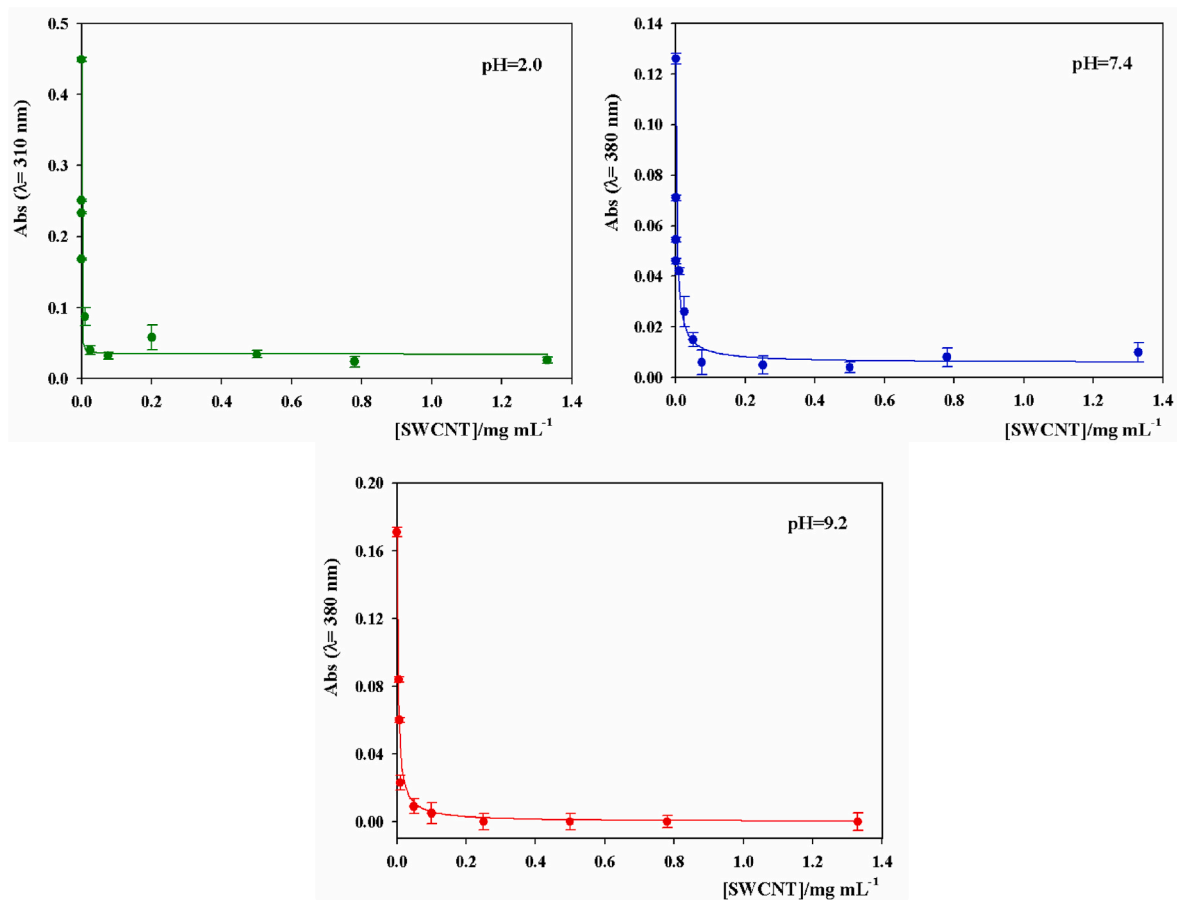


Fig. 2. Dependence of 7,8-DHF absorbance present in the supernatant on SWCNTs concentration at different pHs. Solid lines are the result of fitting the experimental data to equation (5).  $T = 298.1 \pm 0.1$  K.

value is the average of at least 5 measurements.

## 2.8. Transmission Electron Microscopy

The samples for Transmission Electron Microscopy (TEM) were prepared as follows: a drop of the aqueous dispersion containing CNTs or CNT/flavone complexes was deposited onto a copper grid coated with a carbon film. The grid was then air-dried at room temperature.

