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**Evaluation of solubility enhancement, antioxidant activity, and cytotoxicity studies of kynurenic acid loaded cyclodextrin nanosponge**

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(Article begins on next page)

1 **EVALUATION OF SOLUBILITY ENHANCEMENT, ANTIOXIDANT ACTIVITY, AND**  
2 **CYTOTOXICITY STUDIES OF KYNURENIC ACID LOADED CYCLODEXTRIN**  
3 **NANOSPONGE.**

4  
5 **Nilesh K. Dhakar<sup>1</sup>, Fabrizio Caldera<sup>1</sup>, Federica Bessone<sup>2</sup>, Claudio Cecone<sup>1</sup>, Alberto Rubin**  
6 **Pedrazzo<sup>1</sup>, Roberta Cavalli<sup>2</sup>, Chiara Dianzani<sup>2</sup>, Francesco Trotta<sup>1\*</sup>**

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8 <sup>1</sup>**Department of Chemistry, University of Torino, via P. Giuria 7, 10125, Torino, Italy.**

9 <sup>2</sup>**Department of Drug Science and Technology, University of Torino, via P. Giuria 9, 10125,**  
10 **Torino, Italy.**

11  
12 **\*Corresponding author:**

13 **Email: francesco.trotta@unito.it**

14 **Tel: 0116707550 Fax: 0116707855**

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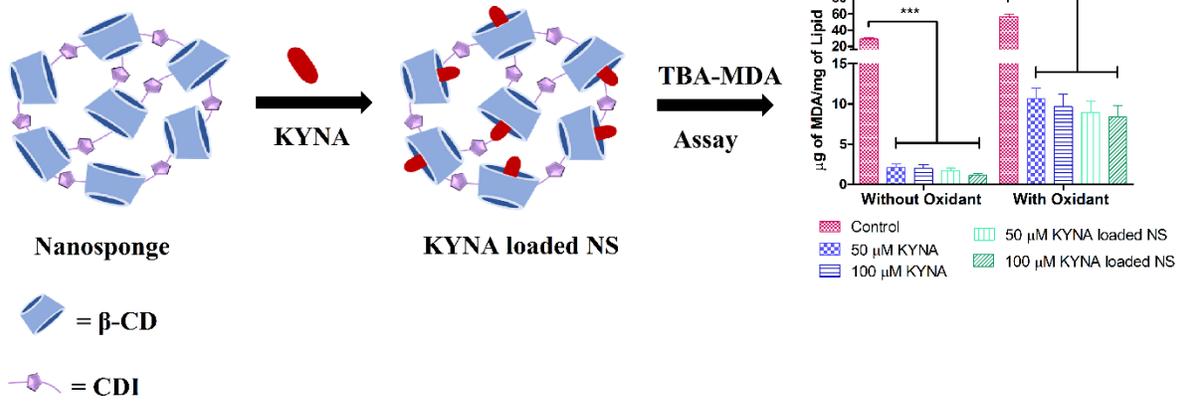
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20 **Graphical Abstract**



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40 **Highlights**

- 41 • The aqueous solubility of KYNA was increased by encapsulation with  $\beta$ -CDNS2.
- 42 • The molar ratio of CDI to  $\beta$ -CD affected the solubilization efficiency of nanosponges.
- 43 • Higher antioxidant activity of KYNA loaded NS was achieved compared to plain KYNA.
- 44 • Cell viability study showed that NS was nontoxic to SHSY-5Y human neuroblastoma cell  
45 lines.
- 46 • The particle size and zeta potential of KYNA loaded NS and blank NS remained  
47 unchanged on storage.

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59 **Abstract**

60 **Kynurenic acid** demonstrates antioxidant, neuroprotective and free radical scavenging  
61 properties. However, low aqueous solubility of **kynurenic acid** limits its therapeutic activity. In  
62 the present study, cyclodextrin nanosponges were used to improve the solubility and therapeutic  
63 activity of **kynurenic acid**. The formation of **kynurenic acid loaded nanosponge** was confirmed  
64 by **different characterization techniques**. The solubility of **kynurenic acid** was significantly  
65 increased with **nanosponge** (111.1  $\mu\text{g/ml}$ ) compared to free kynurenic acid (16.4  $\mu\text{g/ml}$ ) and  **$\beta$ -**  
66 **cyclodextrin** (28.6  $\mu\text{g/ml}$ ). High drug loading (19.06 %) and encapsulation efficiency (95.31 %)   
67 were achieved with NS. The particle size and zeta potential of **kynurenic acid loaded nanosponge**  
68 was around 255.8 nm and -23 mV respectively. Moreover, higher solubilization of **kynurenic**  
69 **acid loaded nanosponge** produced better antioxidant activity compared to free **kynurenic acid**.  
70 The **kynurenic acid loaded nanosponge** and blank **nanosponge** were found nontoxic in the  
71 cytotoxicity assay. Thus, these studies demonstrated that nanosponges can be used as a carrier  
72 for the delivery of **kynurenic acid**.

### 73 **Keywords**

74 Kynurenic acid,  $\beta$ -cyclodextrin, Nanosponges, Solubility Enhancement, Drug-Delivery,  
75 Antioxidant Activity

### 76 **Abbreviations**

77 KYNA: Kynurenic Acid,  $\beta$ -CD: Beta-cyclodextrin, CDI: N,N'-carbonyldiimidazole NS:  
78 Nanosponge, DPPH: 2,2-diphenyl-1-picrylhydrazyl, TBA: Thiobarbituric acid, MDA:  
79 Malondialdehyde

80

## 81 1. Introduction

82 Kynurenic acid (4-Hydroxyquinoline-2-carboxylic acid; KYNA; Fig. 1A) is an endogenous  
83 substance that is formed from tryptophan via kynurenine metabolic pathway. However, KYNA is  
84 also present in dietary food products such as broccoli, potato, and spices (Turski, Turska, Kocki,  
85 Turski, & Paluszkiewicz, 2015; Turski, Turska, Zgrajka, Kuc, & Turski, 2009). The kynurenine  
86 metabolic pathway endogenously metabolizes tryptophan into KYNA, 3-hydroxykynurenine (3-  
87 HK) and quinolinic acid (QUIN) by kynurenine aminotransferase, kynurenine 3-  
88 monooxygenase, and kynureninase enzymes, respectively. The 3-HK is a potent free radical  
89 generator and QUIN acts as N-methyl-D-aspartate (NMDA) receptor agonist. However, KYNA  
90 is an ionotropic glutamate and alpha 7-nicotinic receptor antagonist (Vécsei, Szalárdy, Fülöp, &  
91 Toldi, 2013).

92 KYNA is an excitatory amino acid antagonist possessing neuroprotective properties. The  
93 neuroprotective effect of KYNA was demonstrated by László and Beal (László & Beal, 1991).  
94 Carpenedo and co-workers showed the antagonistic activity of KYNA on glutamate receptors  
95 (Carpenedo et al., 2001). Several researchers have demonstrated the antioxidant and free radical  
96 scavenging properties of KYNA. KYNA is formed inside the central nervous system (CNS) and  
97 it is reported that KYNA is also present in human blood and peripheral organs. The decreased  
98 concentration of KYNA in the CNS is associated with several neurological disorders such as  
99 Parkinson's disease, multiple sclerosis and Huntington's disease (Schwarcz, Bruno, Muchowski,  
100 & Wu, 2013; Stone & Connick, 1985). However, low aqueous solubility and limited blood-brain  
101 barrier (BBB) permeability limits its therapeutic application (Hornok et al., 2012; Varga et al.,  
102 2016). Moreover, kynurenic acid itself has the ability to cross the blood-brain barrier (BBB) to a  
103 limited extent and accumulates in the brain on systemic administration (Stone, 2001). Moroni et

104 al. demonstrated that the concentration of KYNA in the mammalian brain is around 10 to 150  
105 nM (Moroni, Russi, Lombardi, Beni, & Carlh, 1988). Several researchers have demonstrated the  
106 effect of KYNA upon systemic administration and its permeation across BBB. Varga et al.  
107 prepared KYNA loaded core-shell nanoparticles of bovine serum albumin (BSA) and  
108 demonstrated that peripheral administration of KYNA loaded BSA nanoparticles are sufficient  
109 enough to produce electrophysiological effects within the central nervous system (Varga et al.,  
110 2016). Hornok and co-workers prepared KYNA loaded micelles to demonstrate the  
111 pharmacological activity of KYNA on systemic administration (Hornok et al., 2012). Moreover,  
112 López et al. earlier demonstrated the synthesis of KYNA loaded silica nanoparticles to improve  
113 the solubilization of KYNA (López, Ortiz, Gómez, la Cruz, Verónica Pérez-de Carrillo-Mora, &  
114 Novaro, 2014). Different ester derivatives of KYNA were also prepared to improve its solubility  
115 and permeability (Dalpiaz et al., 2005).

116 Cyclodextrin-based delivery systems provide a promising platform to increase drug solubility,  
117 stability, and enhance the drug release profile. The inclusion complex of poorly water-soluble  
118 drugs with  $\alpha$ -cyclodextrin,  $\beta$ -cyclodextrin, and  $\gamma$ -cyclodextrin is widely reported (A. Singh,  
119 Worku, & Mooter, 2011; Woldum, Larsen, & Madsen, 2008). However, there are certain  
120 drawbacks of native cyclodextrins such as small cavity size of  $\alpha$ -cyclodextrin and  $\gamma$ -cyclodextrin  
121 is expensive.  $\beta$ -cyclodextrin is most commonly used in the drug delivery application  
122 nevertheless,  $\beta$ -cyclodextrin exhibits low aqueous solubility and nephrotoxicity (Challa, Ahuja,  
123 Ali, & Khar, 2006).

