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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
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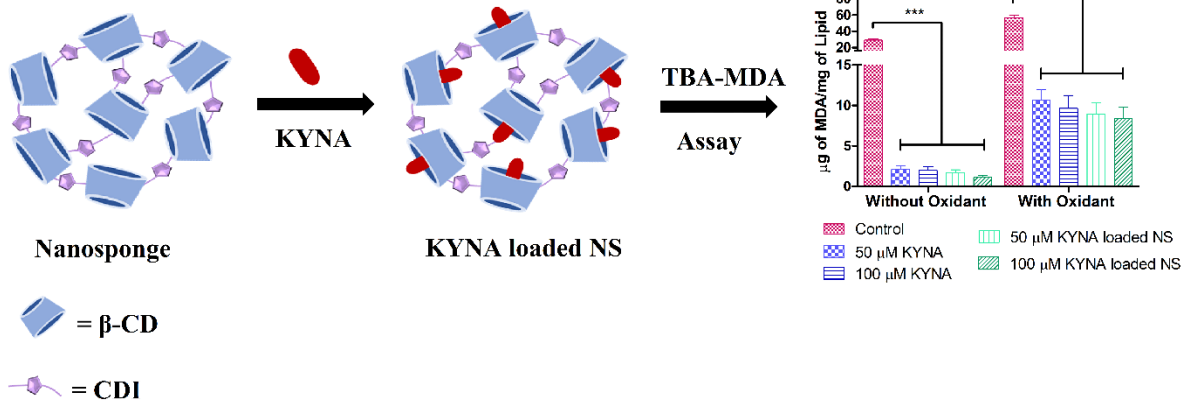
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20 **Graphical Abstract**



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40 **Highlights**

- 41 • The aqueous solubility of KYNA was increased by encapsulation with β -CDNS2.
- 42 • The molar ratio of CDI to β -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 µg/ml) compared to free kynurenic acid (16.4 µg/ml) and **β-**
66 **cyclodextrin** (28.6 µg/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, β-cyclodextrin, Nanosponges, Solubility Enhancement, Drug-Delivery,
75 Antioxidant Activity

76 **Abbreviations**

77 KYNA: Kynurenic Acid, β-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 α -cyclodextrin, β -cyclodextrin, and γ -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 α -cyclodextrin and γ -cyclodextrin
121 is expensive. β -cyclodextrin is most commonly used in the drug delivery application
122 nevertheless, β -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 β -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 β -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 β -cyclodextrin nanosponge (β -CDNS)

153 N,N'-carbonyldiimidazole (CDI) was used as a crosslinker to prepare β -cyclodextrin
154 nanosponge as reported earlier by our group (Trotta et al., 2012). Briefly, β -cyclodextrin (β -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 β -CDNS1,
162 β -CDNS2, and, β -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 (β -CD)		CDI		DMF	β -CD:CDI
	(g)	(mmol)	(g)	(mmol)	(ml)	Molar Ratio
β -CDNS1	5	4.405	1.426	8.794	30	2
β -CDNS2	5	4.405	2.852	17.588	30	4
β -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 β -CD and with nanosponges (β -CDNS1 to β -
175 CDNS3) was studied. An excess quantity of KYNA was suspended in water (2 ml) and a fixed
176 quantity of β -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 μ 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 (β -CDNS1 to β -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- β -CDNS1, KYNA- β -
189 CDNS2, and KYNA- β -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 μ 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 μ 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⁻¹ at a resolution of 4 cm⁻¹ 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 α 1 as a source of radiation. Data was collected over
227 an angular range from 5 to 45 °2 θ at a step size of 0.017 °2 θ 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 ± 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 μ M and 100 μ 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 (KMnO₄) on lipid peroxidation and its inhibition by KYNA or
256 KYNA loaded NS was also studied using additional 0.1 ml of KMnO₄ (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 H₂O₂ Scavenging activity**

269 The H₂O₂ 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 H₂O₂ (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 H₂O₂ 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 % CO₂ 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 % CO₂ 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**

