Original Article

Carbon nanotubes provide longer lasting gastroprotective effects for anandamide in stress-induced gastric ulcer in rat

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Abstract

Introduction: Anandamide (AEA) has shown a wide spectrum of pharmacological activities including the effects against the peptic ulcer, meanwhile, the poor solubility or short half-life may negatively affect the effectiveness of this valuable cannabinoid. Based on the superior properties of carbon nanotubes (CNTs) for controlled drug delivery, we aimed to prepare AEA-CNTs complex and evaluate its therapeutic potential in an experimental model of gastric ulcer.

Methods: Amino-functionalized multi-walled CNTs-AEA (MWCNTs-AEA) complex was prepared using COOH-MWCNTs and then characterized by Fourier transform infrared spectroscopy and transmission electron microscopy. Gastric ulcer was induced by water immersion and restrain stress (WRS) for 3.5 and 6 h in rats and the gastric lesion and oxidative stress were evaluated.

Results: AEA at higher doses reduced the gastric ulcer area and malondialdehyde content and elevated glutathione level and superoxide dismutase and catalase activities after 3.5-h WRS but it was ineffective after 6-h WRS. MWCNTs-AEA complex showed therapeutic effects after both 3.5- and 6-h WRS.

Conclusion: Aminated MWCNTs are suitable carriers for AEA as they provide longer lasting effects for this cannabinoid. The antioxidant mechanism may be involved in the gastroprotective effects of MWCNTs-AEA complex.

Keywords:
Carbon nanotubes; Anandamide; Gastric ulcer; Rat

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Introduction

Over the last few decades, the endocannabinoid system including a group of neuromodulatory lipids and their receptors which regulate the neuronal proliferation, neurotensin neurotransmission, neurotrophin signalling and mediate the action of various psychotropic agents (Di Marzo et al., 1998; Hassanzadeh and Hassanzadeh, 2011; Hassanzadeh and Rostami, 2014), has emerged as a topic of great interest. Arachidonylethanolamide (anandamide, AEA), the first recognized endogenous ligand of cannabinoid receptors, by activating two types of G protein-coupled receptors, cannabinoid CB₁ and CB₂, is implicated in a wide variety of physiological and pathological processes. In this respect, development of the selective AEA
degradation inhibitors or AEA uptake blockers has been the focus of intense research (Boger et al., 2000). According to the protective effects of the endocannabinoid system against the gastric lesions and its regulatory role in feeding behavior and inflammatory bowel disease (Di Sabatino et al., 2011; Orio et al., 2011; Shujaa et al., 2009), this ubiquitous signalling system may be considered as an emerging target for the therapeutic interventions against the gastrointestinal (GI) disorders. AEA with a wide spectrum of pharmacological activities including the effects against the psychological disorders, neurotoxicity and cancer (Adinolfi et al., 2013; Gobbi et al., 2005; Milton, 2002), has also shown therapeutic potential against the peptic ulcer in which the classical drugs have shown limited efficiency or numerous side-effects (Warzecha et al., 2011). Meanwhile, the poor solubility or short half-life of AEA (Jarho et al., 1996) may negatively affect its effectiveness. Over the last few decades, the outstanding breakthroughs in nanotechnology have resulted in the development of novel treatment strategies including the advanced nanovectors for delivery of compounds with poor solubility or short half-life. In this context, carbon nanotubes (CNTs) which may be used for the protection or targeted delivery of a wide variety of compounds, have been the focus of intense research. Indeed, CNTs have been represented as the attractive theranostic agents due to their improved biocompatibility and solubility, high thermoelectrical conductivities and superior mechanical properties (Cellot et al., 2009; Kam and Dai, 2005). These nanostructures may be used for biosensing, high-resolution imaging, tissue engineering and controlled release of drugs or growth factors (Bhirde et al., 2009; Fabbro et al., 2012; Mohammadi et al., 2009; Son et al., 2006). This background prompted us to prepare CNTs-AEA complex and evaluate its suitability to provide longer-lasting effects for AEA in an experimental model of gastric ulcer.