124 Several efforts have been made to overcome drawbacks associated with native cyclodextrins  
125 and, to improve their performance, different types of cyclodextrin nanosponges were prepared  
126 earlier. The nanosponges (NSs) are 3-dimensional hyper crosslinked polymer of native

127 cyclodextrins prepared with a variety of crosslinking agents such as 1,1'-carbonyldiimidazole  
128 (CDI), pyromellitic dianhydride (PMDA), and diphenyl carbonate (DPC) (Trotta, Dianzani,  
129 Caldera, Moggetti, & Cavalli, 2014; Venuti et al., 2017). Moreover, CDI based nanosponges are  
130 more stable at acidic and alkaline pH and comparatively low toxic to their other counterparts  
131 (Trotta, Zanetti, & Cavalli, 2012). Many researchers have also prepared modified nanosponges  
132 such as ionic  $\beta$ -cyclodextrin polymers (Berto et al., 2007) and glutathione responsive  
133 nanosponges (Trotta et al., 2016). Nanosponges have been utilized for a variety of applications  
134 such as enhancement of aqueous solubility and stability of nutraceuticals (Ansari, Vavia, Trotta,  
135 & Cavalli, 2011; Darandale & Vavia, 2013), encapsulation of proteins (Wajs, Caldera, Trotta, &  
136 Fragoso, 2013) and immobilization of enzymes (Di Nardo et al., 2009). It is also reported that  
137 cyclodextrins based nanocarriers facilitate the permeation of drug molecules across BBB on  
138 systemic administration (Shityakov et al., 2016). Furthermore, Cyclodextrin based NSs can be  
139 administered via several routes depending on the applications (Swaminathan et al., 2010; Torne,  
140 Darandale, Vavia, Trotta, & Cavalli, 2013).

141 In the present work, we demonstrated the synthesis and characterization of KYNA loaded  
142 cyclodextrin nanosponges. The enhancement in aqueous solubility, antioxidant activity, and in-  
143 vitro cell toxicity were also studied.

## 144 **2. Materials and Method**

### 145 **2.1 Materials**

146 The  $\beta$ -cyclodextrin was a kind gift from Roquette Italia (Cassano Spinola, Italy). Kynurenic  
147 acid, N,N'-carbonyldiimidazole, Thiobarbituric (TBA) acid and 2,2-diphenyl-1-picrylhydrazyl  
148 (DPPH) were purchased from Sigma-Aldrich (Milan, Italy). Cell culture reagents were

149 purchased from Gibco/Invitrogen (Life Technologies, Paisley, UK). All other chemicals and  
150 reagents used were of analytical grade unless otherwise specified.

## 151 2.2 Methods

### 152 2.2.1 Synthesis of $\beta$ -cyclodextrin nanosponge ( $\beta$ -CDNS)

153 N,N'-carbonyldiimidazole (CDI) was used as a crosslinker to prepare  $\beta$ -cyclodextrin  
154 nanosponge as reported earlier by our group (Trotta et al., 2012). Briefly,  $\beta$ -cyclodextrin ( $\beta$ -CD)  
155 was dissolved in N,N-dimethylformamide (DMF) and CDI was added in a different molar ratio  
156 of 1:2, 1:4 and 1:6 resulting in the formation of three-different types of nanosponges. The  
157 reaction was carried out at 90 °C on a magnetic stirrer for 3 hours  
158 overnight for further crosslinking. The solid monolithic mass of nanosponge was crushed and  
159 washed several times with water to remove unreacted components. The nanosponge was purified  
160 by Soxhlet extraction with ethanol (24 hours), air dried and stored in a desiccator at room  
161 temperature for further use. The nanosponges prepared with CDI were abbreviated as  $\beta$ -CDNS1,  
162  $\beta$ -CDNS2, and,  $\beta$ -CDNS3 respectively.

163 The quantities of chemicals used in the synthesis of three-different types of nanosponges are  
164 listed in table 1.

165 **Table 1.**

Samples	Cyclodextrin ( $\beta$ -CD)		CDI		DMF	$\beta$ -CD:CDI
	(g)	(mmol)	(g)	(mmol)	(ml)	Molar Ratio
$\beta$ -CDNS1	5	4.405	1.426	8.794	30	2
$\beta$ -CDNS2	5	4.405	2.852	17.588	30	4
$\beta$ -CDNS3	5	4.405	4.278	26.382	30	6

166

### 167 **2.2.2 Determination of swelling degree**

168 All the nanosponges were dried overnight and known amounts of different nanosponges were  
169 placed in water. The swollen nanosponges were removed and excess of water was removed by  
170 blotting on filter paper. The weight of nanosponges was recorded and the above procedure was  
171 followed until a constant weight was achieved.

172 The following equation was used to calculate % swelling degree.

$$\% \text{ Swelling Degree} = \frac{\text{Weight in the swollen state} - \text{Weight in the dry state}}{\text{Weight in the dry state}} \times 100$$

### 173 **2.2.3 Solubilization efficiency of nanosponges**

174 The aqueous solubility of KYNA alone, with  $\beta$ -CD and with nanosponges ( $\beta$ -CDNS1 to  $\beta$ -  
175 CDNS3) was studied. An excess quantity of KYNA was suspended in water (2 ml) and a fixed  
176 quantity of  $\beta$ -CD or different nanosponges was added into it. The vials were placed on a  
177 mechanical shaker for 24 hours at room temperature. Later, the suspension was centrifuged at  
178 6000 rpm for 15 minutes and the supernatant was collected. All the samples were filtered using a  
179 0.45  $\mu$ m syringe filter and analyzed on HPLC as mentioned in section 2.2.6.

### 180 **2.2.4 Preparation of KYNA loaded nanosponge**

181 KYNA loaded nanosponge was prepared by the freeze-drying method as established earlier  
182 (Zidan, Ibrahim, Afouna, & Ibrahim, 2018). An aqueous suspension of individual nanosponges  
183 ( $\beta$ -CDNS1 to  $\beta$ -CDNS3) was prepared (10 mg/ml) and the required quantity of KYNA was  
184 added into it at different weight ratio of 1:3, 1:4 and 1:5 (w/w), respectively. The suspension was  
185 sonicated for 10 minutes and stirred for 24 hours at room temperature. Later, the suspension was

186 centrifuged at 6000 rpm for 15 minutes to remove the non-complexed drug. The supernatant was  
187 collected, 5 % trehalose (% w/v) was added as a cryo-protectant and freeze-dried.

188 The freeze-dried formulations of KYNA were abbreviated as KYNA- $\beta$ -CDNS1, KYNA- $\beta$ -  
189 CDNS2, and KYNA- $\beta$ -CDNS3, respectively.

### 190 **2.2.5 Preparation of a physical mixture**

191 Physical mixtures were also prepared by mixing drug (2.5 mg) with different nanosponges (10  
192 mg) by trituration in a mortar for 30 min at room temperature. The physical mixture formulations  
193 were abbreviated as PM1, PM2, and PM3, respectively.

### 194 **2.2.6 Quantitative determination of KYNA by HPLC**

195 The quantitative determination of KYNA was performed by an HPLC system (PerkinElmer,  
196 Waltham, USA) equipped with a UV detector (Flexar UV/Vis LC spectrophotometer) using a  
197 phenomenex C18 analytical column (4.6 mm x 250 mm, 5  $\mu$ m). The mobile phase consisted of a  
198 mixture of 0.14 % (v/v) TFA (trifluoroacetic acid) in water-acetonitrile (90:10 v/v), filtered and  
199 ultrasonically degassed prior to use. The mobile phase was pumped through the column at a flow  
200 rate of 1 ml/min and the samples (20  $\mu$ l) were analyzed at 330 nm using a UV detector (Lesniak  
201 et al., 2013)

### 202 **2.2.7 Determination of KYNA loading efficiency**

203 The KYNA loaded NS was taken into a vial containing 1 ml of DMSO-water mixture (50:50)  
204 and sonicated for 1 hour. Later, it was filtered and suitably diluted with mobile phase and  
205 analyzed on HPLC.

## 206 **2.2.8 Determination of particle size, polydispersity index, and zeta potential**

207 The particle size and polydispersity index were studied by DLS using a Malvern Zetasizer  
208 Nano instrument at a fixed scattering angle of 90°. All the samples were suitably diluted by milli-  
209 Q water and analyzed at 25 °C. The zeta potential of all the samples was calculated on the same  
210 instrument placing an additional electrode. All the measurements were performed in triplicate.

## 211 **2.2.9 Differential Scanning Calorimetry (DSC)**

212 Thermal properties of KYNA, blank NS, physical mixture, and KYNA loaded NS were  
213 evaluated using a TA instruments Q200 DSC (New Castle, DE, USA). The empty aluminum pan  
214 was used as a reference standard and samples (2-3 mg) were scanned from 30 to 300 °C at the  
215 scanning rate of 10 °C/min under a nitrogen purge.

## 216 **2.2.10 Fourier transform infrared spectroscopy (FTIR)**

217 The FTIR spectra of KYNA, blank NS, physical mixture, and KYNA loaded NS were  
218 recorded on PerkinElmer 100 FTIR using an attenuated total reflectance (ATR) accessory. All  
219 the samples were scanned from 4000-650 cm<sup>-1</sup> at a resolution of 4 cm<sup>-1</sup> and 8 scans/spectrum.  
220 The % crosslinking of nanosponges was also determined with some modifications as reported  
221 earlier (Coma, Sebti, Pardon, Pichavant, & Deschamps, 2003; Ghorpade, Yadav, & Dias, 2016).  
222 The detailed information of method and sample preparation is mentioned in the supplementary  
223 information.

## 224 **2.2.11 X-ray powder diffraction studies (PXRD)**

225 The XRD spectra of KYNA, blank NS and KYNA loaded NS were recorded using Malvern  
226 Panalytical X'Pert diffractometer using Cu K $\alpha$ 1 as a source of radiation. Data was collected over  
227 an angular range from 5 to 45 °2 $\theta$  at a step size of 0.017 °2 $\theta$  and a time per step of 100.33 s.

## 228 **2.2.12 Morphology evaluation of nanosponge and drug complex**

229 The surface morphologies of blank NS, and KYNA loaded NS were evaluated on Field  
230 Emission Scanning Electron Microscope (FE-SEM; ZIESS Supra 40). Approximately, 3-4 drops  
231 of NS suspension were placed on a copper stub and air dried later, sputter coated with gold.  
232 Samples were analyzed at 3 kV accelerating voltage at a working distance of 10 mm.

## 233 **2.2.13 The in-vitro drug release profile**

234 The accurate weight amount of KYNA loaded NS (20 mg) was dispersed in 3 ml of phosphate  
235 buffer pH 7.4 and sealed into a dialysis bag (12,400 MWCO). It was submerged into 30 ml of  
236 phosphate buffer pH 7.4 at  $37 \pm 0.5$  °C with rotation speed of 50 rpm. The aliquots (1 ml) were  
237 withdrawn at different time intervals and replaced with the same amount of fresh phosphate  
238 buffer to maintain sink condition. Later, all the samples were analyzed on HPLC.