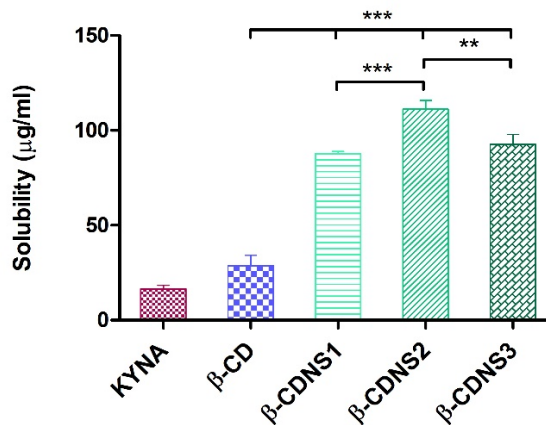
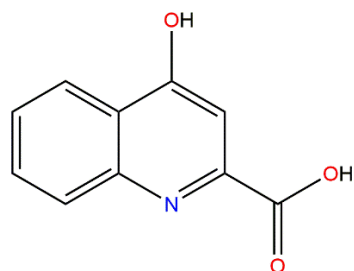
300 **3.1 Physicochemical Characterization of NS and KYNA loaded NS**

301 The presence of a large number of hydroxyl groups of β -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 β -CD. The swelling degree of the prepared NS
304 was determined. **A moderate swelling degree was achieved with β -CDNS2 (251 %), compared to**
305 **β -CDNS1 (200 %) and β -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.

