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ORIGINAL ARTICLE

Nanoassemblies from the aqueous extract of roasted coffee beans modulate the behavioral and molecular effects of smoking withdrawal–induced anxiety in female rats

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Abstract

Antioxidant-rich plant extracts have demonstrated tremendous value as inflammatory modulators and as nanomaterial precursors. Chronic cigarette smoking alters neurotransmitter systems, particularly the glutamatergic system, and produces neuroinflammation. This study aimed to investigate the behavioral and molecular correlates of cigarette smoking withdrawal–induced anxiety-like behavior in rats, and whether these effects could be mitigated by the administration of antioxidant nanoassemblies prepared by spontaneous oxidation of dark-roasted Arabica coffee bean aqueous extracts. Four experimental groups of female Sprague–Dawley rats were randomly assigned to: (i) a control group that was only exposed to room air, (ii) a COF group that was administered 20 mg/kg of the coffee nanoassemblies by oral gavage, (iii) a SMOK group that was exposed to cigarette smoke and was given an oral gavage of distilled water, (iv) and a SMOK + COF group that was exposed to cigarette smoke and administered 20 mg/kg of the coffee nanoassemblies. Animals were exposed to cigarette smoke for 2 h per day, five days per week, with a 2-day withdrawal period each week. At the end of the 4th week, rats began receiving either distilled water or the coffee nanoassemblies before being exposed to cigarette smoke for 21 additional days. Weekly behavioral tests revealed that cigarette smoking withdrawal exacerbated anxiety, while the administration of the coffee nanoassemblies reduced this effect. The effect of cigarette smoking on astroglial glutamate transporters and nuclear factor kappa B (NF-κB) expression in brain subregions was also measured. Smoking reduced the relative mRNA and protein levels of the glutamate transporter 1 (GLT-1) and the cystine/glutamate antiporter (xCT), and increased the levels of NF-κB, but these effects were attenuated by the coffee nanoassemblies. Thus, administration of the antioxidant nanoassemblies decreased the negative effects of cigarette smoke, which included neuroinflammation, changes in glutamate transporters' expression, and a rise in anxiety-like behavior.

Keywords Antioxidants · Anxiety-like behavior · Cigarette smoke exposure · GLT-1 · Nanoassemblies · Neuroinflammation · NF-κB · xCT

Introduction

Cigarette smoking is a chronic and avoidable public health problem. It is a significant risk factor for a number of illnesses such as pulmonary, cardiovascular, and cerebrovascular disorders, as well as a variety of others [1, 2]. Despite the fact that cigarette smoking is a serious health problem, there are around one billion cigarette smokers worldwide. Furthermore, 20% of Americans smoke, resulting in around 400,000 deaths in the USA each year as a direct result of tobacco use [3].

Cigarette smoke is a complex combination of about 4000 known elements, including several reactive chemicals such as reactive aldehydes, free radical species, and a variety of metals [4]. Although studies on the connection between tobacco exposure and anxiety disorders have been inconclusive (for review, see ref. [5]), anxiety disorders, both pre-existing and withdrawal-induced, may lead to tobacco usage. The different time-courses of symptomology during chronic nicotine exposure and withdrawal, as

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well as a complicated and varied etiology, are likely to be the reason for this inconsistency. Additionally, prior research has demonstrated that anxiety is a fundamental, quantifiable symptom of nicotine withdrawal, which has been directly linked to smoking relapse [6].

Exposure to oxidative stress is one of the many pathways that may contribute to the negative effects of cigarette smoking on health. Smoking-related disorders may be exacerbated by the presence and generation of free radicals and other reactive oxygen and nitrogen species from cigarette smoke, which can lead to inflammation [7]. Cigarette smoking also has a significant impact on the immune-inflammatory system [8]. Previous research has shown that smoking has a pro-inflammatory effect, as indicated by elevated levels of inflammatory markers and cytokines in smokers [9]. The key cellular mechanism behind these pro-inflammatory effects appears to be the activation of the nuclear factor-kappa B (NF-κB) pathway by cigarette smoke and/or its contents, which leads to up-regulation of different pro-inflammatory genes [10]. Chronic nicotine use has also been shown to cause oxidative stress and related neuroinflammation. This leads to a decrease in astrocyte glutamate transporters including glutamate transporter 1 (GLT-1) and cystine/glutamate antiporter (xCT), which controls glutamate equilibrium, and increases NF-κB, which, in turn, contributes to the inflammatory cascade [8]. Changes in glutamate homeostasis in the prefrontal cortex (PFC) and nucleus accumbens (NAc) subregions of the brain are linked to the recurrence of drug-seeking behavior [11]. Furthermore, numerous studies have indicated that GLT-1 and xCT overexpression can be an effective smoking cessation target [8, 12–14].