**Materials and methods**

**Preparation of CNTs-AEA complex**

Based on the improved dispersibility and reduced toxicity of amino functionalized multi-walled CNTs (MWCNTs) (Lee et al., 2011), we aimed to prepare aminated MWCNTs-AEA complex. Meanwhile, instead of the direct amination of MWCNTs, we initially used acidified MWCNTs (COOH-MWCNTs) as the carboxylation of CNTs prior to the amination enhances the reactivity of CNTs and facilitates further amination (Hamdi et al., 2015). Amine-functionalization of MWCNTs was performed as previously described (Chen et al., 2014; Hamdi et al., 2015; Lee et al., 2011) with some modifications. In brief, 500 mg of COOH- MWCNTs (Plasmachem GmbH, Berlin, Germany) and 50 ml of 98% thionyl chloride (SOCl₂, Sigma Aldrich, Germany) were sonicated using ultrasonic system (Tecna 6, Tecno-Gaz, Italy) at 70% amplitude for 40 min and stirred using a magnetic stirrer (IKA, Germany) at 25 °C for 48 h. Then, the suspension was filtered with 0.45 μm pore-sized microporous membrane (Sartorius, Germany), washed 5 times with tetrahydrofuran to remove the excess SOCl₂ and vacuumed at 25 °C for 25 min. The residue was reacted with 50 ml of ethylenediamine (EDA) (Sigma Aldrich, Germany) and stirred for 10 h. Afterwards, the suspension was filtered, washed 5 times with tetrahydrofuran, vacuumed for 25 min, dialyzed in the deionized distilled water using a dialysis bag (MW cut-off 14 KD) for 72 h and vacuumed to obtain amine-modified MWCNTs. In order to prepare aminated MWCNTs-AEA complex, AEA (N-arachidonoyl-ethanolamine, Tocris Bioscience, UK) was dissolved in Tween 80 (Sigma-Aldrich, Germany), 98% ethanol and phosphate-buffered saline (PBS) (1:2:18 v/v). Then, AEA (50 μM) was added to the mixture of aminated MWCNTs and PBS (0.25% w/v), stirred at 25 °C for 24 h and centrifuged by sigma-3k30 centrifuge (Sigma, Germany) at 10,000 rpm for 20 min. After the removal of supernatant, the sample was washed with PBS, re-centrifuged at 10,000 rpm for 20 min and dispersed in 10 ml of PBS.

**Characterization of MWCNTs**

Fourier transform infrared (FTIR) spectrophotometer (Shimadzu, Japan) was used to characterize the chemical structures of MWCNTs. The morphologies of MWCNTs were evaluated by transmission electron microscopy (TEM, Philips CM12).

**In vivo experiments**

**Animals**

Male Wistar rats weighing 250-280 g were housed in pairs under the standard laboratory conditions...
(temperature: 22 ± 1 °C, humidity: 55-65%) on a 12-h light/dark cycle. Animals had unlimited access to water but were fasted for 18 h prior to the experiments. The maintenance and care of experimental animals complies with National Institutes of Health guidelines for the humane use of laboratory animals and has been approved by Institutional Ethics Committee.

**Animal groups and induction of gastric ulcer**

Water immersion restraint stress (WRS) is a well-established stress model which mimics the clinical acute gastric ulcerations and is suitable for evaluating the stress ulceration and demonstrating the mechanism of stress-induced gastric injury that might result in the development of novel therapeutic agents (Ernst et al., 1998). Animals were randomly assigned into the following groups; intact (n=6), vehicle-treated without exposure to WRS (n=6), exposed to WRS for 3.5 and 6 h (n=10/group), intraperitoneally (ip) treated with 0.05, 0.5 and 1 mg/kg of AEA (Dembinski et al., 2008; Warzecha et al., 2011) (Tocris Bioscience, UK) dissolved in ethanol, or 0.2, 2 and 4 mg/kg of MWCNTs-AEA complex (containing 0.05, 0.5 and 1 mg/kg of AEA, respectively), COOH-MWCNTs, EDA-MWCNTs or vehicle 30 min before the exposure to WRS (n=10/group). Gastric ulcer was induced by WRS as previously described (Takagi et al., 1964) with some modifications. Animals were individually placed in a cage and immersed in water at 23 °C for 3.5 and 6 h.