## 239 **2.2.14 Evaluation of antioxidant activity**

### 240 **2.2.14.1 TBA Assay**

241 This assay is based on the oxidative decomposition of polyunsaturated fatty acid in acidic  
242 medium to generate malondialdehyde (MDA), which reacts with TBA to form TBA-MDA  
243 adduct (Yen, Chang, & Su, 2003). Two different concentrations (50  $\mu$ M and 100  $\mu$ M) of KYNA  
244 (1 mg/ml stock solution in N-methyl pyrrolidone) and KYNA loaded NS in phosphate buffer pH  
245 7.4 were prepared. 0.1 ml of linoleic acid (1 % w/v) was taken in a test tube, 0.2 ml of sodium  
246 dodecyl sulfate (SDS) (4 % w/v), 1.5 ml of phosphoric acid (1.0 % v/v), 1.0 ml of TBA (0.6 %  
247 w/v), 0.1 ml of water and 0.1 ml of KYNA solution or KYNA loaded NS was added into it. The  
248 mixture was heated at 100 °C for 45 minutes later it was cooled down on an ice bath and mixed  
249 with 1-butanol (4 ml) to extract TBA-MDA adduct. Samples were analyzed by UV-Visible  
250 spectrophotometer (Lambda 25, PerkinElmer, Waltham, USA) at 535 nm and MDA

251 concentration was determined from a calibration curve of a MDA precursor 1,1,3,3-  
252 tetraethoxypropane (TRP), which was recorded under the same experimental conditions. The  
253 antioxidant activity of KYNA was evaluated by determining the reduction in the MDA  
254 generation.

255 The effect of potent oxidant (KMnO<sub>4</sub>) on lipid peroxidation and its inhibition by KYNA or  
256 KYNA loaded NS was also studied using additional 0.1 ml of KMnO<sub>4</sub> (1 mM) solution  
257 following the procedure as mentioned above.

#### 258 **2.2.14.2 DPPH scavenging activity**

259 The DPPH scavenging activity of KYNA loaded NS was studied and compared with the  
260 KYNA solution as demonstrated earlier (Colombo, Figueiró, Dias, Amanda de Fraga Teixeira,  
261 Battastini, & Koester, 2018). Different concentrations of KYNA and KYNA loaded NS (10-100  
262 µM) were prepared. An ethanolic solution of DPPH (0.004 % w/v) was prepared, of which 0.5  
263 ml were mixed with either KYNA solution or KYNA loaded NS (2 ml). Later, the mixture was  
264 incubated for 60 minutes and analyzed by UV-visible spectrophotometer at 525 nm. Ethanol (0.5  
265 ml) was used as control (without drug) and results were compared with L-ascorbic acid as a  
266 positive standard. The percentage of DPPH scavenging activity was calculated using the  
267 following equation.

$$\text{DPPH Inhibition (\%)} = \frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \times 100$$

#### 268 **2.2.14.3 H<sub>2</sub>O<sub>2</sub> Scavenging activity**

269 The H<sub>2</sub>O<sub>2</sub> scavenging activity of KYNA and KYNA loaded NS was evaluated as reported  
270 earlier (Ebadollahinatanzi & Moghadasi, 2016). Briefly, a series of concentrations of KYNA and  
271 KYNA loaded NS (10-100 µM) were prepared. The reaction was carried out between 0.5 ml of

272 KI (1M), 0.5 ml of TCA (0.1% w/v), 0.5 ml of phosphate buffer pH 7.4, 0.5 ml of H<sub>2</sub>O<sub>2</sub> (10 mM)  
273 and KYNA or KYNA loaded NS (0.5 ml) at room temperature for 10 minutes. The phosphate  
274 buffer pH 7.4 (0.5 ml) was added instead of drug solution as a control. The absorbance was  
275 recorded by UV-Vis spectrophotometer at 350 nm.

276 The following equation was used to calculate the percentage of H<sub>2</sub>O<sub>2</sub> scavenging activity.

$$\text{H}_2\text{O}_2 \text{ Scavenging Activity (\%)} = \frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \times 100$$

### 277 **2.2.15 Cell viability studies**

278 SHSY-5Y human neuroblastoma cell lines were purchased from ATCC (Manassas, VA,  
279 USA). The cells were cultured as a monolayer in RPMI 1640 medium accompanied with 10 %  
280 fetal calf serum, 100 U/ml penicillin, and 100 µg/ml streptomycin at 37 °C and maintained under  
281 5 % CO<sub>2</sub> atmosphere. The cell viability of KYNA, KYNA loaded NS and blank NS was  
282 evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.

283 SHSY-5Y cell lines were seeded into a 96-well plate and incubated for 24 hours at 37 °C in a 5  
284 % CO<sub>2</sub> humidified atmosphere. The cells were incubated with increasing concentrations of  
285 KYNA or KYNA loaded NS (1-100 µM) for 24 hours. The Blank NS was also dispersed in 0.9  
286 % NaCl saline solution and treated with the cells as mentioned above. After 24 hours, cell  
287 viability was evaluated using MTT by recording the absorbance at 570 nm according to the  
288 manufacturer's protocol. The cells treated with culture medium alone considered as a control and  
289 the reading obtained from treated cell were expressed as % cell viability.

290 **2.2.16 Stability study of KYNA loaded NS**

291 The in vitro stability of blank NS and KYNA loaded NS in 0.9 % NaCl saline solution was  
292 evaluated. All the samples were incubated at 4 °C for 1 week. The average diameter  
293 and zeta potential of blank NS and KYNA loaded NS were studied at different time intervals.

294 **2.2.17 Statistical analysis**

295 The experiments are expressed as mean  $\pm$  standard deviation (SD). The significance of the  
296 difference was evaluated by one-way ANOVA followed by Bonferroni correction using  
297 GraphPad Prism 5 software (GraphPad Software, USA). A p-value  $< 0.05$  was considered as  
298 statistically significant.

299 **3. Results and discussion**

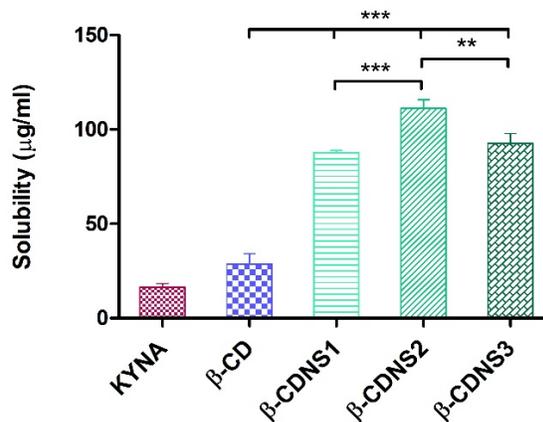
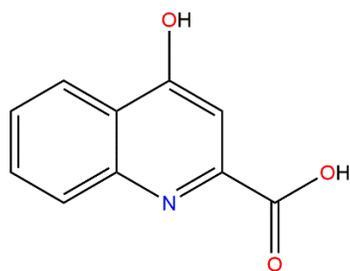
300 **3.1 Physicochemical Characterization of NS and KYNA loaded NS**

301 The presence of a large number of hydroxyl groups of  $\beta$ -CD makes it a suitable candidate for  
302 the crosslinking reaction. The nanosponges were synthesized by the formation of a carbonate  
303 bond between CDI and the hydroxyl groups of  $\beta$ -CD. The swelling degree of the prepared NS  
304 was determined. **A moderate swelling degree was achieved with  $\beta$ -CDNS2 (251 %), compared to**  
305  **$\beta$ -CDNS1 (200 %) and  $\beta$ -CDNS3 (168 %). However, no evident trend was observed.**

306

(A)

(B)



307

308

**Figure 1.**

309 The solubilization of KYNA alone and in the presence of β-CD or different nanosponges was  
 310 also studied as presented in Fig. 1(B). The solubility of KYNA with different nanosponges was  
 311 significantly higher compared to both free KYNA (16.4 µg/ml) and KYNA with β-CD (28.6  
 312 µg/ml). The solubility of KYNA increased to 5.3 folds with β-CDNS1 and 6.77 folds with β-  
 313 CDNS2. However, KYNA solubility further decreased with β-CDNS3 (5.65 folds) because of  
 314 the higher cross-linker concentration that formed more complex nanochannel which hindered the  
 315 drug encapsulation (Torre et al., 2013). **The maximum solubilization of KYNA was achieved**  
 316 **with β-CDNS2 (Fig. S1; Supporting Information), thus β-CDNS2 was selected for drug loading**  
 317 **studies.**

318 Later, drug loading study was carried by taking different KYNA to β-CDNS2 concentrations  
 319 in weight ratios of 1:3, 1:4 and 1:5 w/w. The maximum drug loading of 19.06 % was achieved  
 320 with 1:4 w/w compared to 12.7 % (1:3 w/w) and 19.19 % (1:5 w/w), respectively. A significant  
 321 difference in drug loading was observed in between 1:3 w/w and 1:4 w/w. However, no further

322 change in drug loading was seen in between 1:4 w/w and 1:5 w/w might be due to saturation  
323 solubility of KYNA (Zidan et al., 2018).

324 Depending on the drug loading studies KYNA loaded NS (1:4 w/w) subjected to further  
325 studies. The average particles size and zeta potential of blank nanosponge and KYNA loaded NS  
326 were determined as shown in Table 2.