Coffee is one of the most commonly consumed beverages worldwide [15]. In addition to the unique taste and aroma that vary according to the origin, the roasting degree, and the brewing method, coffee beans are a rich source of bioactive polyphenols, particularly chlorogenic acids, which have been linked to coffee's health benefits [16, 17]. Although the roasting process results in the loss of small phenolics, roasting leads to the formation of other antioxidants including melanoidins. Melanoidins are a brown-colored heterogeneous mixture of Maillard reaction products that result from the condensation of carbohydrates and amino acids at high temperatures. Coffee melanoidins maintain significant antioxidant activity due to the incorporation of chlorogenic acids in their structures [18, 19]. For this reason, roasted coffee bean extracts have been studied as potential therapeutics against diseases in which free radicals are implicated such as cancer, diabetes, and inflammation [16, 17, 20, 21]. Furthermore, melanoidins have been shown to increase intracellular antioxidative capacity by activating the nuclear factor erythroid 2-related factor-2 (Nrf2) in different cell types, leading to increased expression of proteins responsible for cellular defense [22, 23].

We have recently shown that the polyphenol-rich aqueous extracts of coffee beans can form spherical nanoassemblies simply by oxidation-triggered self-polymerization followed by filtration and dialysis. These nanoassemblies varied in size and polydispersity as a function of the coffee beans' roasting degree and the type of oxidant used. Notably, nanoassemblies prepared from air-oxidized coffee bean extracts maintained the most significant antioxidant activity and biocompatibility [24]. Unlike the plain coffee bean extracts (i.e., typical coffee beverages), the purification process of the nanoassemblies removes small molecules such as caffeine, while preserving the antioxidant activity. Inspired by the health benefits of coffee extracts as dietary antioxidants, and their interesting ability to form nanoassemblies, we hypothesized that these nanoassemblies could play a role in modulating the neuroinflammatory response associated with cigarette smoking. This would in turn mitigate the behavioral effects of smoking withdrawal, particularly withdrawal-induced anxiety. To test this hypothesis, we established a cigarette smoke exposure animal model where animals were treated with nanoassemblies from the air-oxidized dark-roasted coffee bean extract. A series of behavioral tests associated with anxiety were conducted, and the results were recorded and compared to the control groups. The behavioral findings were paralleled by molecular assays of markers implicated in anxiety-like behavior such as GLT-1, xCT, and NF-κB in the PFC and NAc subregions of the rat brains. These findings establish an association between antioxidant consumption and mitigation of smoking withdrawal-induced anxiety and, putatively, neuroinflammation.

Experimental

Materials

Dark-roasted ground Arabica beans were obtained from Al-Ameed Coffee (Amman, Jordan). LD blue cigarettes cruise (Liggett Ducat, 0.6 mg nicotine, 0.8 mg tar, and 0.01 mg carbon monoxide) were obtained from a local supermarket (Amman, Jordan).

Preparation of nanoassemblies from dark-roasted coffee beans

Nanoassemblies form the aqueous extract of dark-roasted ground coffee beans were prepared by air oxidation as previously described [24]. In a typical procedure, 5 g of ground beans was placed in a 500-mL Erlenmeyer flask, to which 200 mL of boiling distilled water was added,

followed by vigorous stirring on a magnetic stirring plate at room temperature for 15 min. The mixture was then filtered using normal filter paper and left to cool at room temperature. The extract was further purified by dialysis against deionized water using a 12–14 kDa regenerated cellulose membrane, changing the water twice a day for 5 days. Next, the product was lyophilized using a Labconco FreeZone 4.5 L Benchtop Freeze Dryer (Kansas City, MO, USA) and stored in an air-tight container at room temperature.

Characterization of the coffee nanoassemblies

Particle size and morphology

The formation of nanoassemblies was confirmed by dynamic light scattering (DLS) by measuring the particle size and polydispersity index (PDI) of freshly prepared coffee extracts. Measurements were conducted using a Nicomp Nano Z3000 instrument (Entegris, Billerica, MA, USA) after diluting the sample 1:1 with distilled water. The intensity-weighed particle size and PDI were measured from three different batches of the nanoassemblies and the results were reported as the mean ± standard deviation (SD). The nanoassemblies were also visualized by transmission electron microscopy (TEM) using a Morgagni 268 microscope (FEI, Netherlands) at 60-kV accelerating voltage to confirm their morphology.

Total phenol content of the nanoassemblies

The total phenol content of fresh nanoassemblies was determined based on the Folin-Ciocalteu method as previously described with some modification [24, 25]. For the assay, lyophilized coffee nanoassemblies were dissolved in distilled water at 1 mg/mL. Gallic acid was used as a standard and prepared at concentrations within the range 6.25–100 µg/mL. The Folin-Ciocalteu reagent (Sigma-Aldrich, St Louis, MO, USA) was diluted 10 × in distilled water, and then 1 mL of the reagent was added to 250 µL of the nanoassemblies and each gallic acid dilution. Sodium bicarbonate (1 mL; 10% w/v) was added to each sample 5 min later and the samples were further incubated for 30 min in the dark. Then, 100 µL of each sample was transferred to a 96-well plate and the absorbance was measured at 765 nm using a Synergy HTX multimode microplate reader (Biotek, Winooski, VT, USA). A calibration curve was constructed from the absorbance of the gallic acid samples versus gallic acid concentration, from which the total phenol content of the nanoassemblies was determined and expressed as gallic acid equivalents (GAE) per milligram nanoassemblies. The experiment was repeated three times, and the final value was reported as the mean ± SD.