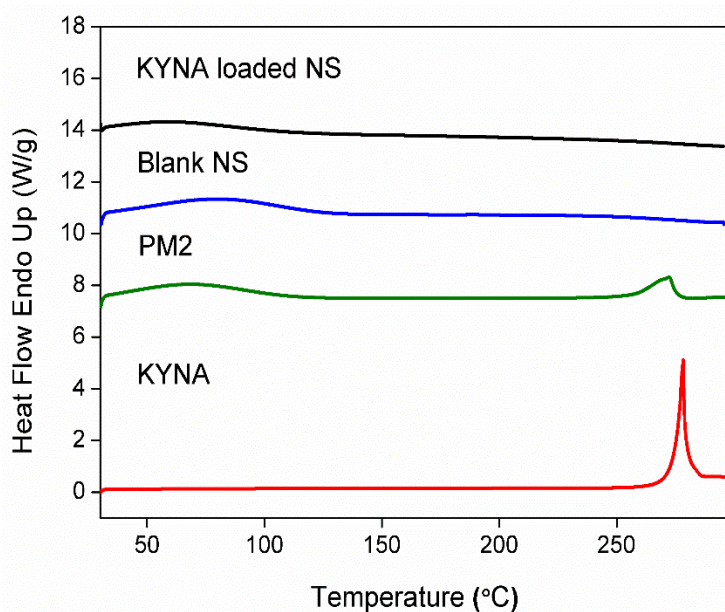
327 **Table 2.**

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

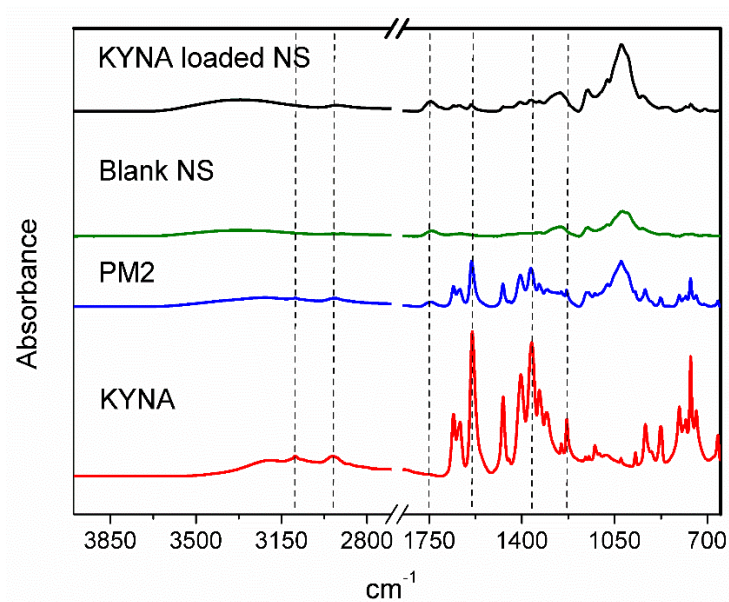
328
329 The DSC thermogram of KYNA showed an endothermic peak at 277.15 °C that
330 corresponds to its melting temperature as shown in Fig. 2(I). The nanosponges are stable thus do
331 not undergo any thermal transition and physical mixture showed endothermic transition similar
332 to KYNA with lesser 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 cm^{-1} (-N-H
339 stretching), 2940 cm^{-1} (-C-H stretching), 1660 cm^{-1} (-C=C stretching), 1362 cm^{-1} (-OH bending),
340 1121 cm^{-1} (-C-O stretching). The occurrence of carbonate bond peak at 1739 cm^{-1} in the FTIR
341 spectrum is the characteristic feature of NS (Lembo et al., 2013).

(I)



(II)

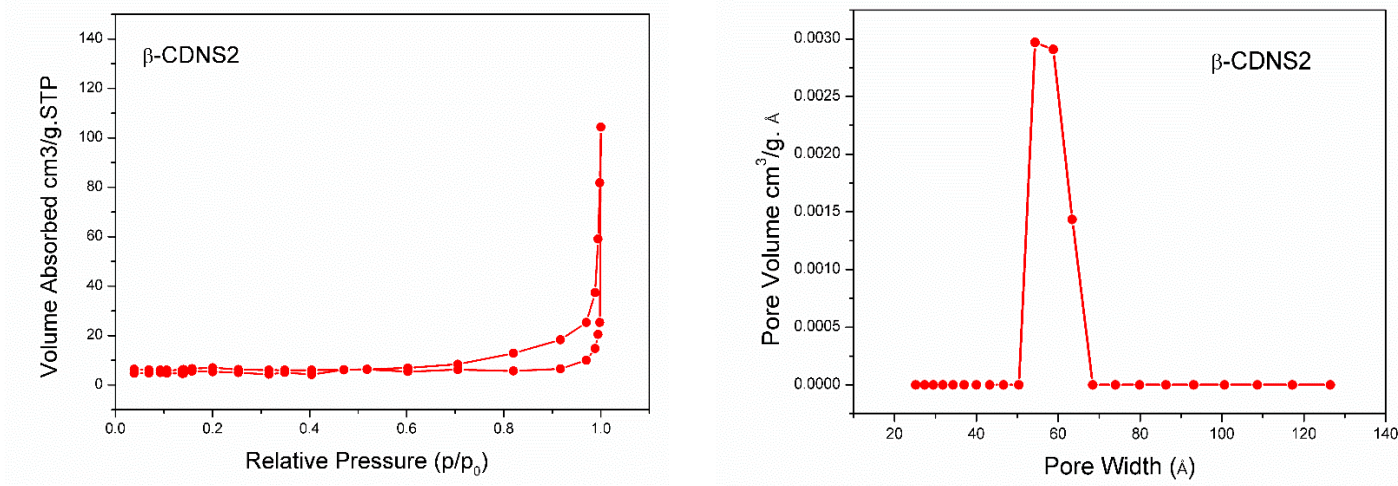


355 suggested that higher crosslinker concentration leads to higher crosslinking of the nanosponges.
356 β -CDNS1 showed lowest crosslinking of 59.49 % compared to β -CDNS2 (79.42 %) and β -
357 CDNS3 (83.7 %) (Fig. S2-S4; Supporting Information). The % crosslinking values were similar
358 to those reported 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 2θ 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 β -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)