The images were visualized with a Talos S200 High-Resolution Transmission Electron Microscope. The concentrations used were  $[CNT] = 0.05 \text{ mg mL}^{-1}$  or  $[CNT] = 0.05 \text{ mg mL}^{-1} / [7,8\text{-DHF}] = 3 \cdot 10^{-5} \text{ mol dm}^{-3}$ . All the dispersions were prepared, in aqueous buffer solution at  $\text{pH} = 7.4$ .

## 2.9. Statistical analysis

Values are expressed as the mean  $\pm$  standard errors of separate experiments. Statistical analysis was performed with Student's t-test and One-way analysis of variance (ANOVA). When  $p < 0.05$  (95% confidence) the differences were considered as significant.

## 3. Results and discussion

### 3.1. Encapsulation of 7,8-DHF in pristine single- and multi-walled carbon nanotubes

The encapsulation process of 7,8-DHF by adsorption at the CNTs walls has been studied by using spectrophotometric measurements, as was described above. The influence of pH, the type of CNTs and their

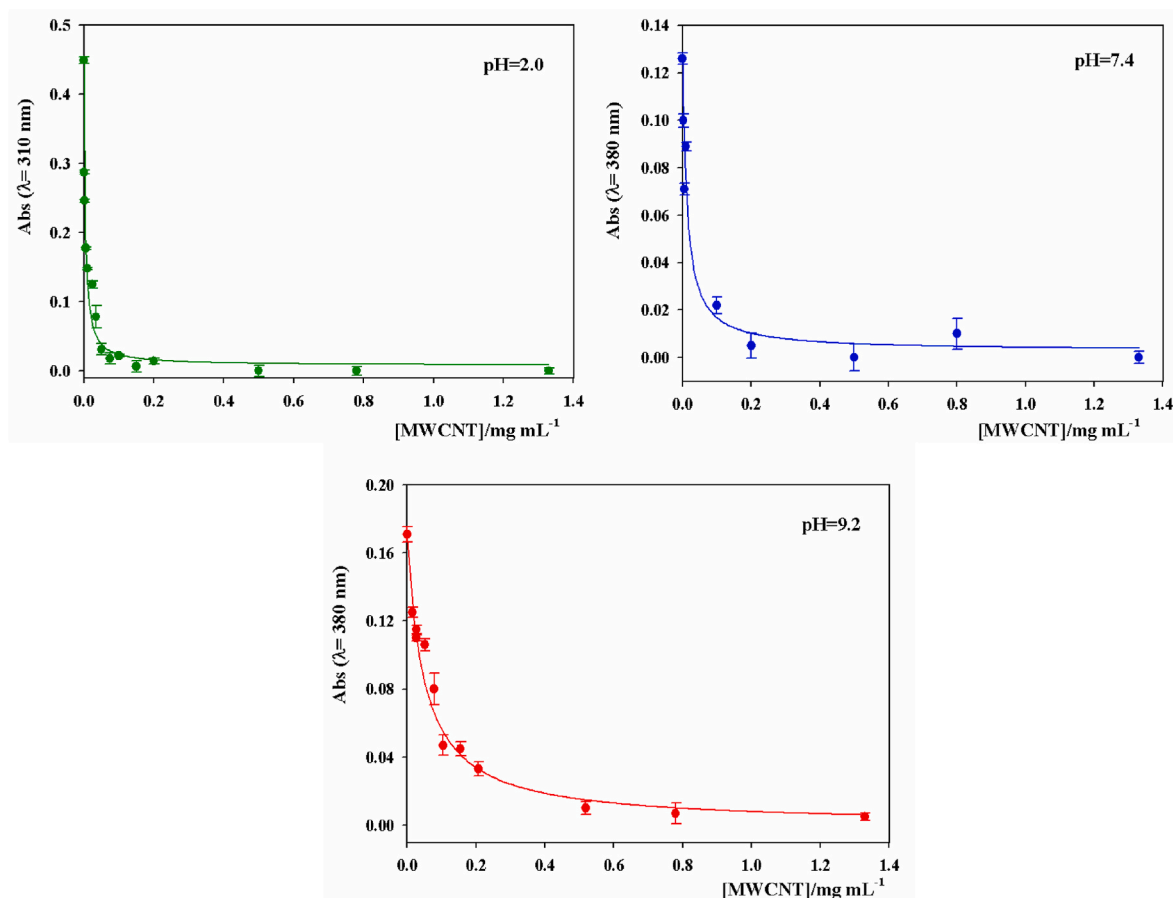
concentration on the association process was investigated at pHs 2.0, 7.4 and 9.2.