In vitro antioxidant activity

The antioxidant activity of the nanoassemblies was determined using a 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) assay. The reagent was obtained from Abcam (Cambridge, UK) and activated as previously described [24, 26]. The ABTS working solution (1 mL) was added to 200 µL of the nanoassemblies prepared at various concentrations (0–5000 µg/mL in water). The samples were incubated in the dark for 30 min, followed by reading the absorbance at 743 nm (BioTek Synergy HTX multimode microplate reader). Antioxidant activity was calculated according to the following equation:

$$\text{Antioxidant activity}(\%) = \frac{[\text{Absorbance of ABTS alone} - \text{Absorbance of the sample}]}{\text{Absorbance of ABTS alone}} \times 100\%$$

The values were reported as the mean ± SD from three independent experiments. The half-maximal antioxidant activity (IC_{50}) was obtained by non-linear regression analysis of the antioxidant activity % versus concentration in GraphPad Prism 9.0.

Whole-body cigarette smoke exposure animal model

Animals

Thirty-four female Sprague–Dawley rats, weighing 180 – 250 g, were inbred (around 15 generations) at Al-Zaytoonah University of Jordan. The housing and treatments of animals were in accordance with the Declaration of Helsinki [27]. Animals had free access to food and water in the rat home vivarium and were kept on a 12-h light/dark cycle (lights on from 07:00 to 19:00) throughout the experiment. All experiments (cigarette smoke exposure and behavioral tests) were conducted during the light cycle.

Animal groups and treatments

The animal groups and the timeline for cigarette smoke exposure, coffee nanoassemblies treatment, and behavioral tests are illustrated in Fig. 1A. After an acclimation period of three weeks (at which time the rats were nine weeks old