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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 N₂ 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 m²/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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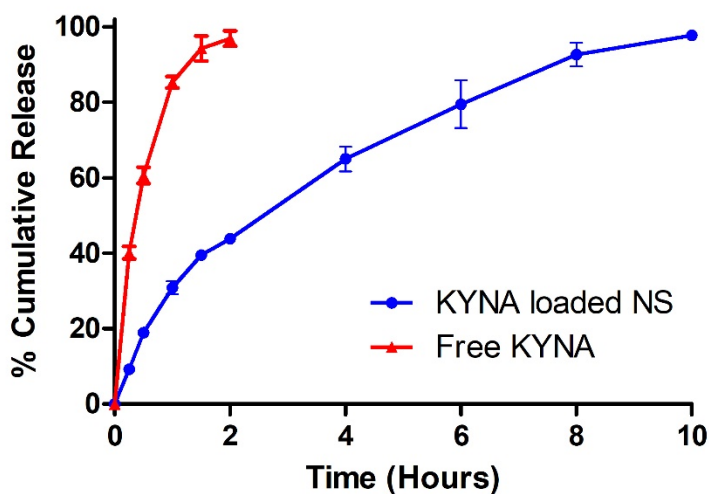
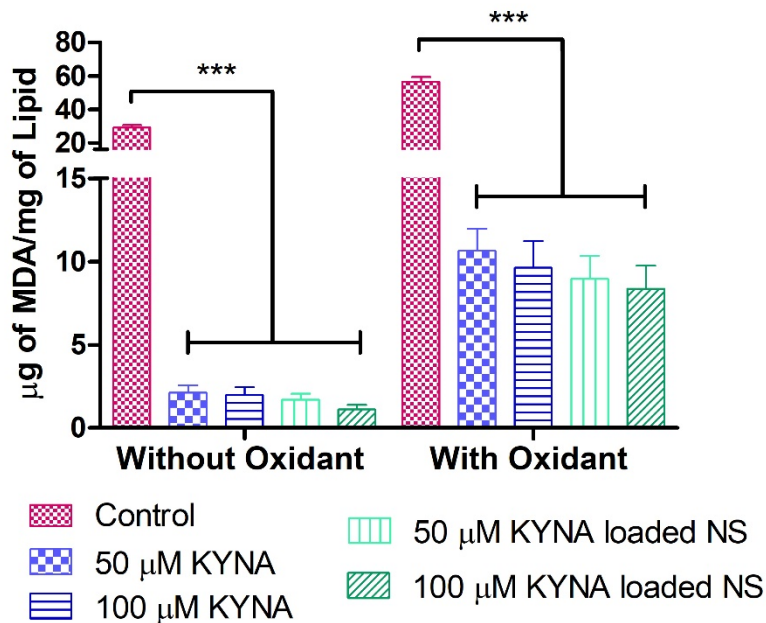


Figure 4.

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.



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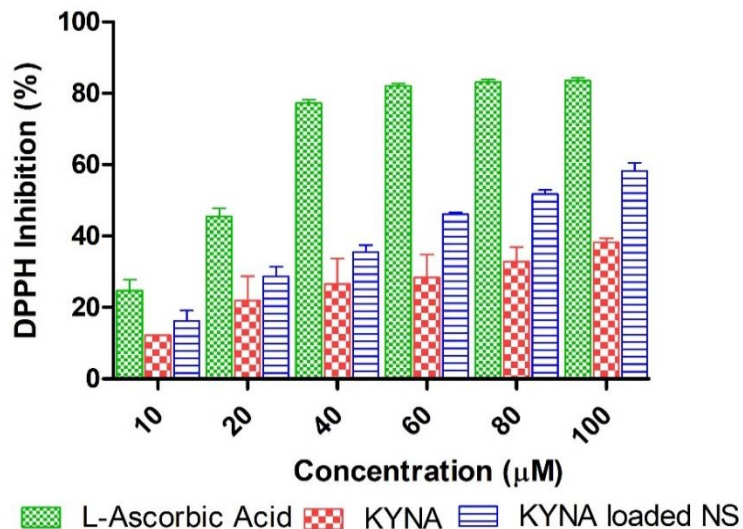
418

Figure 5.