**Evaluations of the gastric ulcer and histological alterations**

Following 3.5- or 6-h WRS, each animal was sacrificed with 100 mg/kg of thiopental (Altana, Wesel, Germany), abdomen was incised, stomach was removed and opened along the greater curvature, washed by ice-cold normal saline, and the area of lesions of the oxyntic mucosa were determined using the computerized planimeter (Morphomat, Carl Zeiss, Germany). For the histological evaluations, gastric tissue samples were fixed in 10% buffered formalin for 24 h, embedded in paraffin, sectioned at 5 μm and stained with hematoxylin and eosin for further assessments by light microscope (Olympus Bx 10, Japan) equipped with a digital camera (Olympus DP12, Japan) (Dembinski et al., 2005).

**Biochemical assays**

Gastric tissue homogenates [10% w/v in ice-cold phosphate-buffered saline (0.1 M/L)] were provided using a homogenizer (Polytron, Heidolph RZR 1, Germany), centrifuged at 10,000 g at 4 °C for 15 min and then the pure supernatant was used for the measurement of malondialdehyde (MDA) and reduced glutathione (GSH) contents and superoxide dismutase (SOD) and catalase (CAT) activities according to the manufacturer’s instruction (Sigma Aldrich, Germany). In brief, the absorption of a pink-coloured chromophore (due to the reaction of MDA with thiobarbituric acid) was determined at 532 nm using a spectrophotometer (UV-1601, Shimadzu, Japan) and MDA content was expressed as nM/mg tissue (Esterbauer et al., 1990). GSH content was measured based on its reaction with 5,5′-dithiobis (2-nitrobenzoic acid) leading to the formation of a yellow-coloured product with an absorbance at 412 nm and expressed as nM/mg tissue (Jollow et al., 1974). CAT activity was assessed based on the rate of hydrogen peroxide degradation at 240 nm and expressed as U/mg tissue (Aebi, 1984). SOD activity was evaluated based on the extent of the inhibition of amino blue tetrazolium formazan formation in the mixture of nicotinamide adenine dinucleotide, phenazine methosulphate and nitroblue tetrazolium. The colour intensity was determined at 560 nm and the enzyme activity was expressed as U/mg tissue (Kakkar et al., 1984).

**Statistical analysis**

Three-way analysis of variance (ANOVA) followed by Tukey's post hoc test was used for data analysis. Data are presented as mean±SEM (standard error of the mean) and the level of significance was set at *P*<0.05.

**Results**

**Characterization of MWCNTs**

In COOH-MWCNTs, FTIR spectroscopy demonstrated the carboxyl groups at 3168 and 1732 cm⁻¹ and C-H stretching at 2939 and 2865 cm⁻¹ (Fig. 1, curve A). Peaks at 1645 and 1248 cm⁻¹ corresponded to C=O and C–O stretching, respectively (Fig. 1, curve A). After treatment with EDA, two peaks at 3391 and 3194 cm⁻¹ were observed which corresponded to N–H stretching (Fig.
Table 1: The effects of AEA solution and various types of MWCNTs on MDA and GSH contents and SOD and CAT activities in the gastric mucosa exposed to WRS for 3.5 and 6 h