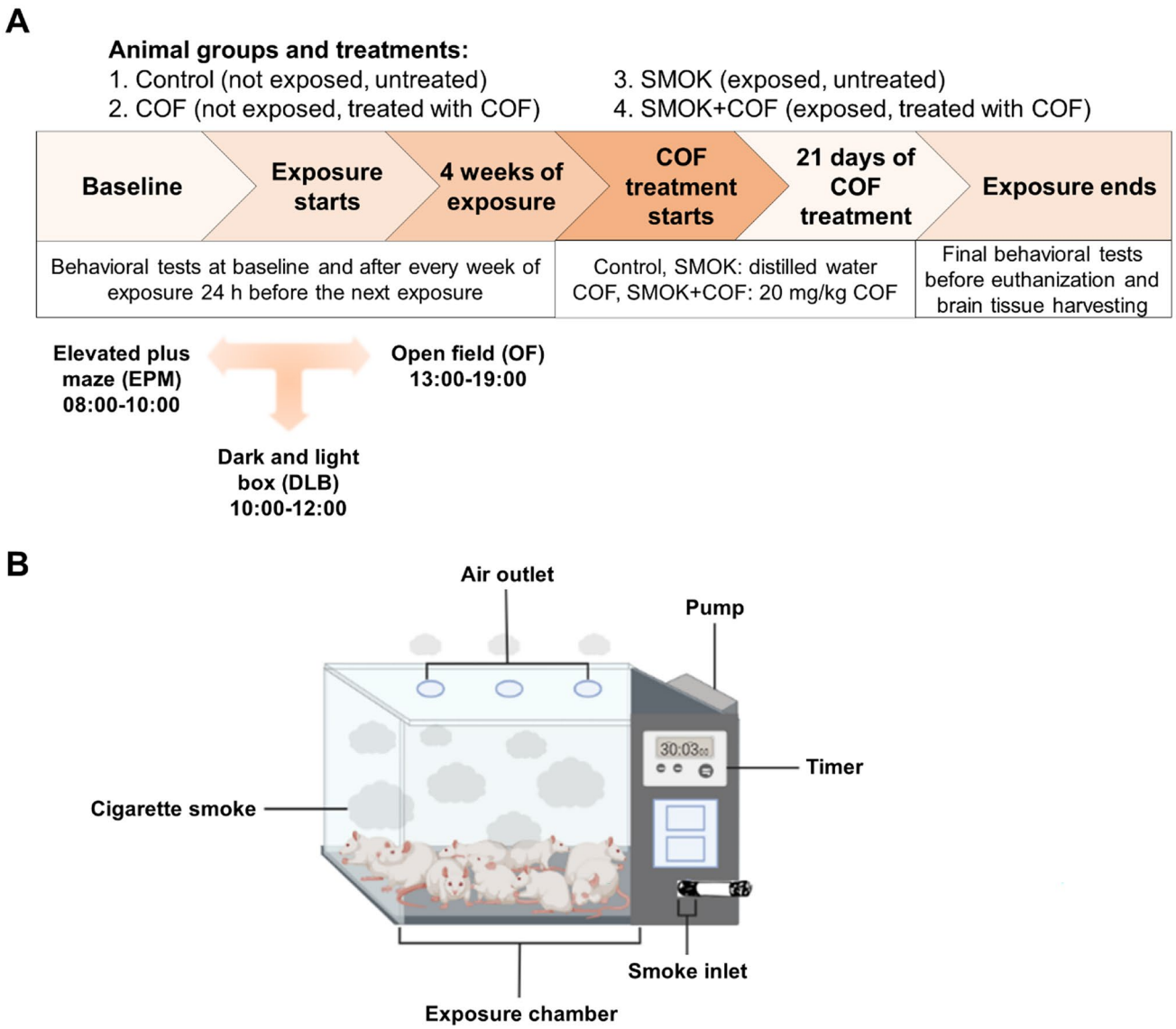

Fig. 1 **A** Experimental groups and timeline for the cigarette smoke exposure animal model (COF, coffee nanoassemblies; SMOK, exposed to cigarette smoke); **B** diagram demonstrating the apparatus used for cigarette smoke exposure

and weighed about 180 g), baseline behavioral tests were performed (see below) and then rats were randomly assigned to one of four groups: the control group, which was exposed only to room air during the experiment and given distilled water by oral gavage (at 08:00, around 1 mL) during the last 21 days of the experiment; the coffee group (COF), which was exposed to room air during the experiment and received a daily oral gavage of 20 mg/kg of the coffee nanoassemblies dissolved in distilled water (at 09:00, around 1 mL) during the last 21 days of the experiment; the cigarette smoke–exposed group (SMOK), which was exposed to cigarette smoke for two consecutive hours per day, five consecutive days per week, followed by two days without exposure, for the first four weeks (20 days) and received a daily oral gavage of distilled water (at 10:30, around 1 mL) 45 min before cigarette exposure for an additional 21 days of the experiment; and the cigarette smoke–exposed and coffee group (SMOK + COF), which was exposed to cigarette smoke for two consecutive hours per day, five consecutive days per week, followed by two days without exposure for the first four weeks (20 days) and received a daily oral gavage of 20 mg/kg (at 10:00, around 1 mL) of the coffee nanoassemblies dissolved in distilled water 45 min before exposure to cigarette smoke for an additional 21 days of the experiment. Behavioral tests were conducted on the days without exposure.

Cigarette smoke exposure

Animals were exposed to cigarette smoke in a specially designed 40 × 40 × 40 cm acrylic box (Fig. 1B) in which the animals (SMOK and SMOK + COF groups; 16 rats) were confined for 2 h during cigarette smoke exposure. Smoke was pulled from the lit cigarettes using a pump and pushed toward the inner chamber where rats were exposed to the cigarette smoke. The exposure chamber was programmed to inhale and exhale at regular intervals with each cigarette lasting 3 – 5 min, and a total of 14 cigarettes were consumed per exposure session to mimic an average smoker's cigarette consumption [28]. Liggett Ducat cigarettes (LD blue) with 0.6 mg of nicotine per cigarette were used in this study. The cigarette smoke exposure was monitored by measuring carbon monoxide (CO) levels which were kept between (600 – 800 ppm) [29].

Behavioral tests

Baseline behavioral tests were conducted before the first cigarette smoke exposure session, and rats were then divided into the four experimental groups according to their performance to ensure similar baseline behaviors in all the groups. Tests were also conducted at the end of each week of cigarette smoke exposure 24 h before the following exposure started, which continued until the end of the study. At the end of the treatment phase, the last set of behavioral tests was performed before euthanizing the animals. Behavioral scoring was done by an observer who was not aware of the experiment or its hypothesis. The following behavioral tests were performed.

Open field (OF)

The open field (OF) test was performed in a white polyvinyl chloride plastic board square arena (50 × 40 × 25 cm), and a video camera was used to capture the test sessions. The overall distance traveled and the time spent in the center zone were measured. Prior to the trial, all rats were habituated to the apparatus for 10 min, and each session lasted 20 min. The open field area was cleaned with water between trials.

The dark and light box (DLB) test

The dark and light box (DLB) apparatus was made up of two linked compartments (40 × 40 × 30 cm), one lit (aversion region) and the other darkened (safe area), with a 7.5 × 5.0 cm entrance between them. The dark chamber was covered with a wooden lid. The lit area was illuminated by a table lamp (80-W light bulb, 50 cm above the floor, illumination in the center of the light compartment ~ 1000 lx). A tripod was used to mount the camera above the DLB. Rats were placed in the center of the light compartment facing away from the exit and then allowed to move around the edges of the compartment until they discovered the dark compartment's entrance. During the tests, rats were placed in the apparatus for 10 min, and the duration spent in the light chamber and the latency to enter the dark chamber were recorded. All compartments were cleaned with water after each session.