Based on the results of the calibration lines (Fig. S1 in Supplementary Material), a flavone solution with  $[7,8\text{-DHF}] = 3 \cdot 10^{-5} \text{ mol dm}^{-3}$  was selected for the encapsulation study in all buffered media. The adsorption of 7,8-DHF on SWCNTs and MWCNTs, was described in section 2.2. Once the solution containing 7,8-DHF and the CNTs is centrifuged, the supernatant is filtered and the absorbance of the resulting solution is measured at the maximum absorbance wavelength of the flavone at the different pHs. Figs. 2 and 3 show the dependence of the absorbance on the CNTs concentration at the three pHs investigated. Since the encapsulation percentage of 7,8-DHF bound to the CNTs is related to the absorbance of 7,8-DHF free, data in Figs. 2 and 3 indicate that the amount of 7,8-DHF associated depends on the  $[CNTs]$ : as can be seen, a decrease in the 7,8-DHF absorbance in the supernatant is observed as the amount of nanotube increases in all the CNTs and media studied. This fact indicates that more and more flavone is adsorbed (encapsulated) at the walls of the CNTs as the concentration of the nanotubes augments.

Table 1 summarizes the CNTs concentrations for which a complete encapsulation of 7,8-DHF is observed at the three pHs studied. The encapsulation efficiency,  $\%7,8\text{-DHF}_{\text{encapsulated}}$ , was determined by using the following equation:

$$\%7,8\text{-DHF}_{\text{encapsulated}} = \frac{m_{7,8\text{-DHF}}^{\text{total}} - m_{7,8\text{-DHF}}^{\text{supernatant}}}{m_{7,8\text{-DHF}}^{\text{total}}} \cdot 100 \quad (2)$$

where  $m_{7,8\text{-DHF}}^{\text{supernatant}}$  is the amount of the flavone remaining in the supernatant, and  $m_{7,8\text{-DHF}}^{\text{total}}$  is the amount of flavone initially added (see section 2.2).



**Fig. 3.** Dependence of 7,8-DHF absorbance present in the supernatant on MWCNTs concentration at different pHs. Solid lines are the result of fitting the experimental data to equation (5).  $T = 298.1 \pm 0.1$  K.

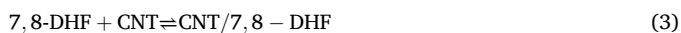
**Table 1**

CNTs concentration required to completely encapsulate 7,8-DHF at different pHs.

[7,8-DHF] = $3 \cdot 10^{-5}$ mol dm <sup>-3</sup>		
pH	[SWCNT] <sub>100% 7,8-DHFencapsulated</sub> /mg mL <sup>-1</sup>	[MWCNT] <sub>100% 7,8-DHFencapsulated</sub> /mg mL <sup>-1</sup>
2.0	$0.025 \pm 0.002$	$0.150 \pm 0.011$
7.4	$0.075 \pm 0.006$	$0.200 \pm 0.009$
9.2	$0.100 \pm 0.005$	$0.780 \pm 0.013$

As can be seen, both the pH and the type of nanotube have an influence on the encapsulation of 7,8-DHF: i) the CNTs concentration required to reach the 100 % of 7,8-DHF<sub>encapsulated</sub> diminishes as the pH of the media decreases, and ii) for all pHs, it is observed that  $[MWCNT]_{100\% \text{ 7,8-DHFencapsulated}} > [SWCNT]_{100\% \text{ 7,8-DHFencapsulated}}$ .