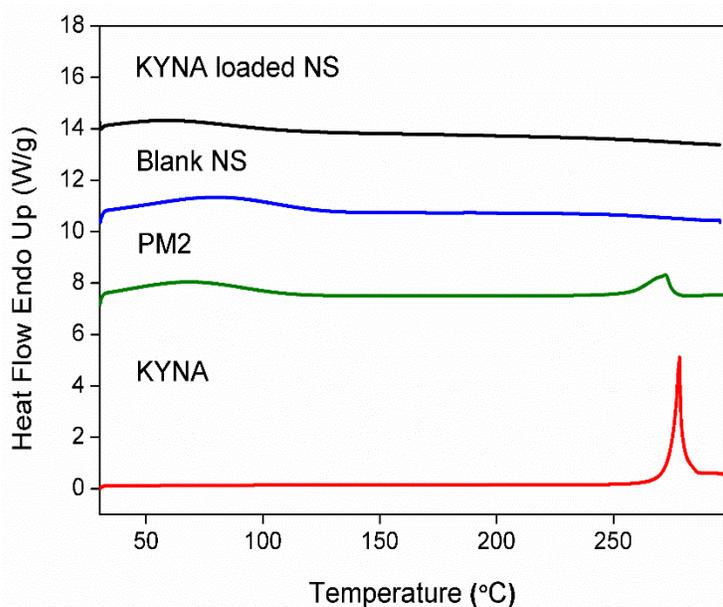
327 **Table 2.**

<b>Properties</b>	<b>Blank NS (<math>\beta</math>-CDNS2)</b>	<b>KYNA loaded NS (KYNA-<math>\beta</math>-CDNS2)</b>
<b>Particle Size (nm)</b>	224.43 $\pm$ 3.72	255.8 $\pm$ 7.88
<b>PDI</b>	0.35 $\pm$ 0.040	0.32 $\pm$ 0.043
<b>Zeta Potential (mV)</b>	-26.3 $\pm$ 1.91	-23 $\pm$ 0.945
<b>Encapsulation Efficiency (%)</b>	-	95.31 %

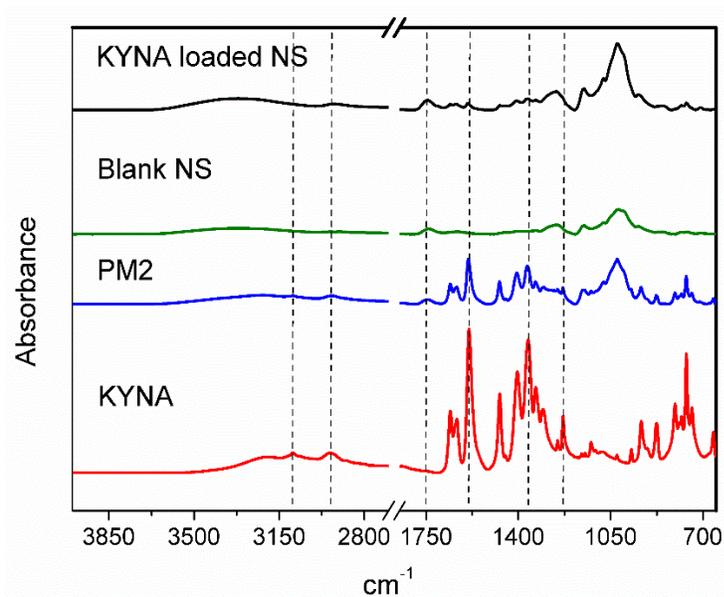
328  
329 The DSC thermogram of KYNA showed an endothermic peak at 277.15 °C that  
330 corresponds to its melting temperature as shown in Fig. 2(I). The nanosponges are stable thus do  
331 not undergo any thermal transition and physical mixture showed endothermic transition similar  
332 to KYNA with lesser intensity because of the possible dilution with NS. It is reported that  
333 encapsulation of drug molecules within the NS leads to the formation of an amorphous system  
334 (Ansari et al., 2011). A similar pattern was observed with KYNA loaded NS which did not  
335 produce any significant endothermic melting because of possible amorphization of KYNA, thus  
336 confirming the encapsulation of KYNA within the nanosponges.

337 Fig. 2(II) shows the FTIR spectra of KYNA, blank NS, physical mixture and KYNA  
338 loaded NS. The FTIR spectrum of KYNA showed strong characteristic peaks at 3095  $\text{cm}^{-1}$  (-N-H  
339 stretching), 2940  $\text{cm}^{-1}$  (-C-H stretching), 1660  $\text{cm}^{-1}$  (-C=C stretching), 1362  $\text{cm}^{-1}$  (-OH bending),  
340 1121  $\text{cm}^{-1}$  (-C-O stretching). The occurrence of carbonate bond peak at 1739  $\text{cm}^{-1}$  in the FTIR  
341 spectrum is the characteristic feature of NS (Lembo et al., 2013).

(I)



(II)



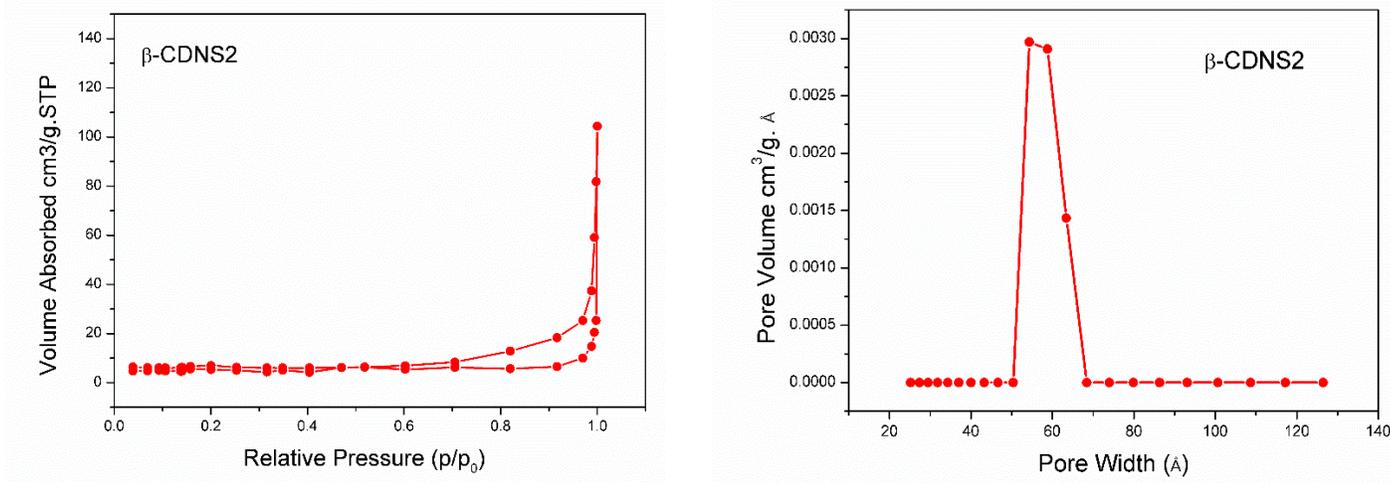


355 suggested that higher crosslinker concentration leads to higher crosslinking of the nanosponges.  
356  $\beta$ -CDNS1 showed lowest crosslinking of 59.49 % compared to  $\beta$ -CDNS2 (79.42 %) and  $\beta$ -  
357 CDNS3 (83.7 %) (Fig. S2-S4; Supporting Information). The % crosslinking values were similar  
358 to those reported earlier for other cyclodextrins nanosponges (Gholibegloo et al., 2019; P. Singh  
359 et al., 2018).

360 The PXRD pattern of KYNA showed sharp and intense peaks at 8.01, 9.90, 12.62, 16.07,  
361 24.79, 28.22, 31.68, and 39.89  $2\theta$  values that indicates crystalline behavior of KYNA as shown  
362 in Fig. 2(III). The PXRD of blank NS did not show any sharp peaks in agreement with its  
363 amorphous nature. The disappearance of characteristic peaks of KYNA in KYNA loaded NSs  
364 indicate a loss in the crystallinity and consequent amorphization of KYNA because of the  
365 encapsulation of KYNA inside the NSs. Such evidence suggested the formation of an inclusion  
366 complex (Darandale & Vavia, 2013; Dora et al., 2016). However, in case of physical mixture  
367 characteristic peaks of KYNA were clearly visible with lesser intensity, suggesting no change in  
368 the property of KYNA, which remained in the crystalline form. **The PXRD pattern of  $\beta$ -CD was  
369 also determined and the presence of sharp peaks clearly indicated crystalline structure of it in  
370 contrast to the NSs (Fig. S5; Supporting Information).**

(A)

(B)



371

**Figure 3.**

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Fig. 2(IV) shows the FE-SEM images of blank NS and KYNA loaded NS in which both showed small spherical shape particles of diameter less than 200 nm with appropriate size distribution. Furthermore, fig. 3 presents the N<sub>2</sub> adsorption-desorption isotherm and pore distribution curve of blank NS. As shown in fig. 3(A), a type IV isotherm with hysteresis loop was observed for NS. The presence of hysteresis loop could be attributed to the mesoporous structure of the NS. Moreover, fig. 3(B) displayed the pore distribution of NS which further suggest the mesoporosity of NS as the pore diameter was 50-70 Å (5-7 nm). The surface area of blank NS was less than 1 m<sup>2</sup>/g. It is also reported that the surface area and porosity can be tailored depending upon the cross-linker concentration. The results obtained were in agreement with those previously reported in the literature for other cyclodextrin nanosponges (Gholibegloo et al., 2019; Pushpalatha, Selvamuthukumar, & Kilimozhi, 2018). Moreover, BET analysis of KYNA loaded NS showed a type IV isotherm with hysteresis loop similar to blank NS. However, porosity of KYNA loaded NS (35-45 Å) was reduced which might be due to encapsulation of KYNA within the NSs (Fig. S6; Supporting Information).

386 The in-vitro release profile of KYNA loaded NS was studied in phosphate buffer pH 7.4  
387 (Fig. 4). A slow and uniform drug release profile of KYNA was observed without any initial  
388 burst effect. The slower drug release profile can be attributed to the presence of KYNA inside  
389 the cavities of nanosponges. Moreover, the absence of an initial burst release further confirmed  
390 that the drug was not partially encapsulated or adsorbed on the surface of nanosponges. The  
391 different drug release kinetics models (such as zero order, first order, Higuchi-Connors, Hixen-  
392 Crowell, and Korsmeyer-Peppas) were applied and the release profile of KYNA was best fitted  
393 to Higuchi-Connors release kinetic model ( $R^2 = 0.995$ ) indicating that drug release was carried  
394 out by diffusion from the nanosponges (Machín, Isasi, & Vélaz, 2012; Zainuddin, Zaheer,  
395 Sangshetti, & Momin, 2017).