<table>
<thead>
<tr>
<th>Groups</th>
<th>MDA (nM/mg tissue)</th>
<th>GSH (nM/mg tissue)</th>
<th>CAT (U/mg tissue)</th>
<th>SOD (U/mg tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3.5 h</td>
<td>6 h</td>
<td>3.5 h</td>
<td>6 h</td>
</tr>
<tr>
<td>Intact</td>
<td>3.65±0.22</td>
<td>4.11±0.27</td>
<td>6.03±0.44</td>
<td>6.11±0.27</td>
</tr>
<tr>
<td>Vehicle</td>
<td>3.74±0.38</td>
<td>4.18±0.30</td>
<td>6.17±0.26</td>
<td>6.07±0.31</td>
</tr>
<tr>
<td>WRS+ vehicle</td>
<td>13.59±0.65</td>
<td>16.95±1.26</td>
<td>2.33±0.29</td>
<td>1.85±0.06</td>
</tr>
<tr>
<td>WRS</td>
<td>13.10±0.82</td>
<td>17.13±0.71</td>
<td>2.17±0.13</td>
<td>1.96±0.04</td>
</tr>
<tr>
<td><strong>AEA solution:</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>0.05 mg/kg + WRS</td>
<td>12.98±0.91</td>
<td>15.37±1.66</td>
<td>2.85±0.29</td>
<td>2.01±0.07</td>
</tr>
<tr>
<td>0.5 mg/kg + WRS</td>
<td>6.83±0.70</td>
<td>15.78±0.75</td>
<td>5.23±0.21</td>
<td>1.99±0.05</td>
</tr>
<tr>
<td>1 mg/kg + WRS</td>
<td>5.59±0.43</td>
<td>13.55±0.89</td>
<td>5.58±0.46</td>
<td>2.21±0.18</td>
</tr>
<tr>
<td><strong>MWCNTs-AEA complex:</strong></td>
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<tr>
<td>0.2 mg/kg + WRS</td>
<td>13.48±0.73</td>
<td>12.65±1.11</td>
<td>2.76±0.09</td>
<td>2.51±0.14</td>
</tr>
<tr>
<td>2 mg/kg + WRS</td>
<td>7.49±0.96</td>
<td>9.50±0.76</td>
<td>4.66±0.25</td>
<td>3.74±0.27</td>
</tr>
<tr>
<td>4 mg/kg + WRS</td>
<td>5.64±0.39</td>
<td>7.69±0.48</td>
<td>5.03±0.22</td>
<td>4.33±0.16</td>
</tr>
<tr>
<td><strong>COOH-MWCNTs:</strong></td>
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<tr>
<td>0.2 mg/kg + WRS</td>
<td>13.79±0.86</td>
<td>16.18±0.83</td>
<td>2.17±0.09</td>
<td>1.75±0.14</td>
</tr>
<tr>
<td>2 mg/kg + WRS</td>
<td>12.28±0.79</td>
<td>16.09±1.04</td>
<td>2.05±0.16</td>
<td>1.65±0.21</td>
</tr>
<tr>
<td>4 mg/kg + WRS</td>
<td>12.90±0.41</td>
<td>17.33±0.72</td>
<td>2.01±0.15</td>
<td>1.87±0.12</td>
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<tr>
<td><strong>EDA-MWCNTs:</strong></td>
<td></td>
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<td></td>
<td></td>
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<tr>
<td>0.2 mg/kg + WRS</td>
<td>13.65±0.47</td>
<td>15.83±0.78</td>
<td>2.83±0.29</td>
<td>1.77±0.09</td>
</tr>
<tr>
<td>2 mg/kg + WRS</td>
<td>13.44±1.12</td>
<td>15.67±0.49</td>
<td>2.78±0.18</td>
<td>2.06±0.12</td>
</tr>
<tr>
<td>4 mg/kg + WRS</td>
<td>12.69±0.57</td>
<td>17.06±1.09</td>
<td>2.95±0.11</td>
<td>1.93±0.14</td>
</tr>
</tbody>
</table>

The 0.2, 2 and 4 mg/kg of MWCNTs-AEA complex contain 0.05, 0.5 and 1 mg/kg of AEA, respectively. Each value represents mean±SEM of six independent experiments.

*P<0.001 vs. the intact or vehicle group, †P<0.05, ‡P<0.01, §P<0.001 vs. WRS or WRS + vehicle group. MWCNTs: multi-walled carbon nanotubes, EDA: ethylenediamine, AEA: anandamide, MDA: malondialdehyde, GSH: reduced glutathione, SOD: superoxide dismutase, CAT: catalase.
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Fig. 1. FTIR spectra of various types of MWCNTs. A, B and C represent the FTIR spectra of COOH-MWCNTs, EDA-MWCNTs and EDA-MWCNTs-AEA complex, respectively. FTIR: Fourier transform infrared, MWCNTs: multi-walled carbon nanotubes, EDA: ethylenediamine, AEA: anandamide.