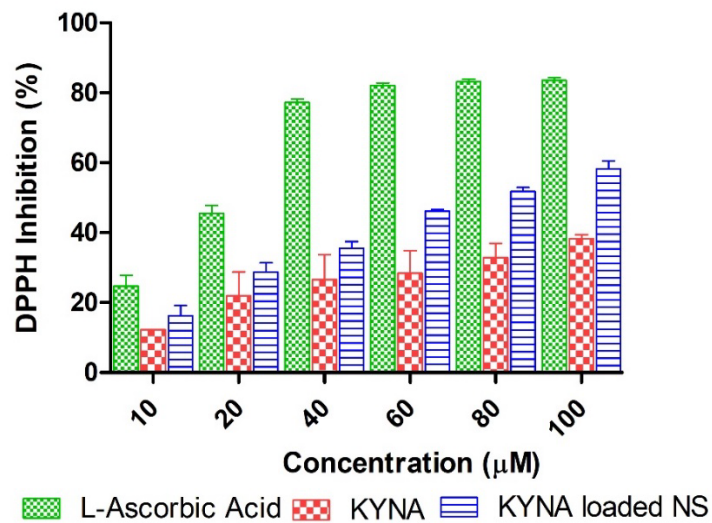
419 The effect of KYNA on MDA generation in the presence of the strong oxidizing agent
 420 (i.e. KMnO_4) was also studied and it was observed that oxidizing agent generated a large amount
 421 of MDA because of the rapid and higher oxidation of fatty acid. However, free KYNA and
 422 KYNA loaded NS 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.

(A)



(B)



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 H₂O₂

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

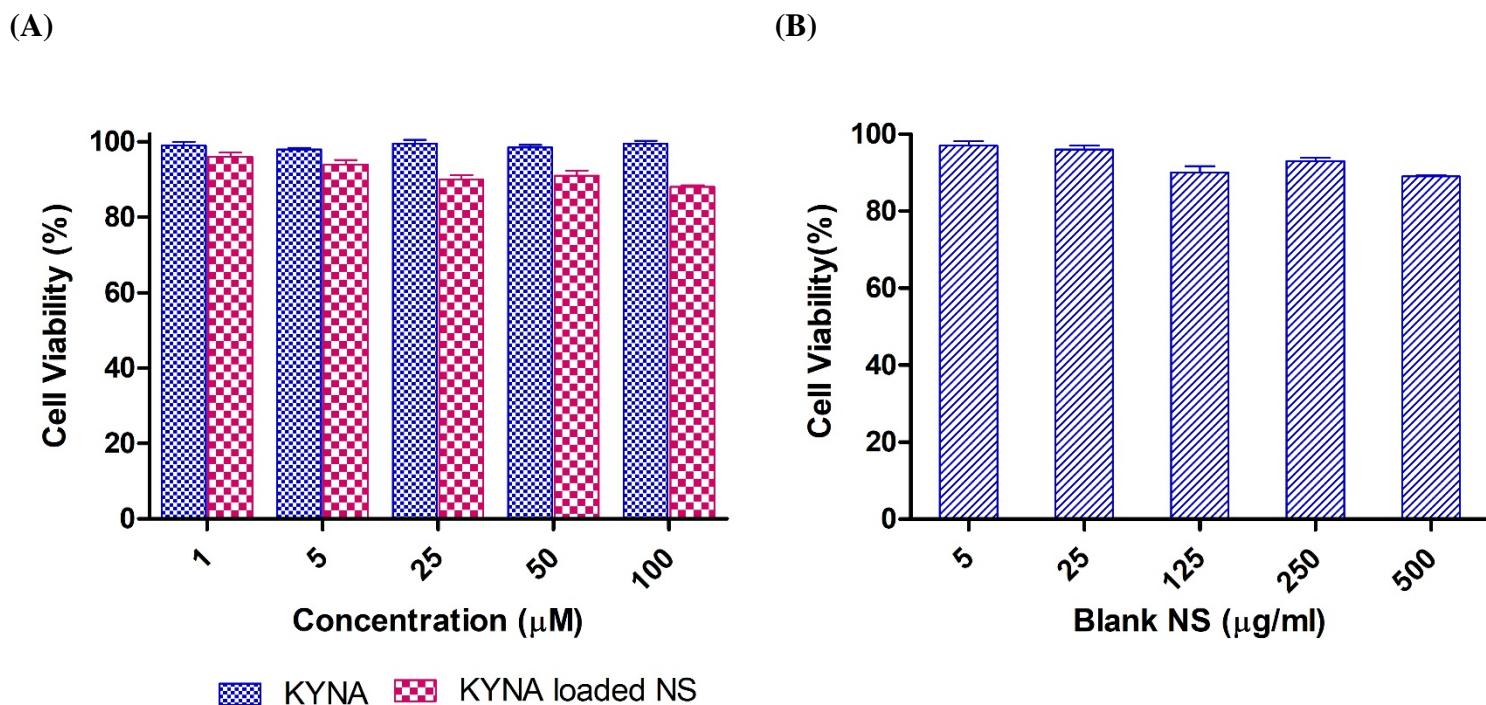
445 All the samples showed concentration-dependent H₂O₂ inhibition. The KYNA loaded NS
446 showed better H₂O₂ inhibition compared to free KYNA ranging from 17 % to 78 % at the tested
447 concentration range. It might be possible that KYNA loaded NS reacts rapidly with peroxide
448 intermediates to exhibit better H₂O₂ 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.**