With the goal of quantifying the CNT/7,8-DHF interactions, the equilibrium binding constant of 7,8-DHF to the pristine CNTs, K, was calculated. The equilibrium can be written as:



Therefore:

$$K = \frac{[\text{CNT}/7,8\text{-DHF}]}{[7,8\text{-DHF}][\text{CNT}]} \quad (4)$$

The dependence of the absorbance on [CNTs] can be expressed, considering the Pseudophase Model, as follows [50]:

**Table 2**

Values of K obtained by fitting the absorbance data (Figs. 2 and 3) using equation (5).  $T = 298.1 \pm 0.1$  K.

K/g <sup>-1</sup> L		
pH	SWCNT/7,8-DHF	MWCNT/7,8-DHF
2.0	$(4.6 \pm 1.1) \cdot 10^3$	$307 \pm 62$
7.4	$(5 \pm 2) \cdot 10^3$	$77 \pm 27$
9.2	$257 \pm 53$	$20 \pm 5$

$$A = \frac{(A)_f + (A)_b K[\text{CNT}]}{1 + K[\text{CNT}]} \quad (5)$$

where  $(A)_f$  and  $(A)_b$  are the absorbance of free and bound 7,8-DHF, respectively.

The experimental data shown in Figs. 2 and 3 were fitted by using equation (5). The results of the fittings are represented by the solid lines in these figures. One can see that the agreement between the experimental and the theoretical data is good. The values of K obtained from the fittings are listed in Table 2.

Data listed in Table 2 show that 7,8-DHF interacts more strongly with SWCNTs than with MWCNTs. Besides, the strength of these interactions increases upon decreasing the pH of the medium. At this point, it is worth noting that the encapsulation (adsorption) of 7,8-DHF at the CNTs surfaces is expected to occur by  $\pi$ - $\pi$  interactions. However, since these interactions would be operative in the both CNTs and all pHs studied, the differences observed point out that other interactions must also be at work. In regard to the pH influence, 7,8-DHF, as many flavones, is a weakly acidic compound. That is, the 7,8-DHF molecule is uncharged at