1, curve B). Peaks at 2917 and 2841 cm\(^{-1}\) corresponded to C–H stretching and peaks at 1716 and 1641 cm\(^{-1}\) attributed to C=O stretching due to the formation of amide linkage (Fig. 1, curve B). The peak at 1192 cm\(^{-1}\) corresponded to C–N stretching of amide group (Fig. 1, curve B). The immobilization of AEA on the amine-modified MWCNTs has been shown in the curve C (Fig. 1) with characteristic
peaks at 1495, 1276 and 1233 cm\(^{-1}\). Furthermore, other peaks were observed in the curve C including those at 3432 and 3194 cm\(^{-1}\) (N–H stretching), 2951 and 2820 cm\(^{-1}\) (C–H stretching), 1786 and 1642 cm\(^{-1}\) (C=O stretching), and 1141 cm\(^{-1}\) (C–N stretching).

The morphology of MWCNTs
TEM assessments showed no remarkable difference between the morphologies of acid- and amine-modified MWCNTs (Figs. 2A and B, respectively); however, MWCNTs-AEA complex showed rougher and thicker appearance (Fig. 2C).

The effects of AEA solution and various types of MWCNTs on WRS-induced gastric lesion
Exposure to 3.5 or 6 h WRS led to the significant enhancement of gastric ulcer area (Figs. 3A and B, \(P<0.001\) vs. the intact or vehicle-treated group without exposure to WRS). AEA solution at higher doses reduced the gastric ulcer area induced by 3.5-h WRS (Fig. 3A, \(P<0.05\), \(P<0.001\)), while, it was ineffective after 6-h WRS (Fig. 3B, \(P>0.05\)). MWCNTs-AEA complex significantly reduced the gastric ulcer area after both 3.5- and 6-h WRS (Figs. 3A and B, \(P<0.05\), \(P<0.01\)). WRS-induced gastric mucosal damage was not affected by COOH- or EDA-MWCNTs (Figs. 3A and B, \(P>0.05\)). As shown in the representative photomicrographs of the gastric mucosa, WRS led to the loss of the surface epithelial cells, ulceration of the gastric mucosa and edematous submucosa (Fig. 4B) which were not effectively prevented by free AEA (Fig. 4C), while, healing of the ulcers and increased thickness of gastric mucosa were observed following treatment with MWCNTs-AEA complex (Fig. 4D).

The effects of AEA and various types of MWCNTs on MDA and GSH contents and SOD and CAT activities in the gastric mucosa exposed to WRS for 3.5 and 6 h
WRS led to a significant enhancement of MDA level and reduced GSH content and SOD and CAT activities (Table 1, \(P<0.001\) vs. intact or vehicle group). Following 3.5-h WRS, AEA solution at higher doses reduced MDA (\(P<0.001\)) and elevated GSH (\(P<0.001\)), CAT (\(P<0.05\), \(P<0.01\)) and SOD (\(P<0.05\), \(P<0.01\)) as compared to WRS or WRS + vehicle group, but it was ineffective after 6-h WRS (\(P>0.05\)). While, MWCNTs-AEA complex at higher doses showed therapeutic effects after both 3.5- and 6-h WRS (MDA: \(P<0.001\), GSH: \(P<0.001\), CAT: \(P<0.05\), \(P<0.01\), \(P<0.001\), SOD: \(P<0.05\), \(P<0.01\), \(P<0.001\) vs. WRS or WRS + vehicle group). COOH-MWCNTs or EDA-MWCNTs showed no significant effect (\(P>0.05\) vs. WRS or WRS + vehicle group).