396 Plain KYNA was found to be dissolved very rapidly and uncontrolled release was  
397 observed. Plain KYNA showed that equilibrium was achieved within a few minutes during in-  
398 vitro release profile. **Moreover, rapid drug release is associated with dose related toxic effects.**  
399 **Therefore, controlled drug delivery, which can be achieved by encapsulation of drug within NSs,**  
400 **is required (Wen, Jung, & Li, 2015).**

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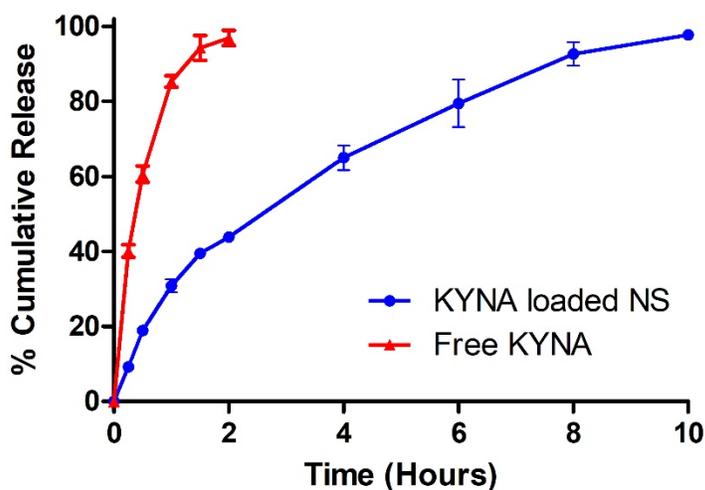
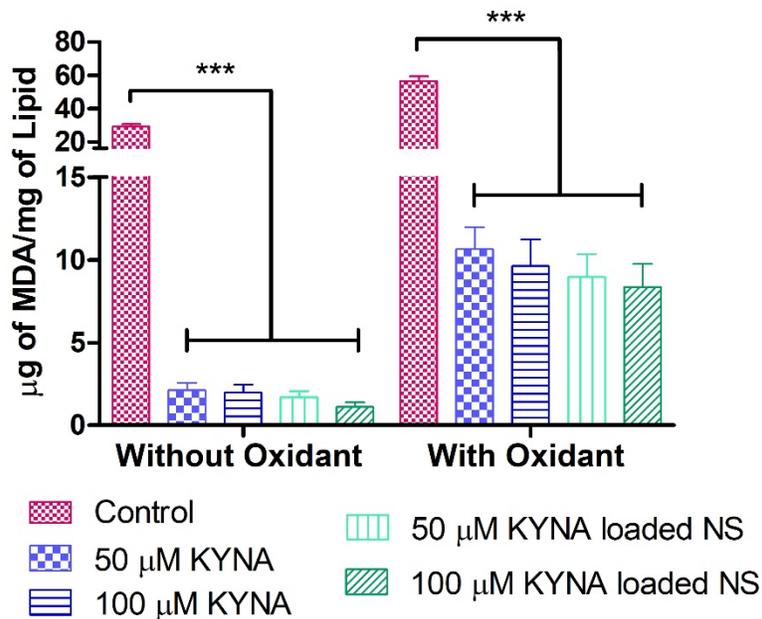


Figure 4.

### 3.2 Evaluation of antioxidant activity

Phenolic compounds exhibit antioxidant activity because of their ability to react with free radicals at a faster rate. The antioxidant activity of KYNA might be because of the presence of an aromatic hydroxyl group that readily provides protons for the reaction with free radicals. Moreover, the presence of nitrogen in the aromatic ring or carboxylic acid acting as an electron withdrawing group helps to stabilize the free radical produced by donation of the proton (Zhuravlev, Zakharov, Shchegolev, & Savvateeva-Popova, 2016).

The generation of MDA because of the degradation of fatty acid is a common indicator for determining the degree of lipid peroxidation. MDA reacts with TBA to produce a pink TBA-MDA adduct. The production of MDA is shown in fig. 5 and it was observed that KYNA loaded NS produced a lesser amount of MDA ( $P < 0.001$ ) compared to free KYNA. A decreased production of MDA might be because of the inhibiting and scavenging effect of KYNA on ROS produced during oxidation process.



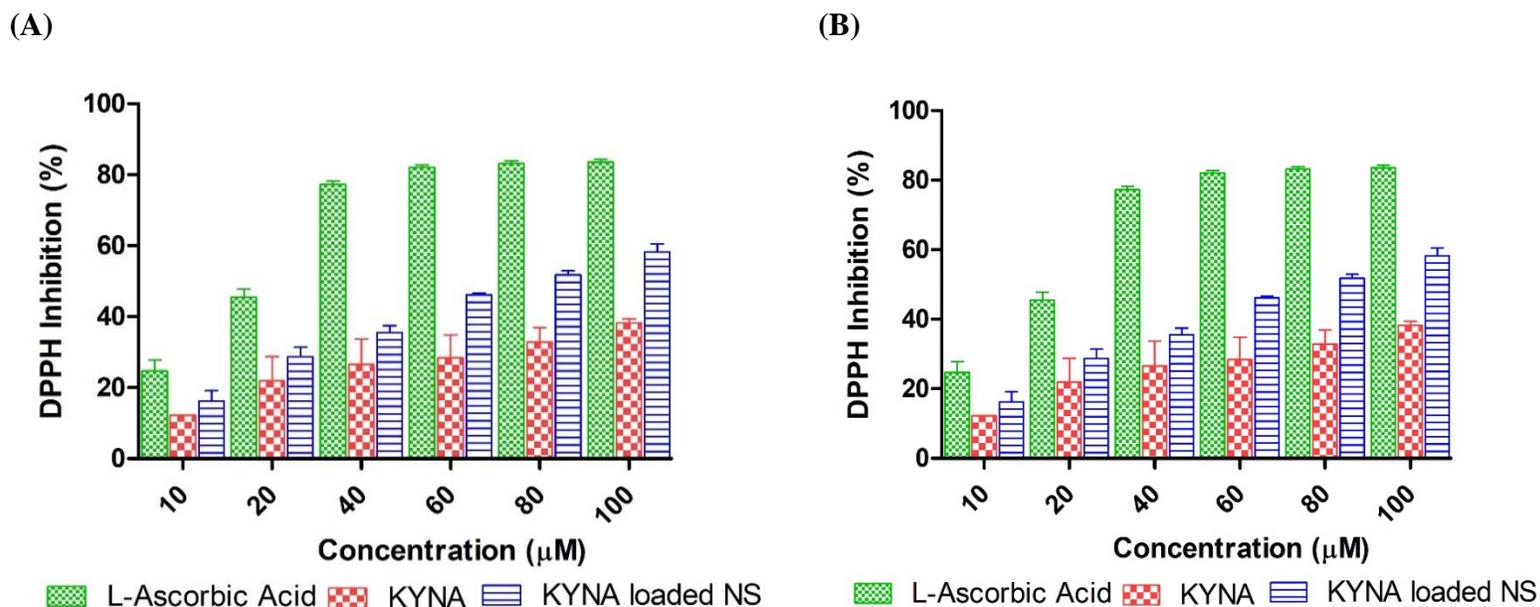
417

418

**Figure 5.**

419 The effect of KYNA on MDA generation in the presence of the strong oxidizing agent  
 420 (i.e.  $\text{KMnO}_4$ ) was also studied and it was observed that oxidizing agent generated a large amount  
 421 of MDA because of the rapid and higher oxidation of fatty acid. However, free KYNA and  
 422 KYNA loaded NS significantly decreased the production of MDA even in the presence of the  
 423 oxidizing agent, demonstrating its antioxidant potential. Moreover, KYNA loaded NS was more  
 424 effective compared to free KYNA, this might be because of the higher solubilization of KYNA  
 425 with NS.

426 The DPPH assay is based on the reduction of its absorbance at 517 nm because of the  
 427 acceptance of proton. Fig. 6(A) illustrates the DPPH inhibition effect of KYNA at different  
 428 concentrations. It was observed that the DPPH inhibition effect of KYNA was increased in a  
 429 dose-dependent manner. Moreover, the KYNA loaded NS showed a higher reduction ( $P < 0.001$ )  
 430 in the DPPH concentration compared to free KYNA at higher concentration.



432

**Figure 6.**

433 The higher antioxidant activity of KYNA loaded NS can be attributed to enhanced  
 434 solubilization of KYNA with nanosponge thus readily provided protons to DPPH. The results  
 435 obtained are in agreement with those reported in the literature. Sundararajan et al. demonstrated  
 436 that chrysin loaded NS produced more than 2 fold DPPH inhibition compared to free chrysin.  
 437 (Sundararajan, Thomas, Venkadeswaran, Jeganathan, & Geraldine, 2017).

438 Moreover, the antioxidant property of KYNA was further evaluated by hydrogen  
 439 peroxide scavenging activity. The hydrogen peroxide is a very reactive compound that generates  
 440 hydroxyl radicals. The hydroxyl radicals are the most common reactive oxygen species (ROS)  
 441 that can cause cell death because of the oxidative degradation of biomolecules such as DNA,  
 442 RNA, and proteins. Thus, to demonstrate the antioxidant potential of KYNA, its H<sub>2</sub>O<sub>2</sub>

443 scavenging effect was studied. Fig. 6(B), shows the hydrogen peroxide scavenging effect of  
444 KYNA, KYNA loaded NS and L-ascorbic acid.