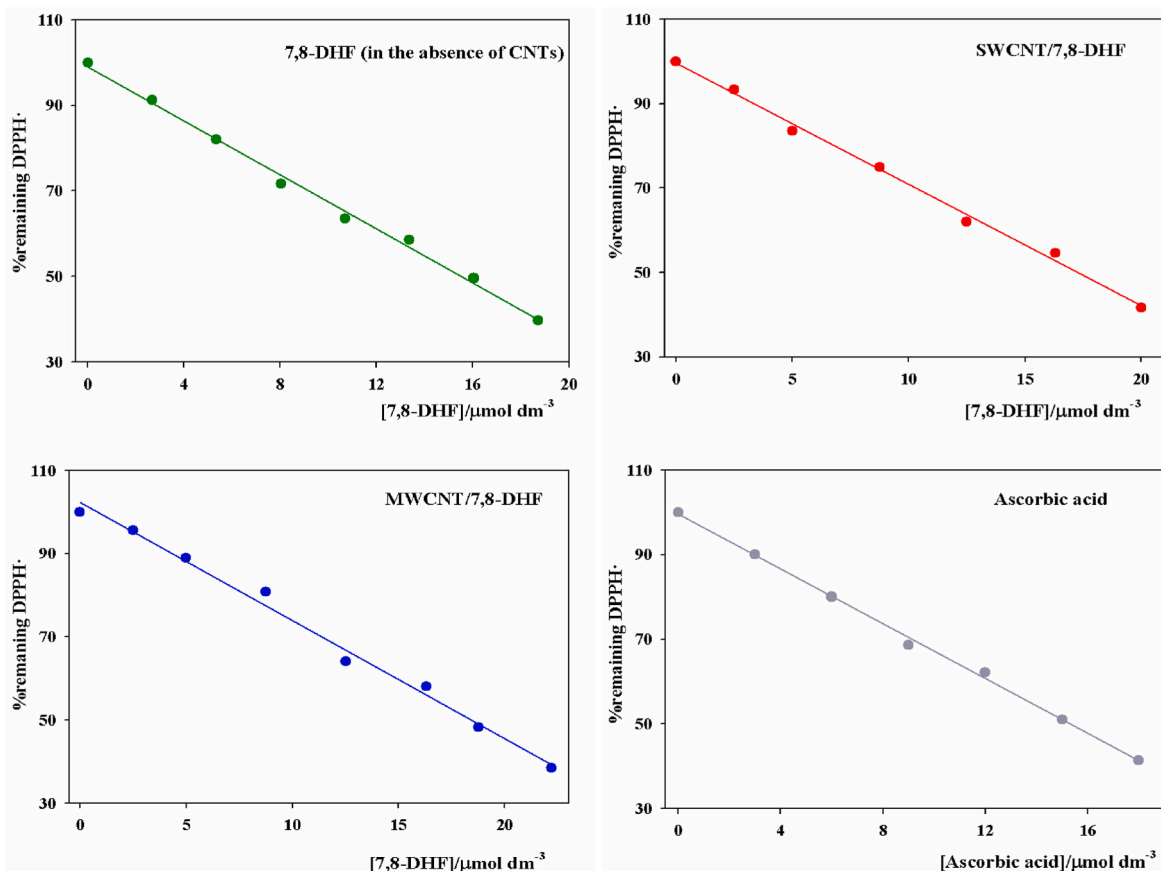


Fig. 4. Dependence of the % remaining DPPH· on the concentration of the antioxidant species in methanol.

acidic pH, whereas it will be negatively charged at basic pH [51–53]. In a previous work, the authors have shown that the adsorption of ionic surfactants at the surface of different CNTs occurs through hydrophobic interactions; adsorption becomes stronger as the lipophilic character of the surfactant increases [54]. Since the non-ionic form of 7,8-DHF is much more lipophilic than the anionic form, one would expect the hydrophobic interactions to follow the trend  $\text{pH} = 2.0 > \text{pH} = 7.4 > \text{pH} = 9.2$ . Therefore,  $K$  values are also expected to follow the same trend, as is observed.

In addition, changes in pH affect the distribution of  $\pi$  electrons in the aromatic rings of the flavone. The resonance energy of 7,8-DHF in its anionic form is lower than that of its neutral form. This will lead to a weakening of the  $\pi$ - $\pi$  interactions of the anionic flavone molecules with the surface of CNTs compared to those of the neutral one. This behaviour also explains the observed dependence of  $K$  on the pH of the medium.

In relation to the influence of the CNT nature on  $K$ , the strength of the CNTs/7,8-DHF interactions depends on the CNTs adsorption capacity. This capacity is strongly determined by the surface area,  $S$  ( $\text{m}^2 \text{g}^{-1}$ ), of the CNTs. In general, SWCNTs have a larger surface area, and consequently a higher adsorption capacity, than MWCNTs [55,56]. This explains the experimental observation:  $K_{\text{SWCNT/7,8-DHF}} > K_{\text{MWCNT/7,8-DHF}}$ .

### 3.2. Stability and antioxidant activity of CNTs/7,8-DHF: role of SWCNTs and MWCNTs as nanocarriers of 7,8-DHF

The stability of SWCNTs/7,8-DHF and MWCNTs/7,8-DHF complexes has been studied for 24 and 48 h as is described in section 2.3. According to the results, the flavone release from the nanotube surface is negligible in all buffered aqueous media, so that the complexes formed are stable for at least two days.

As was mentioned above, flavones have an aromatic ring linked to at

Table 3

Values of  $\text{EC}_{50}$  of 7,8-DHF free and encapsulated in CNTs and ascorbic acid in methanol.  $T = 298.1 \pm 0.1$  K.