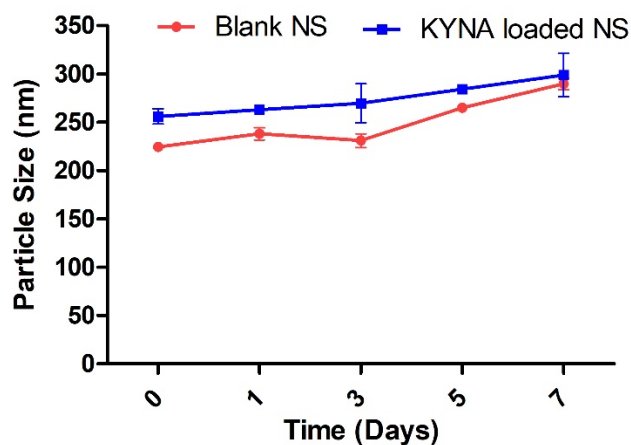
468 The effect of KYNA and KYNA loaded NS was also evaluated on the survival of SHSY-5Y
469 cell lines. Both KYNA and KYNA loaded NS do not produce any significant toxic effects on cell
470 lines at the concentration of 1-100 µM. Furthermore, 90 % or more cell survival was observed at
471 all the tested 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.

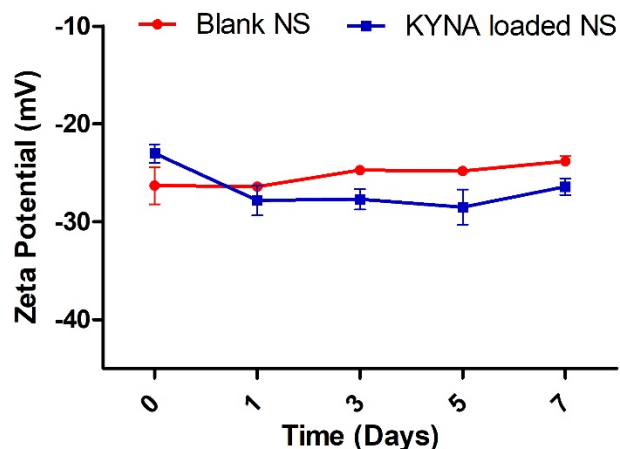
484 3.4 Stability study

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 β -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, β -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 β -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 ($n = 3$).

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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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