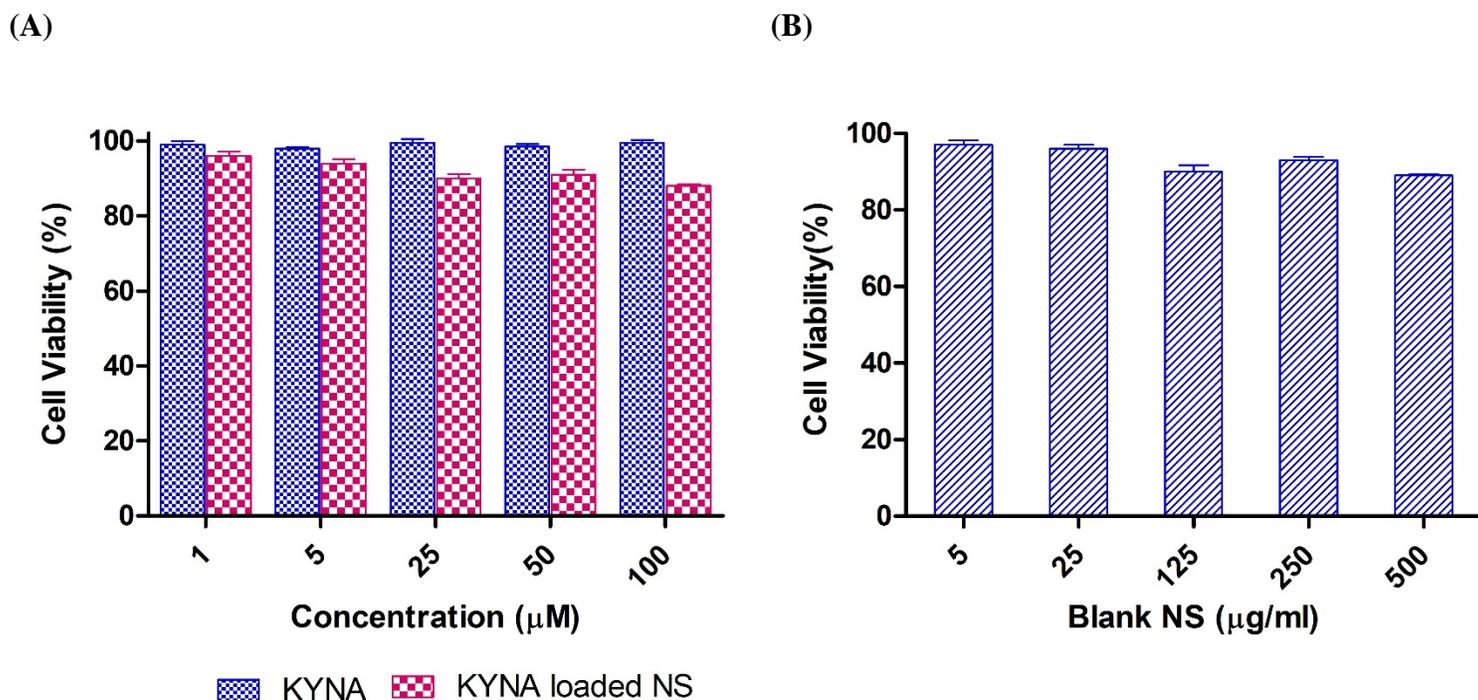
445 All the samples showed concentration-dependent H<sub>2</sub>O<sub>2</sub> inhibition. The KYNA loaded NS  
446 showed better H<sub>2</sub>O<sub>2</sub> inhibition compared to free KYNA ranging from 17 % to 78 % at the tested  
447 concentration range. It might be possible that KYNA loaded NS reacts rapidly with peroxide  
448 intermediates to exhibit better H<sub>2</sub>O<sub>2</sub> inhibition due to high KYNA solubilization. It is also  
449 evident that the standard solution of L-ascorbic acid is more effective compared to KYNA and  
450 KYNA loaded NS. The above-mentioned tests were also carried out on blank NS to determine  
451 the antioxidant activity of NS alone. However, no significant results were obtained as they do not  
452 exhibit antioxidant activity (Fig. S7; Supporting Information).

453 The results observed are in agreement with those reported in the literature. Lugo-Huitrón  
454 et al. demonstrated the scavenging capacity of KYNA compared to known reference compounds  
455 and suggested that KYNA acts as a potent inhibitor of ROS (Lugo-Huitrón et al., 2011). In  
456 another study, Genestet and co-workers demonstrated the superoxide scavenging activity of  
457 KYNA. They demonstrated that KYNA showed better antioxidant activity compared to other  
458 tryptophan metabolites (Genestet et al., 2014).

### 459 **3.3 Cell Viability**

460 The biocompatibility of SHSY-5Y cell lines with blank NS was determined using a MTT  
461 assay at the concentration of 5-500 µg/ml indicated that blank nanosponge does not produce any  
462 significant toxic effect even at higher concentration thus confirming the safety of our nanocarrier  
463 (fig. 7). The minimum cell viability was 89 % at the concentration of 500 µg/ml after 24 hours.  
464 The results obtained are in agreement with previously reported data indicating that NS does not

465 produce any significant toxicity on different cell lines (Gholibegloo et al., 2019; Mognetti et al.,  
466 2012).



467 **Figure 7.**

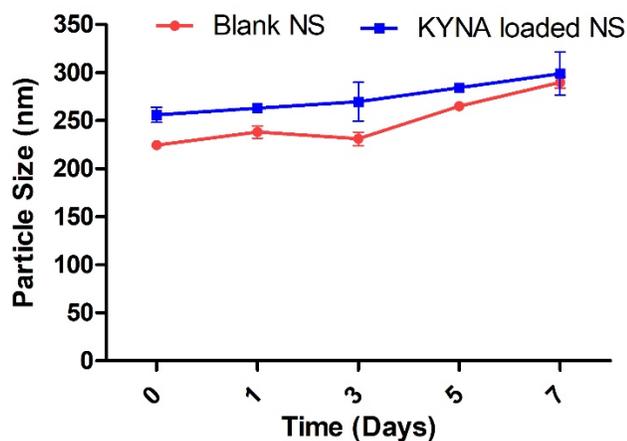
468 The effect of KYNA and KYNA loaded NS was also evaluated on the survival of SHSY-5Y  
469 cell lines. Both KYNA and KYNA loaded NS do not produce any significant toxic effects on cell  
470 lines at the concentration of 1-100 μM. Furthermore, 90 % or more cell survival was observed at  
471 all the tested concentrations with KYNA and KYNA loaded NS. Klein et al. earlier demonstrated  
472 the effect of KYNA and its derivatives on the survival of SHSY-5Y cell lines which suggested  
473 that KYNA promotes higher cell survival of SHSY-5Y cell lines compared to the structurally  
474 modified derivative of KYNA (Klein et al., 2013). Researchers have also tried to prepare water-  
475 soluble salts of KYNA to improve the performance in the neurological applications. However,  
476 selectivity and stability of salts of KYNA are always the major concerns. Dalpiaz et al., prepared

477 6-bromo-ascorbic acid derivative of KYNA and reported that it showed the affinity to the  
478 vitamin C transporters, however, does not show any permeation because of the enzymatic  
479 hydrolysis (Dalpiaz et al., 2005). In another study Baron et al., prepared a KYNA analog and  
480 showed that higher concentration of KYNA analog was needed to produce glutamate inhibition  
481 compared to native KYNA (Baron et al., 1992). It suggested that native KYNA shows better  
482 neurological effects compared to its derivatives, therefore, NSs can be employed as a delivery  
483 vehicle for KYNA without any undesirable effects.

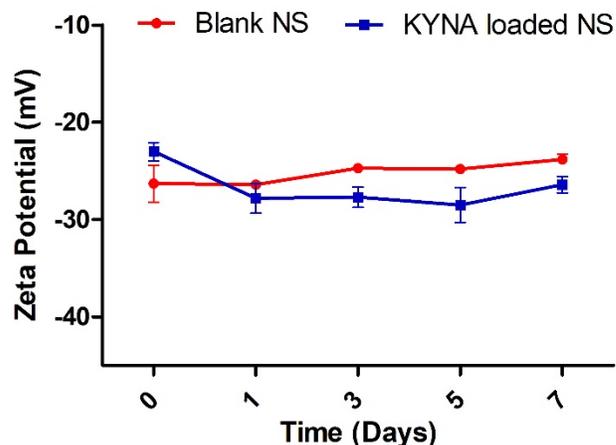
### 484 3.4 Stability study

485 The stability of blank NS and KYNA loaded NS was evaluated at 4 °C for a week.  
486 particle size and zeta potential were studied to assess the stability of the blank NS and KYNA  
487 loaded NS. The change in the particle size and zeta potential is demonstrated in fig. 8(A) and  
488 8(B), respectively.

(A)



(B)



489

Figure 8.

490 It was observed that the particle size of blank NS was increased from 224.4 nm to 289.7  
491 nm compared to KYNA loaded NS that showed a slight increase from 255.8 nm to 298.8 nm.  
492 Moreover, the zeta potential was also observed in the desired range without any significant  
493 change. Argenziano et al. earlier demonstrated that particle size of drug loaded NSs do not show  
494 any significant increment on storage for 24 hours (Argenziano et al., 2018). However, another  
495 study revealed that other hyper cross-linked polymer of cyclodextrin showed more than 2-fold  
496 increment in the particles size on storage within a week (Gref et al., 2006). On the basis of the  
497 above results, it is clear that these NSs provide better protection from aggregation and remained  
498 stable for the period of a week at 4 °C

#### 499 **4. Conclusion**

500 In the present study, we demonstrated the synthesis of KYNA loaded NS and it was  
501 observed that high solubilization and drug loading of KYNA was achieved with  $\beta$ -CDNS2. The  
502 higher solubilization of KYNA was obtained because of the encapsulation of KYNA in the  
503 cyclodextrin cavities and porous matrix of NS. The KYNA loaded NS was characterized by  
504 FTIR, DSC, and PXRD which confirmed the formation of an inclusion complex of KYNA with  
505 NS. The antioxidant potential of KYNA and KYNA loaded NS was studied which further  
506 confirmed that KYNA loaded NS shows better antioxidant activity compared to free KYNA  
507 which can be attributed to the change in the physiochemical property and higher solubilization of  
508 KYNA with NS. The cytotoxicity of KYNA and KYNA loaded NS was also evaluated on  
509 SHSY-5Y cell lines which demonstrated that KYNA alone and in the presence of NS does not  
510 produce any significant toxic effect. Moreover, nanosponge alone was found to be non-toxic.  
511 Thus, the above study demonstrated that cyclodextrin nanosponge acts as a promising carrier for

512 the delivery of KYNA and can possibly be employed in biological systems for its antioxidant  
513 potential.

514 **Conflict of interest**

515 The authors declare no conflict of interest.

516 **Acknowledgments**

517 We would like to thank the University of Turin (ex-60 %) for providing the funds and Roquette  
518 Italia for their support.

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## Figure Captions

**Fig 1.** (A) Structure of KYNA. (B) Solubilization efficiency of KYNA,  $\beta$ -CD and different nanosponges. All the values present data in triplicate (mean  $\pm$  SD); \* indicates  $P < 0.05$  and \*\*\* indicates  $P < 0.0001$ .

**Fig 2.** (I) DSC thermograms of KYNA, physical mixture, blank NS and KYNA loaded NS. (II) FTIR spectra of KYNA, physical mixture, blank NS and KYNA loaded NS. (III) PXRD pattern of KYNA, physical mixture, blank NS and KYNA loaded NS. (IV) FE-SEM images of blank NS (A), and KYNA loaded NS (B) (scale bar = 200 nm).