The elevated plus maze (EPM) test

The elevated plus maze (EPM) apparatus was made up of four cross-shaped arms with a central zone in the middle rising ~ 50 cm above the ground. The EPM consisted of two open arms (without walls; 50 × 10 cm; L × W) and two closed arms (with black walls; 50 × 10 × 30 cm; L × W × H). Rats avoid open, well-lit areas, yet they also like to discover new places. As a result, the ratio of these conflicting impulses was assessed. The number of crossings through the center zone, as well as the total time spent in the open arms, was recorded. During the test (one session per rat on each test day), rats were placed at the crossroad of the EPM's four arms, and their behavior was observed for 5 min. The apparatus was cleaned with water after each session.

Brain tissue harvesting

At the end of the treatment phase, rats were euthanized with diethyl ether followed by cervical dislocation. Brains were immediately placed in liquid nitrogen and stored at – 80 °C. The PFC and the NAc were then dissected using a cryostat apparatus (JUNG CM 1500, Leica, Germany) set at – 20 °C. Brain regions were identified according to the Rat Brain Atlas [30]. Extracted brain regions were stored at – 80 °C for further analysis.

Real-time quantitative polymerase chain reaction (qPCR)

RNA samples were isolated from the PFC and the NAc using a kit from ZYMO Research (cat# R2052), and reverse-transcribed into cDNA using a PrimeScript™ RT Master Mix kit (Takara Bio, Japan). Primer sequences used for the PCR reactions are listed in the Supplementary Information (SI; Table S1). qPCR was performed using a Prime-Pro iCycler system (Cole-Parmer, UK). For each gene, the mRNA expression measured in each sample was normalized to β-actin mRNA levels, and relative mRNA for the

gene-of-interest expression versus the housekeeping gene was calculated by calculating $2^{-\Delta\Delta CT}$ for each sample [31].

Western blot

Brain tissue samples from the PFC and NAc brain regions were lysed using a lysis buffer. Equal quantities of isolated proteins were then mixed with 5 × laemmli loading dye. Proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) (10%) and electrophoretically transferred at a constant current of 25 V for 50 min using a Trans-Blot Turbo™ Transfer System (Bio-Rad, USA) onto a polyvinylidene difluoride membrane (PVDF, Santa Cruz Biotechnology, USA). After blocking with 3% w/v, 0% fat Regilait milk diluted in TBST (50 mM Tris–HCl; 150 mM NaCl, pH 7.4; 0.1% Tween 20) for 30 min at 4 °C, the membrane was incubated overnight at 4 °C with one of the following primary antibodies: Recombinant anti-EAAT2 (GLT-1) antibody [EPR19794] (ab205247; Abcam, UK), recombinant anti-xCT antibody [EPR8290 (2)] (ab175186, Abcam), or recombinant anti-NF-κB p65 antibody [EP2161Y] (ab76311, Abcam). Anti-β-tubulin antibody was used as a loading control (ab6046, Abcam). The amount of each antibody used was according to the manufacturer's instructions. The next day, the primary antibody was removed, and the washing and blocking processes were completed for 15 and 30 min, respectively. After that, the secondary antibody (goat anti-rabbit IgG H&L (HRP)) was added for 90 min. The antibodies used in this experiment are listed in the SI (Table S2). The secondary antibody was removed after 90 min, and the membrane was washed for 15 min to prepare it for imaging using a ChemiDoc™ Imaging System (Bio-Rad, USA). The bands were quantified using the Image Lab 6.0.1 system, and the results were expressed as a ratio of the measured protein to β-tubulin. In each gel run, the control group was set to 100%, and the protein expression for each treatment group was calculated relative to the control group [32].

Statistical analysis

Data are presented in the form of means and standard errors of the means (SEM). The results from the behavioral tests were compared by two-way repeated measures analysis of variance (ANOVA), followed by Bonferroni's multiple comparisons test. The relative mRNA and protein expression levels were compared by one-way ANOVA, followed by Tukey's multiple comparisons test. In addition, an unpaired *t*-test was performed to compare the levels of relative mRNA expression and relative protein expression between the control and COF groups. All statistical analyses were based on $p < 0.05$ level of significance, using GraphPad Prism 9.0.