Antioxidant	$\text{EC}_{50}/\mu\text{mol dm}^{-3}$
7,8-DHF (in the absence of CNTs)	$13.8 \pm 1.8$
SWCNT/7,8-DHF	$13.8 \pm 1.9^a$
MWCNT/7,8-DHF	$12 \pm 2^b$
Ascorbic acid	$15.9 \pm 1.1$

<sup>a</sup>  $\text{EC}_{50}^{24\text{h}} = 13.0 \pm 1.6 \mu\text{mol dm}^{-3}$  and  $\text{EC}_{50}^{48\text{h}} = 13.3 \pm 1.8 \mu\text{mol dm}^{-3}$ .

<sup>b</sup>  $\text{EC}_{50}^{24\text{h}} = 12.2 \pm 1.9 \mu\text{mol dm}^{-3}$  and  $\text{EC}_{50}^{48\text{h}} = 12.0 \pm 1.2 \mu\text{mol dm}^{-3}$ .

least one hydroxyl functional group in their molecular structure. The most interesting property of the hydroxyl groups is their susceptibility to oxidation. This antioxidant capacity makes them capable of capturing a wide range of oxygen, nitrogen or chlorine centered radical species. The antioxidant activity of the free and bound 7,8-DHF has been evaluated by using the 2,2-diphenyl-1-picrylhydrazyl, DPPH·, method as is described in sections 2.4 and 2.5. DPPH· is a nitrogen centered radical, which shows a characteristic UV–vis spectrum with a maximum of absorbance close to  $\lambda = 515$  nm in methanol. The experiments were performed with an excess of DPPH· radical to exhaust the H-donating capacity of the flavone. The final concentration of DPPH· was  $6.3 \cdot 10^{-5} \text{ mol dm}^{-3}$ , while the flavone was added approximately in the range  $0$ – $22.2 \mu\text{mol dm}^{-3}$  for both free and bound 7,8-DHF. The results obtained are shown in Fig. 4. The antioxidant capacity of ascorbic acid was also determined under the same conditions as a reference antioxidant.

The antioxidant activity is evaluated using the  $\text{EC}_{50}$  index, defined as the antioxidant concentration needed to reduce by half the amount of DPPH·. This parameter was obtained from the data shown in Fig. 4. The results are listed in Table 3.

The free 7,8-DHF has an  $\text{EC}_{50}$  similar to that of ascorbic acid,

**Table 4**

Zeta-potential ( $\xi$ ), hydrodynamic diameter ( $d_H$ ) and polydispersity index (PDI) values for CNTs/7,8-DHF complexes in aqueous buffered solution at pH= 7.4. T= 298.1±0.1 K.

Complex	$\xi$ /mV	$d_H$ /nm	PDI
SWCNT/7,8-DHF	-11 ± 4	540 ± 70	0.435 ± 0.017
MWCNT/7,8-DHF	-16 ± 7	608 ± 65	0.452 ± 0.011

The  $\xi$  values are expressed as the mean ± standard deviation (SD) (n = 5). The  $d_H$  and PDI values are expressed as the mean ± SD (n = 10).

indicating that its antioxidant activity is very high and comparable to that of other potent antioxidants [49]. Results shown in Table 3 indicate that the 7,8-DHF encapsulated in both types of CNTs maintains its antioxidant activity unchanged for the freshly prepared complex. In addition, the antioxidant activity of the complexes was determined after they were maintained in buffer pH 7.4 for 24 h and 48 h with constant stirring to simulate what happens to this pharmacological property after administration of the encapsulated drug. The EC<sub>50</sub> values were similar within the experimental error to those obtained for the freshly prepared complexes, showing that the antioxidant activity of 7,8-DHF is preserved over time. Furthermore, it is shown that the encapsulation in CNTs confers a high stability to 7,8-DHF in the aqueous media studied.