**Fig 3.**  $N_2$  absorption-desorption (A) and porosity curve of  $\beta$ -CDNS2 (B).

**Fig 4.** In-vitro release kinetics of KYNA from NS.

**Fig 5.** The inhibition of lipid peroxidation by KYNA loaded NS in the absence of oxidizing agent (left) and in the presence of the oxidizing agent (right). Statistical significance \*\*\* indicates  $P < 0.0001$ .

**Fig 6.** The Percentage DPPH inhibition (A) and  $H_2O_2$  scavenging activity (B) by KYNA, KYNA loaded NS and L-ascorbic acid.

**Fig 7.** The evaluation of in-vitro cytotoxicity study of KYNA and KYNA loaded NS (A) and blank NS (B) on SHSY-5Y cell lines after 24 hours.

**Fig 8.** The in-vitro stability study of blank NS and KYNA loaded NS to determine particles size (A) and zeta potential (B). All the values present in terms of mean  $\pm$  SD ( $n = 3$ ).

540

### **Table Captions**

541 **Table 1.** Quantities of chemicals used for preparing nanosponges.

542 **Table 2.** Physicochemical characteristics of Blank and KYNA loaded NS.

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545 **References**

546 Ansari, K. A., Vavia, P. R., Trotta, F., & Cavalli, R. (2011). Cyclodextrin-Based Nanosponges  
547 for Delivery of Resveratrol: In Vitro Characterisation, Stability, Cytotoxicity and Permeation  
548 Study. *AAPS PharmSciTech*, *12*(1), 279–286. <https://doi.org/10.1208/s12249-011-9584-3>.

549 Argenziano, M., Lombardi, C., Ferrara, B., Trotta, F., Caldera, F., Blangetti, M., Cavalli, R.  
550 (2018). Glutathione/pH-responsive nanosponges enhance strigolactone delivery to prostate  
551 cancer cells. *Oncotarget*, *9*(88), 35813–35829. <https://doi.org/10.18632/oncotarget.26287>.

552 Baron, B. M., Harrison, B. L., McDonald, I. A., Meldrum, B. S., Palfreyman, M. G., Salituro, F.  
553 G., White, H. S. (1992). Potent indole- and quinoline-containing N-methyl-D-aspartate  
554 antagonists acting at the strychnine-insensitive glycine binding site. *J Pharmacol Exp Ther*,  
555 *262*(3), 947–956.

556 Berto, S., Bruzzoniti, M. C., Cavalli, R., Perrachon, D., Prenesti, E., Sarzanini, C., Tumiatti, W.  
557 (2007). Synthesis of new ionic  $\beta$ -cyclodextrin polymers and characterization of their heavy  
558 metals retention. *Journal of Inclusion Phenomena and Macrocyclic Chemistry*, *57*(1–4), 631–  
559 636. <https://doi.org/10.1007/s10847-006-9273-0>.

560 Carpenedo, R., Pittaluga, A., Cozzi, A., Attucci, S., Galli, A., Raiteri, M., & Moroni, F. (2001).  
561 Presynaptic kynurenate-sensitive receptors inhibit glutamate release. *European Journal of*  
562 *Neuroscience*, *13*, 2141–2147. <https://doi.org/10.1046/j.0953-816x.2001.01592.x>.

563 Challa, R., Ahuja, A., Ali, J., & Khar, R. K. (2006). Cyclodextrins in drug delivery: An updated  
564 review. *AAPS PharmSciTech*, *6*(2), E329–E357. <https://doi.org/10.1208/pt060243>.

565 Colombo, M., Figueiró, F., Dias, Amanda de Fraga Teixeira, H. F., Battastini, A. M. O., &

566 Koester, L. S. (2018). Kaempferol-loaded mucoadhesive nanoemulsion for intranasal  
567 administration reduces glioma growth in vitro. *International Journal of Pharmaceutics*, 543(1–  
568 2), 214–223. <https://doi.org/10.1016/j.ijpharm.2018.03.055>.

569 Coma, V., Sebti, I., Pardon, P., Pichavant, F. H., & Deschamps, A. (2003). Film properties from  
570 crosslinking of cellulosic derivatives with a polyfunctional carboxylic acid. *Carbohydrate*  
571 *Polymers*, 51(3), 265–271. [https://doi.org/10.1016/S0144-8617\(02\)00191-1](https://doi.org/10.1016/S0144-8617(02)00191-1).

572 Dalpiaz, A., Pavan, B., Vertuani, S., Vitali, F., Scaglianti, M., Bortolotti, F., Manfredini, S.  
573 (2005). Ascorbic and 6-Br-ascorbic acid conjugates as a tool to increase the therapeutic effects of  
574 potentially central active drugs. *European Journal of Pharmaceutical Sciences*, 24(4), 259–269.  
575 <https://doi.org/10.1016/j.ejps.2004.10.014>.

576 Darandale, S. S., & Vavia, P. R. (2013). Cyclodextrin-based nanosponges of curcumin:  
577 Formulation and physicochemical characterization. *Journal of Inclusion Phenomena and*  
578 *Macrocyclic Chemistry*, 75(3–4), 315–322. <https://doi.org/10.1007/s10847-012-0186-9>.

579 Di Nardo, G., Roggero, C., Campolongo, S., Valetti, F., Trotta, F., & Gilardi, G. (2009).  
580 Catalytic properties of catechol 1,2-dioxygenase from *Acinetobacter radioresistens* S13  
581 immobilized on nanosponges. *Dalton Transactions*, 2(33), 6507–6512.  
582 <https://doi.org/10.1039/b903105g>.

583 Dora, C. P., Trotta, F., Kushwah, V., Devasari, N., Singh, C., Suresh, S., & Jain, S. (2016).  
584 Potential of erlotinib cyclodextrin nanosponge complex to enhance solubility, dissolution rate, in  
585 vitro cytotoxicity and oral bioavailability. *Carbohydrate Polymers*, 137, 339–349.  
586 <https://doi.org/10.1016/j.carbpol.2015.10.080>.

587 Ebadollahinatanz, A., & Moghadasi, H. (2016). Hydrogen peroxide scavenging activity of two

588 different infusions made from black tea. *Toxicology Letters*, 258, S192–S193.  
589 <https://doi.org/10.1016/j.toxlet.2016.06.1706>.

590 Genestet, C., Le Gouellec, A., Chaker, H., Polack, B., Guery, B., Toussaint, B., & Stasia, M. J.  
591 (2014). Scavenging of reactive oxygen species by tryptophan metabolites helps *Pseudomonas*  
592 *aeruginosa* escape neutrophil killing. *Free Radical Biology and Medicine*, 73, 400–410.  
593 <https://doi.org/10.1016/j.freeradbiomed.2014.06.003>.

594 Gholibegloo, E., Mortezaazadeh, T., Salehian, F., Ramazani, A., Amanlou, M., & Khoobi, M.  
595 (2019). Improved curcumin loading, release, solubility and toxicity by tuning the molar ratio of  
596 cross-linker to  $\beta$ -cyclodextrin. *Carbohydrate Polymers*, (213), 70–78.  
597 <https://doi.org/10.1016/j.carbpol.2019.02.075>.

598 Ghorpade, V. S., Yadav, A. V., & Dias, R. J. (2016). Citric acid crosslinked  
599 cyclodextrin/hydroxypropylmethylcellulose hydrogel films for hydrophobic drug delivery.  
600 *International Journal of Biological Macromolecules*, 93, 75–86.  
601 <https://doi.org/10.1016/j.ijbiomac.2016.08.072>.

602 Gref, R., Amiel, C., Molinard, K., Daoud-Mahammed, S., Sébille, B., Gillet, B., Couvreur, P.  
603 (2006). New self-assembled nanogels based on host-guest interactions: Characterization and  
604 drug loading. *Journal of Controlled Release*, 111(3), 316–324.  
605 <https://doi.org/10.1016/j.jconrel.2005.12.025>.

606 Hornok, V., Bujdosó, T., Toldi, J., Nagy, K., Demeter, I., Fazakas, C., Dékány, I. (2012).  
607 Preparation and properties of nanoscale containers for biomedical application in drug delivery:  
608 Preliminary studies with kynurenic acid. *Journal of Neural Transmission*, 119(2), 115–121.  
609 <https://doi.org/10.1007/s00702-011-0726-2>.

610 Klein, C., Patte-mensah, C., Taleb, O., Bourguignon, J., Schmitt, M., Bihel, F., Mensah-nyagan,  
611 A. G. (2013). The neuroprotector kynurenic acid increases neuronal cell survival through  
612 neprilysin induction. *Neuropharmacology*, 70, 254–260.  
613 <https://doi.org/10.1016/j.neuropharm.2013.02.006>.

614 László, V., & Beal, M. F. (1991). Comparative behavioral and pharmacological studies with  
615 centrally administered kynurenine and kynurenic acid in rats. *European Journal of*  
616 *Pharmacology*, 196(3), 239–246. [https://doi.org/10.1016/0014-2999\(91\)90436-T](https://doi.org/10.1016/0014-2999(91)90436-T).

617 Lembo, D., Swaminathan, S., Donalisio, M., Civra, A., Pastero, L., Aquilano, D., Cavalli, R.  
618 (2013). Encapsulation of Acyclovir in new carboxylated cyclodextrin-based nanosponges  
619 improves the agent's antiviral efficacy. *International Journal of Pharmaceutics*, 443(1–2), 262–  
620 272. <https://doi.org/10.1016/j.ijpharm.2012.12.031>.

621 Lesniak, W. G., Jyoti, A., Mishra, M. K., Louissaint, N., Romero, R., Chugani, D. C., Kannan,  
622 R. M. (2013). Concurrent quantification of tryptophan and its major metabolites. *Anal Biochem*,  
623 443(2), 222–231. <https://doi.org/10.1016/j.ab.2013.09.001>.Concurrent.

624 López, T., Ortiz, E., Gómez, E., la Cruz, Verónica Pérez-de Carrillo-Mora, P., & Novaro, O.  
625 (2014). Preparation and Characterization of Kynurenic Acid Occluded in Sol-Gel Silica and  
626 SBA-15 Silica as Release Reservoirs. *Journal of Nanomaterials*, 2014(Article ID 507178), 1–9.  
627 <https://doi.org/10.1155/2014/507178>.