Discussion
Gastric ulcer has been recognized as one of the major GI disorders which affects about 1.2% of the people in developed countries (Malfertheiner et al., 2009). Various factors including the consumption of steroidal or non-steroidal drugs, haemorrhagic shock, pulmonary or liver disease, sepsis, trauma, stress and imbalance between the oxidant/anti-oxidant mechanisms or gastric defensive/aggressive factors may be involved in the pathomechanism of this polyetiological chronic disease (Kisaoglu et al., 2013;
Malfertheiner et al., 2009). The limited efficiency of the currently available drugs (Kisaoglu et al., 2013; Malfertheiner et al., 2009) has provoked increasing research efforts towards the development of more efficient treatment strategies. Over the last decade, the regulatory role of the endocannabinoid signalling in the feeding behavior or GI disorders has been well-documented and it has been considered an emerging target for therapeutic interventions in GI disorders (Di Sabatino et al., 2011; Orio et al., 2011; Warzecha et al., 2011). In this respect, the endocannabinoid, AEA, with a wide variety of pharmacological actions including the gastroprotective effects has attracted a growing interest; however, the short half-life and poor solubility (Jarho et al., 1996) may limit its effectiveness. In the present study, we have designed functionalized MWCNTs as the nanoreservoirs to provide longer-lasting effects for AEA and investigated the therapeutic potential of AEA-MWCNTs complex in an experimental model of gastric ulcer. Using FTIR spectroscopy, a powerful tool for comprehensive characterization of the...
chemical structures of MWCNTs, amino functionalization of COOH-MWCNTs (the presence of N-H and C-N bands; Fig. 1, curve B) and immobilization of AEA on the aminated MWCNTs (the presence of characteristic peaks; Fig. 1, curve C) were confirmed. As previously shown, aminated CNTs induce less cytotoxicity than carboxylated MWCNTs (Hassanzadeh et al., 2015) suggesting that amine modification improves the biocompatibility of MWCNTs leading to the enhanced cell viability. TEM images revealed that proper functionalization of MWCNTs does not damage their structures and AEA-MWCNTs complex may be easily recognized from acid- or amine-modified MWCNTs by its rougher appearance and increased diameter (Fig. 2).

MWCNTs-AEA complex, but not free AEA, showed ameliorative effects against the gastric lesions after longer exposure to WRS (Figs. 3 and 4) indicating the ability of this nanostructure to provide a sustained concentration of AEA. The short half-life of free AEA (Jarho et al., 1996) and therefore lack of a sustained concentration of this cannabinoid might be involved in

**Fig. 4.** The representative photomicrographs of the gastric mucosa stained with hematoxylin and eosin. A: represents the normal gastric mucosa, B: loss of the surface epithelial cells, necrosis of the superficial layers, hemorrhage and ulceration of the gastric mucosa due to 6-h WRS, C: inflammatory cells in the oedematous submucosa, focal loss of the superficial mucous cells and haemorrhagic spots following the treatment with free AEA (1 mg/kg), D: healing of the ulcer and increased mucosal thickness due to the treatment with 4 mg/kg of MWCNTs-AEA complex (containing 1 mg/kg of AEA), scale bars = 200 μm. WRS: water immersion restraint stress, AEA: anandamide, MWCNTs: multi-walled carbon nanotubes.
its inefficiency against the deleterious effects induced by 6-h WRS.

As shown in Table 1, AEA solution and MWCNTs-AEA complex at higher doses suppressed WRS-induced oxidative stress. It was revealed by reduced MDA, an indicator of lipid peroxidation and oxidative damage (Draper and Hadley, 1990), increased GSH content, an intracellular antioxidant compound and free radical scavenger (Spitz et al., 1991), increased SOD activity, an essential inhibitor of lipid peroxidation which protects the cells against the oxidative injury (Warner et al., 2004), and increased CAT, the scavenger of hydrogen peroxide (Fukumoto and Mazza, 2000). These findings suggest the implication of antioxidant mechanisms in the gastroprotective effect of AEA and MWCNTs-AEA complex. Since oxidative stress contributes to the pathophysiology of GI disorders (Yoshikawa et al., 1993), therefore, prevention of WRS-induced oxidative stress by AEA or MWCNTs-AEA complex might be of therapeutic value in GI pathologies including the peptic ulcer. MWCNTs-AEA complex, but not AEA solution, showed beneficial effects even after 6-h WRS (Figs. 3 and 4, Table 1) suggesting that this nanocomplex prolongs the action of AEA leading to the longer lasting cytoprotective activity and suppressive effects against WRS-induced oxidative damage and lipid peroxidation. Noteworthy, providing the longer lasting effects for AEA using the aminated MWCNTs may result in the reduced dosage frequency and therefore the reduced side effects which may be observed following the chronic treatment with cannabinoids (Whan et al., 2006).

**Conclusion**

Aminated MWCNTs are suitable carriers for AEA as they provide a sustained concentration of AEA leading to the longer-lasting therapeutic effects. MWCNTs-AEA complex by prolonged attenuation of the gastric ulcer formation and oxidative stress appears as a valuable gastroprotective nanoformulation.

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**Conflict of interest**

None of the authors has any conflict of interest to disclose.

**References**