### 3.3. Physicochemical characterization of CNTs/7,8-DHF complexes

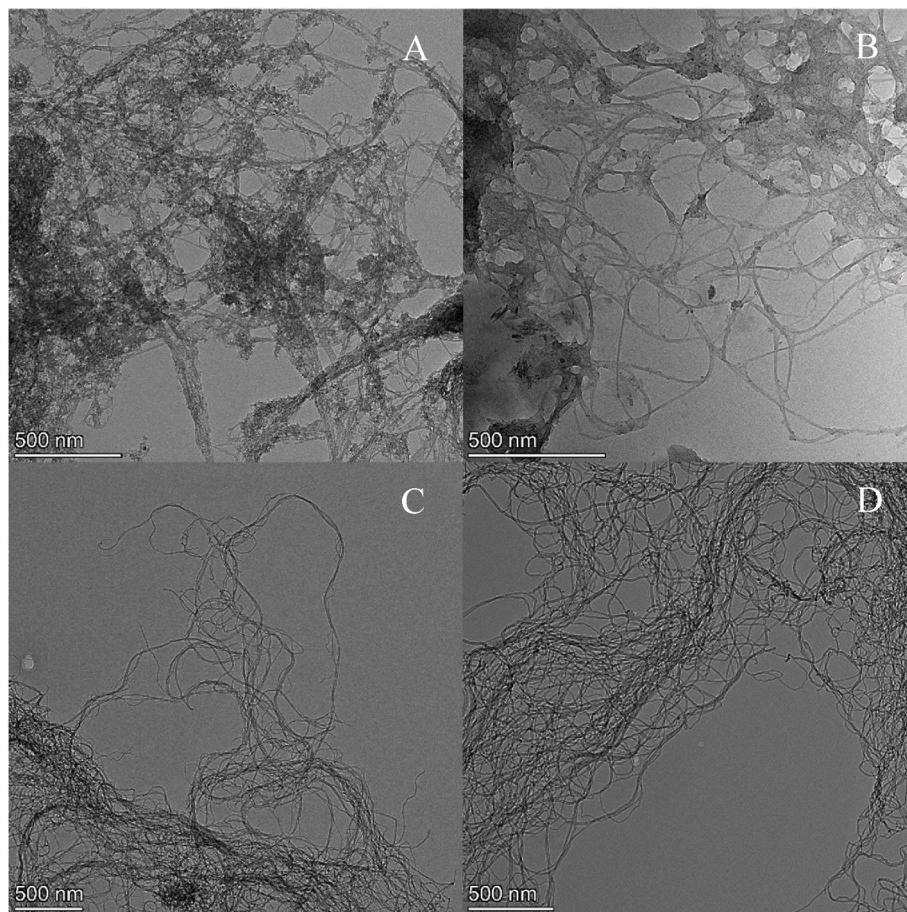
As was mentioned in the introduction, CNTs show exceptional properties that allow them to be applied in different fields, including medicine. CNTs applications are limited due to a low and poor solubility

in solution caused by the existence of strong intermolecular interactions as van der Waals forces and  $\pi$ - $\pi$  stacking interactions, which provoke the agglomeration of the tubes into ropes and/or bundles. The CNTs dispersion in solution has been the subject of exhaustive study by the scientific community. Besides, their applications, including their role as drug nanocarriers, are all the more effective the better the quality of the dispersion is [54,57]. The dispersion quality of these and other nanomaterials depends on their distribution of particle size: the smaller the hydrodynamic radius and polydispersity index, the better the dispersion of nanomaterials in solution [54,58].

With this in mind and given the good performance of CNTs as 7,8-DHF nanocarriers, the study of the size distribution of the CNTs/7,8-DHF complexes was carried out by the dynamic light scattering technique, DLS, as is described in section 2.6. The results are shown in Table 4.

The values obtained for  $d_H$  and PDI show that the size distribution of both complexes in aqueous solution are small, indicating that the adsorption of 7,8-DHF at the surface of CNTs prevents the aggregation of the carbon nanostructures; that is, the adsorption of the drug favors the CNTs dispersion. This fact has been confirmed by Transmission Electron Microscopy (TEM). Fig. 5A–D shows the dispersion of carbon nanotubes in the absence and presence of 7,8-DHF. In both types of CNTs, an improvement in their dispersion is observed in the presence of the flavone. Such dispersion ensures the biodistribution of CNTs/Flavone complexes and, given their stability, the bioavailability of 7,8-DHF would be increased [57].