Results

Preparation and characterization of nanoassemblies from the air-oxidized aqueous extract of dark coffee beans

As reported earlier, the aqueous extract of dark-roasted coffee beans was found to form nanoassemblies under various oxidative conditions [24]. Even when exposed to air only, the polyphenols present in the coffee extract undergo oxidative coupling reactions. In the case of the dark-roasted coffee bean extract, this resulted in the formation of nanoassemblies averaging 89 ± 25 nm in diameter with a polydispersity index (PDI) of 0.17 ± 0.03 and a spherical morphology (Fig. 2A). The nanoassemblies were associated with a broad UV–Vis absorption spectrum between 200–500 nm (Fig. 2B). The FT-IR spectrum showed characteristic stretching bands of O–H/ = C–H, C–H, and C = C/C = O functional groups at 3700–3000, 2900–2700, and 1700–1500 cm^{-1}, respectively (Fig. 2C). As for the total phenol content and in vitro antioxidant activity, the nanoassemblies were found to contain 69.5 ± 13.3 µg GAE/mg, and a half-maximal antioxidant activity (IC_{50}) of 149.7 ± 30.4 µg/mL (Fig. 2D).

Effect of whole-body cigarette smoke exposure and coffee nanoassemblies treatment on anxiety-like behavioral tests

After recording the baseline behavior for the animals, the SMOK and SMOK + COF groups were both exposed to cigarette smoke for four consecutive weeks. As shown in Figs. 3, 4, and 5, the animals began to exhibit anxiety-like behavior starting from the second to third weeks of exposure. The exposure was continued for four weeks to ensure that the animals exhibited similar anxiety-like behavior in all the behavioral tests performed.

The results of the OF test (total traveled distance and time spent in center zone) are shown in Fig. 3. Overall, the total traveled distance and time spent in center zone decreased over the four weeks of exposure in SMOK and SMOK + COF groups compared to the control group. This decrease was reversed after administering the coffee nanoassemblies for 21 days to the SMOK + COF group. In case of the total traveled distance, a repeated measures two-way ANOVA revealed a significant main effect of time [$F(5, 105) = 11.92$, $p < 0.0001$], a significant main effect of treatment [$F(2, 21) = 56.72$, $p < 0.0001$], and a significant time × treatment interaction [$F(10, 105) = 6.287$, $p < 0.0001$]. Bonferroni's multiple comparisons test confirmed a significant decrease in total traveled distance at the 2nd, 3rd, and 4th weeks of exposure in the SMOK and SMOK + COF groups compared to the control group. This effect was reversed by giving the rats 21 daily