628 Lugo-Huitrón, R., Blanco-Ayala, T., Ugalde-Muñiz, P., Carrillo-Mora, P., Pedraza-Chaverri, J.,  
629 Silva-Adaya, D., La Cruz, V. P. (2011). On the antioxidant properties of kynurenic acid: Free  
630 radical scavenging activity and inhibition of oxidative stress. *Neurotoxicology and Teratology*,  
631 33(5), 538–547. <https://doi.org/10.1016/j.ntt.2011.07.002>.

632 Machín, R., Isasi, J. R., & Vélaz, I. (2012).  $\beta$ -Cyclodextrin hydrogels as potential drug delivery  
633 systems. *Carbohydrate Polymers*, 87(3), 2024–2030.  
634 <https://doi.org/10.1016/j.carbpol.2011.10.024>.

635 Mognetti, B., Barberis, A., Marino, S., Berta, G., De Francia, S., Trotta, F., & Cavalli, R. (2012).  
636 In vitro enhancement of anticancer activity of paclitaxel by a Cremophor free cyclodextrin-based  
637 nanosponge formulation. *Journal of Inclusion Phenomena and Macrocyclic Chemistry*, 74(1–4),  
638 201–210. <https://doi.org/10.1007/s10847-011-0101-9>.

639 Moroni, F., Russi, P., Lombardi, G., Beni, M., & Carlh, V. (1988). Presence of Kynurenic Acid  
640 in the Mammalian Brain. *Journal of Neurochemistry*, 51, 177–180.  
641 <https://doi.org/10.1111/j.1471-4159.1988.tb04852.x>.

642 Pushpalatha, R., Selvamuthukumar, S., & Kilimozhi, D. (2018). Cross-linked, cyclodextrin-  
643 based nanosponges for curcumin delivery -Physicochemical characterization, drug release,  
644 stability and cytotoxicity. *Journal of Drug Delivery Science and Technology*, 45, 45–53.  
645 <https://doi.org/10.1016/j.jddst.2018.03.004>.

646 Schwarcz, R., Bruno, J. P., Muchowski, P. P., & Wu, H.-Q. (2013). Kynurenines in the  
647 Mammalian Brain: When Physiology Meets Pathology. *Nature Review Neuroscience*, 13(7),  
648 465–477. <https://doi.org/10.1038/nrn3257.KYNURENINES>.

649 Shityakov, S., Salmas, R. E., Durdagi, S., Salvador, E., Pápai, K., Yáñez-Gascón, M. J.,  
650 Broscheit, J.-A. (2016). Characterization, in Vivo Evaluation, and Molecular Modeling of  
651 Different Propofol-Cyclodextrin Complexes To Assess Their Drug Delivery Potential at the  
652 Blood-Brain Barrier Level. *J. Chem. Inf. Model.*, 56(10), 1914–1922.  
653 <https://doi.org/10.1021/acs.jcim.6b00215>.

654 Singh, A., Worku, Z. A., & Mooter, G. Van Den. (2011). Oral formulation strategies to improve  
655 solubility of poorly water-soluble drugs Oral formulation strategies to improve solubility of  
656 poorly water-soluble drugs. *Expert Opinion on Drug Delivery*, 8(10), 1361–1378.  
657 <https://doi.org/10.1517/17425247.2011.606808>.

658 Singh, P., Ren, X., Guo, T., Wu, L., Shakya, S., He, Y., Zhang, J. (2018). Biofunctionalization  
659 of  $\beta$ -cyclodextrin nanosponges using cholesterol. *Carbohydrate Polymers*, 190, 23–30.  
660 <https://doi.org/10.1016/j.carbpol.2018.02.044> CARP.

661 Stone, T. W. (2001). Endogenous neurotoxins from tryptophan. *Toxicol*, 39(1), 61–73.  
662 [https://doi.org/10.1016/S0041-0101\(00\)00156-2](https://doi.org/10.1016/S0041-0101(00)00156-2).

663 Stone, T. W., & Connick, J. H. (1985). Quinolinic acid and other kynurenines in the central  
664 nervous system. *Neuroscience*, 15(3), 597–617. [https://doi.org/10.1016/0306-4522\(85\)90063-6](https://doi.org/10.1016/0306-4522(85)90063-6).

665 Sundararajan, M., Thomas, P. A., Venkadeswaran, K., Jeganathan, K., & Geraldine, P. (2017).  
666 Synthesis and Characterization of Chrysin-Loaded  $\beta$ -Cyclodextrin-Based Nanosponges to  
667 Enhance In-Vitro Solubility, Photostability, Drug Release, Antioxidant Effects and Antitumorous  
668 Efficacy. *Journal of Nanoscience and Nanotechnology*, 17(12), 8742–8751.  
669 <https://doi.org/10.1166/jnn.2017.13911>.

670 Swaminathan, S., Pastero, L., Serpe, L., Trotta, F., Vavia, P., Aquilano, D., Cavalli, R. (2010).  
671 Cyclodextrin-based nanosponges encapsulating camptothecin: Physicochemical characterization,  
672 stability and cytotoxicity. *European Journal of Pharmaceutics and Biopharmaceutics*, 74(2),  
673 193–201. <https://doi.org/10.1016/j.ejpb.2009.11.003>.

674 Torne, S., Darandale, S., Vavia, P., Trotta, F., & Cavalli, R. (2013). Cyclodextrin-based  
675 nanosponges: effective nanocarrier for tamoxifen delivery. *Pharmaceutical Development and*

676 *Technology*, 18(3), 619–625. <https://doi.org/10.3109/10837450.2011.649855>.

677 Trotta, F., Caldera, F., Dianzani, C., Argenziano, M., Barrera, G., & Cavalli, R. (2016).  
678 Glutathione Bioresponsive Cyclodextrin Nanosponges. *ChemPlusChem*, 81(5), 439–443.  
679 <https://doi.org/10.1002/cplu.201500531>.

680 Trotta, F., Dianzani, C., Caldera, F., Moggetti, B., & Cavalli, R. (2014). The application of  
681 nanosponges to cancer drug delivery. *Expert Opinion on Drug Delivery*, 11(6), 931–941.  
682 <https://doi.org/10.1517/17425247.2014.911729>.

683 Trotta, F., Zanetti, M., & Cavalli, R. (2012). Cyclodextrin-based nanosponges as drug carriers.  
684 *Beilstein Journal of Organic Chemistry*, 8, 2091–2099. <https://doi.org/10.3762/bjoc.8.235>.

685 Turski, M. P., Turska, M., Kocki, T., Turski, W. A., & Paluszkiewicz, P. (2015). Kynurenic Acid  
686 Content in Selected Culinary Herbs and Spices. *Journal of Chemistry*, 2015, 1–6.  
687 <https://doi.org/10.1155/2015/617571>.

688 Turski, M. P., Turska, M., Zgrajka, W., Kuc, D., & Turski, W. A. (2009). Presence of kynurenic  
689 acid in food and honeybee products. *Amino Acids*, 36(1), 75–80. [https://doi.org/10.1007/s00726-](https://doi.org/10.1007/s00726-008-0031-z)  
690 008-0031-z.

691 Varga, N., Csapó, E., Majláth, Z., Ilisz, I., Krizbai, I. A., Wilhelm, I., Dékány, I. (2016).  
692 Targeting of the kynurenic acid across the blood-brain barrier by core-shell nanoparticles.  
693 *European Journal of Pharmaceutical Sciences*, 86, 67–74.  
694 <https://doi.org/10.1016/j.ejps.2016.02.012>.

695 Vécsei, L., Szalárdy, L., Fülöp, F., & Toldi, J. (2013). Kynurenines in the CNS: recent advances  
696 and new questions. *Nature Reviews Drug Discovery*, 12(1), 64–82.

697 <https://doi.org/10.1038/nrd3793>.

698 Venuti, V., Rossi, B., Mele, A., Melone, L., Punta, C., Majolino, D., Trotta, F. (2017). Tuning  
699 structural parameters for the optimization of drug delivery performance of cyclodextrin-based  
700 nanosponges. *Expert Opinion on Drug Delivery*, 14(3), 331–340.  
701 <https://doi.org/10.1080/17425247.2016.1215301>.

702 Wajs, E., Caldera, F., Trotta, F., & Fragoso, A. (2013). Peroxidase-encapsulated cyclodextrin  
703 nanosponge immunoconjugates as a signal enhancement tool in optical and electrochemical  
704 assays. *Analyst*, 139(2), 375–380. <https://doi.org/10.1039/c3an01643a>.

705 Wen, H., Jung, H., & Li, X. (2015). Drug Delivery Approaches in Addressing Clinical  
706 Pharmacology-Related Issues: Opportunities and Challenges. *The AAPS Journal*, 17(6), 1327–  
707 1340. <https://doi.org/10.1208/s12248-015-9814-9>.

708 Woldum, H. S., Larsen, K. L., & Madsen, F. (2008). Cyclodextrin Controlled Release of Poorly  
709 Water-Soluble Drugs from Hydrogels. *Drug Delivery*, 15(1), 69–80.  
710 <https://doi.org/10.1080/10717540701829267>.

711 Yen, G. C., Chang, Y. C., & Su, S. W. (2003). Antioxidant activity and active compounds of rice  
712 koji fermented with *Aspergillus candidus*. *Food Chemistry*, 83(1), 49–54.  
713 [https://doi.org/10.1016/S0308-8146\(03\)00035-9](https://doi.org/10.1016/S0308-8146(03)00035-9).

714 Zainuddin, R., Zaheer, Z., Sangshetti, J. N., & Momin, M. (2017). Enhancement of oral  
715 bioavailability of anti-HIV drug rilpivirine HCl through nanosponge formulation. *Drug*  
716 *Development and Industrial Pharmacy*, 43(12), 2076–2084.  
717 <https://doi.org/10.1080/03639045.2017.1371732>.

718 Zhuravlev, A. V., Zakharov, G. A., Shchegolev, B. F., & Savvateeva-Popova, E. V. (2016).  
719 Antioxidant Properties of Kynurenines: Density Functional Theory Calculations. *PLoS*  
720 *Computational Biology*, *12*(11), 1–31. <https://doi.org/10.1371/journal.pcbi.1005213>.

721 Zidan, M. F., Ibrahim, H. M., Afouna, M. I., & Ibrahim, E. A. (2018). In vitro and in vivo  
722 evaluation of cyclodextrin-based nanosponges for enhancing oral bioavailability of atorvastatin  
723 calcium. *Drug Development and Industrial Pharmacy*, *44*(8), 1243–1253.  
724 <https://doi.org/10.1080/03639045.2018.1442844>.

725