The Z-potential of the complexes SWCNTs/7,8-DHF and MWCNTs/7,8-DHF in aqueous solution at pH 7.4 has also been measured (see section 2.7). Both complexes show a low negative Z-potential value in



**Fig. 5.** Transmission electron microscopic images of SWCNT (A), SWCNT/7,8-DHF (B), MWCNT (C) and MWCNT/7,8-DHF (D) with scale bars of 500 nm. The samples were prepared in an aqueous buffer solution Tris/HCl pH= 7.4.

good agreement to that obtained for pristine carbon nanotubes at neutral pH [59]. This result was expected since the most of the flavone is neutral at pH 7.4, although there is also a small percentage of the anionic form.

Based on all these results and the lack of cytotoxicity shown in normal and cancer cell lines by the SWCNTs and MWCNTs in the range of concentrations used [60], pristine carbon nanotubes could be suitable nanocarriers for the controlled delivery of 7,8-DHF, as its encapsulation in these nanotubes does not affect its pharmacological properties and increases its stability, biodistribution, and bioavailability.

#### 4. Conclusions

In the present work, the encapsulation of 7,8-dihydroxyflavone, 7,8-DHF, within non-functionalized single- and multi-walled carbon nanotubes, SWCNTs and MWCNTs, respectively, has been investigated. Results show that the 100 % of the flavone was encapsulated (adsorbed) by the CNTs and the equilibrium constant,  $K$ , for the binding CNTs/Flavone, depended on the pH of the medium and the carbon nanotube nature: i)  $K$  increases as the pH of the media decreases and ii)  $K_{\text{SWCNT}/7,8\text{-DHF}}$  is higher than  $K_{\text{MWCNT}/7,8\text{-DHF}}$  for all the pH values studied.

A plausible explanation for rationalizing the influence of the pH on the formation of CNT/7,8-DHF complex would be the existence of hydrophobic interactions, in addition to the  $\pi-\pi$  interactions, between the flavone molecules and the CNTs surface, being more important at acid and neutral pHs where the drug molecule is mostly neutral. In regard to the influence of the type of nanotubes, the effect of surface chemistry and morphology of carbon nanotubes on their adsorption capacity towards a given molecule could explain the results obtained: SWCNTs have a larger surface area and, consequently, a higher adsorption capacity than MWCNTs.

SWCNTs/7,8-DHF and MWCNTs/7,8-DHF complexes have shown a high stability during at least 48 h, improving the poor stability of 7,8-DHF molecules in water. The flavone encapsulation on these carbon nanomaterials does not affect its pharmacological properties: 7,8-DHF encapsulated in both types of CNTs maintains unchanged its antioxidant activity and is preserved over time after its administration.

The physicochemical characterization of the CNTs/7,8-DHF complexes and their stability show that the adsorption favors the dispersion of the CNTs, improves the biodistribution of the nanocarriers with the cargo and increases the bioavailability of the drug.

Based on these results and the lack of cytotoxicity shown by the SWCNTs and MWCNTs in the range of concentrations used, it can be concluded that pristine carbon nanotubes, MWCNTs and SWCNTs, are suitable nanocarriers for the controlled delivery of 7,8-DHF.

#### CRedit authorship contribution statement

**Cecilia Espíndola:** Writing – review & editing, Methodology, Investigation. **Ana Gómez-Méndez:** Writing – review & editing, Methodology, Investigation. **María Luisa Moyá:** Writing – review & editing, Methodology, Investigation, Funding acquisition. **Fabián López-Vallejo:** Writing – review & editing, Methodology, Investigation. **Eva Bernal:** Writing – review & editing, Methodology, Investigation. **José Antonio Lebrón:** Writing – review & editing, Methodology, Investigation. **Victoria Isabel Martín:** Writing – review & editing, Methodology, Investigation. **Francisco José Ostos:** Writing – review & editing, Methodology, Investigation. **Pilar López-Cornejo:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Manuel López-López:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Investigation, Formal analysis, Conceptualization.

#### Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

#### Data availability

Data will be made available on request.

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#### Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jddst.2024.106068>.

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