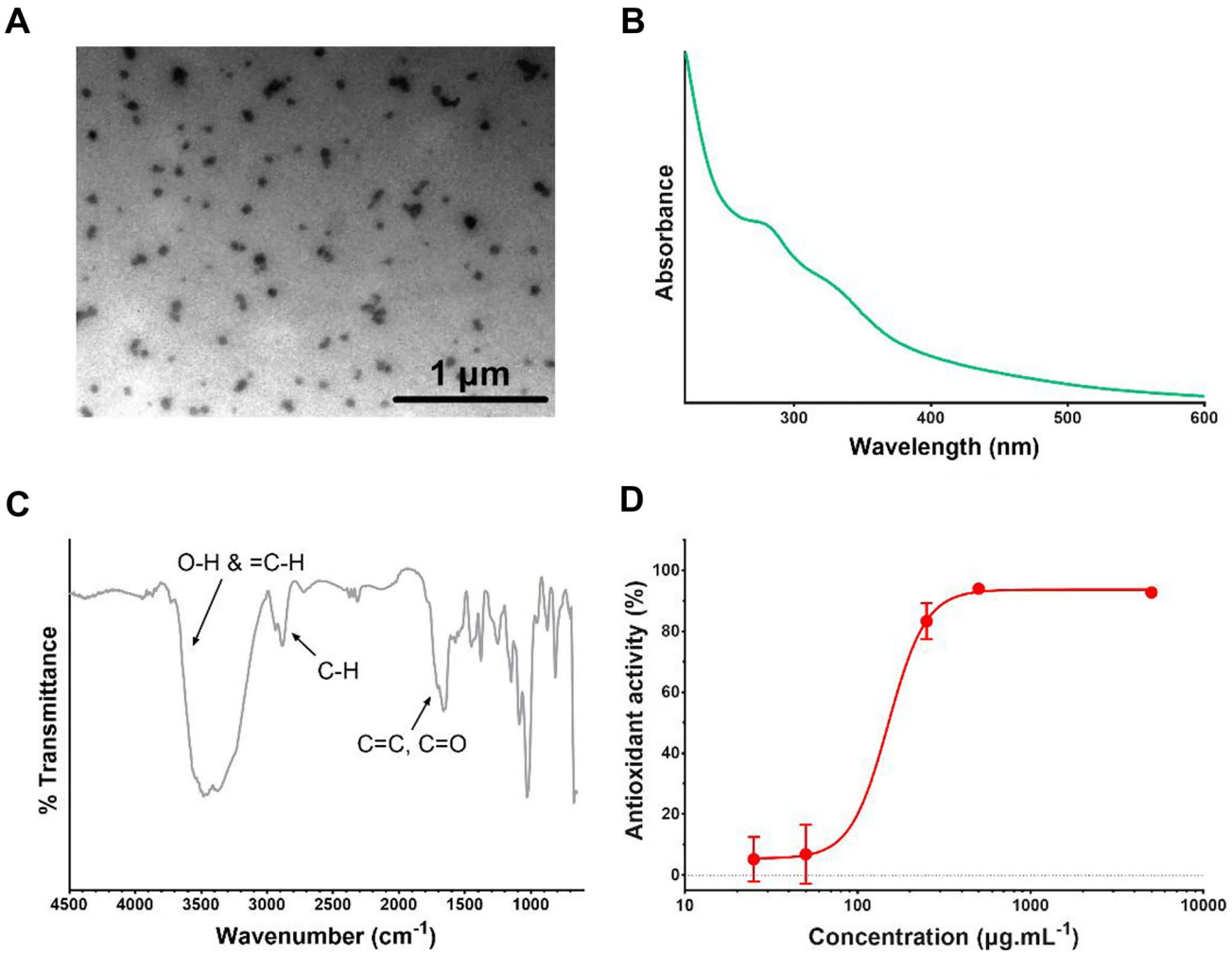

Fig. 2 Characterization of the coffee nanoassemblies by **A** TEM, **B** UV–Vis, **C** FT-IR, and **D** in vitro antioxidant activity

doses of the coffee nanoassemblies. A significant increase was observed in distance traveled with the SMOK + COF group compared to the SMOK group (Fig. 3A), confirming the recovery of locomotion by 21 daily doses of 20 mg/kg of the coffee nanoassemblies.

A similar pattern was observed for time spent in center of the OF. Repeated measures two-way ANOVA revealed a significant main effect of time [F (5, 105) = 43.26, $p < 0.0001$], a significant effect of treatment [F (2, 21) = 10.77, $p = 0.0006$], and a significant effect of time × treatment interaction [F (10,105) = 12.70, $p < 0.0001$]. Bonferroni's multiple comparisons test confirmed that there was a significant decrease in time spent in center zone of the OF at the 2nd, 3rd, and 4th weeks of exposure in the SMOK and SMOK + COF groups compared to the control group. This effect was reversed by giving the rats 21 daily doses of 20 mg/kg of the coffee nanoassemblies. Furthermore, the results showed a significant difference in time spent in center zone of the OF between the SMOK and SMOK + COF groups (Fig. 3B). Moreover, animals treated with 20 mg/kg of the coffee nanoassemblies for 21 days but without prior cigarette smoke exposure showed no significant difference in the total traveled distance and time spent in center zone of the OF in comparison with the control group (SI; Fig. S1).

In the DLB test, cigarette smoke exposure gradually increased withdrawal-induced anxiety throughout the first four weeks of exposure, as shown by the time spent in the light chamber and latency to enter the dark chamber (Fig. 4). As with the OF test, this effect was reversed by 21 daily doses of 20 mg/kg of the coffee nanoassemblies. For the time spent in the light chamber test (Fig. 4A), a repeated measures two-way ANOVA revealed a significant main effect of time [F (5, 105) = 32.79, $p < 0.0001$], a significant main effect of treatment [F (2, 21) = 8.830, $p = 0.0016$], and a significant time × treatment interaction [F (10, 105) = 9.631, $p < 0.0001$]. Bonferroni's multiple comparisons test showed a significant decrease in time spent in the light chamber at

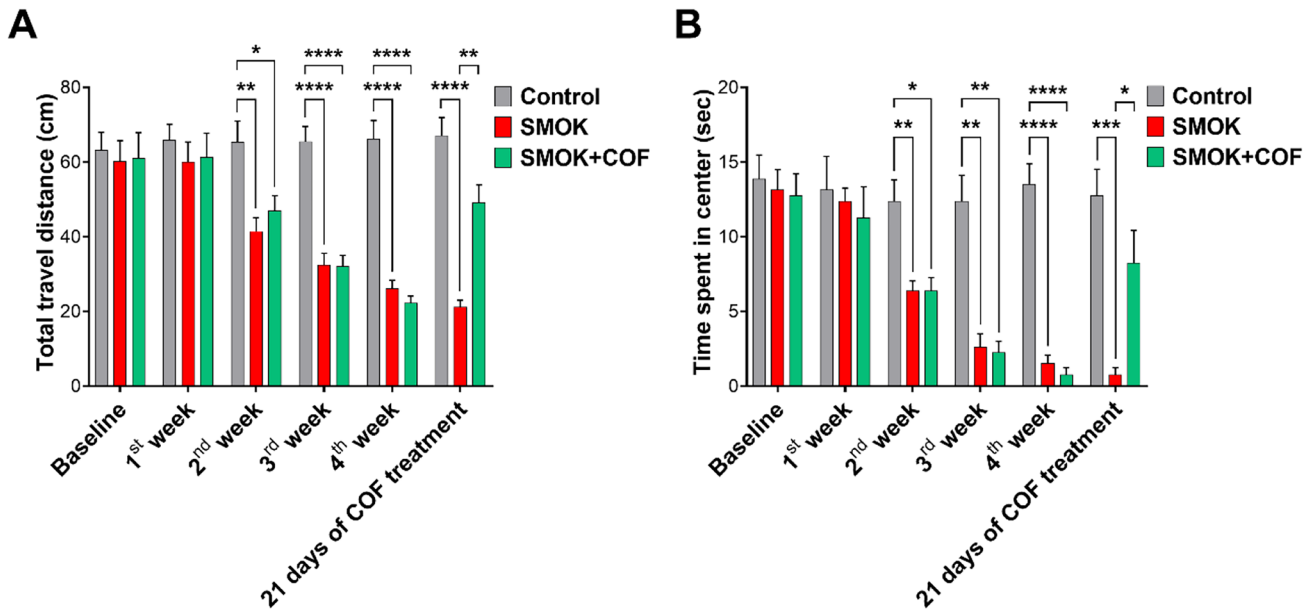

Fig. 3 Results of the open field (OF) behavioral test. **A** Total distance traveled in the OF, and **B** time spent in the center of the OF, in control, SMOK, and SMOK + COF groups. Data are presented as mean ± SEM (n = 6); *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$ (COF, coffee nanoassemblies; SMOK, exposed to cigarette smoke)

the 2nd, 3rd, and 4th weeks of exposure in the SMOK and SMOK + COF groups compared to the control group. This effect was reversed after 21 daily doses of 20 mg/kg of the coffee nanoassemblies. In addition, there was a significant difference in time spent in the light chamber of the DLB in the SMOK group compared to the SMOK + COF and control groups (Fig. 4A).

A similar pattern was observed for the latency to enter the dark chamber. Repeated measures two-way ANOVA revealed a significant main effect of time [F (2, 15) = 4.104, $p = 0.0184$], a significant effect of treatment [F (2, 15) = 4.310, $p = 0.0332$], and a significant effect of time × treatment interaction [F (10, 75) = 2.290, $p = 0.0210$]. Bonferroni's multiple comparisons test confirmed that there was a significant

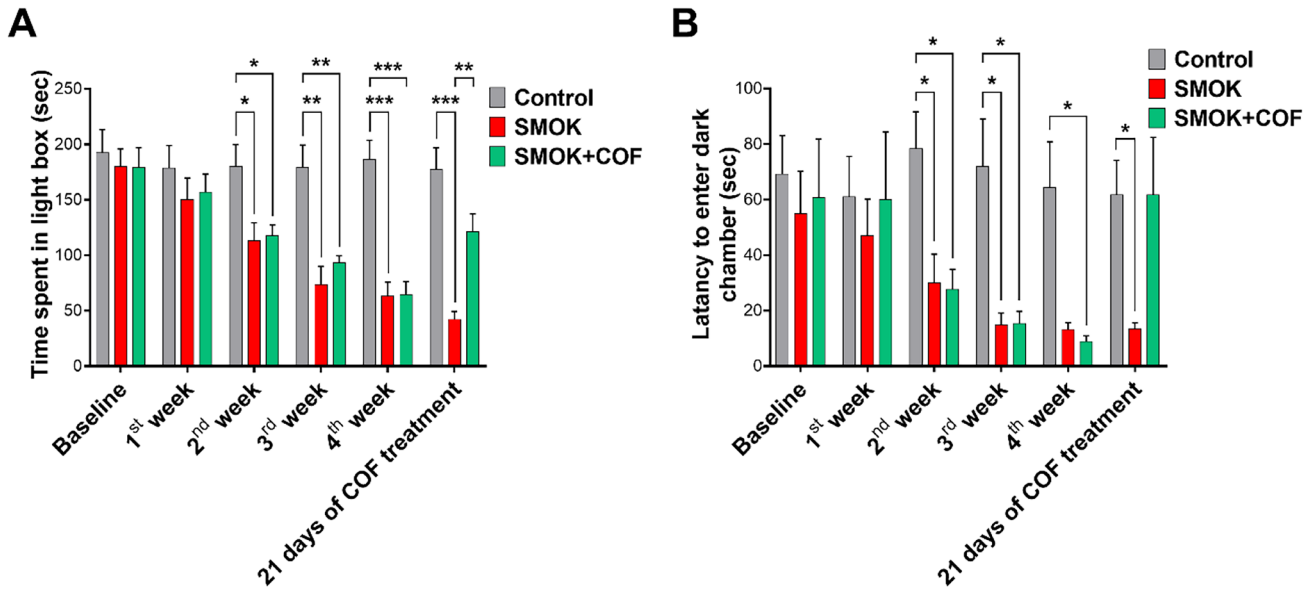

Fig. 4 Results from the dark and light box (DLB) behavioral test. **A** Time spent in the light chamber of the DLB, and **B** latency to enter the dark chamber, in control, SMOK, and SMOK + COF groups. Data are presented as mean ± SEM ($n = 6$); *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ (COF, coffee nanoassemblies; SMOK, exposed to cigarette smoke)

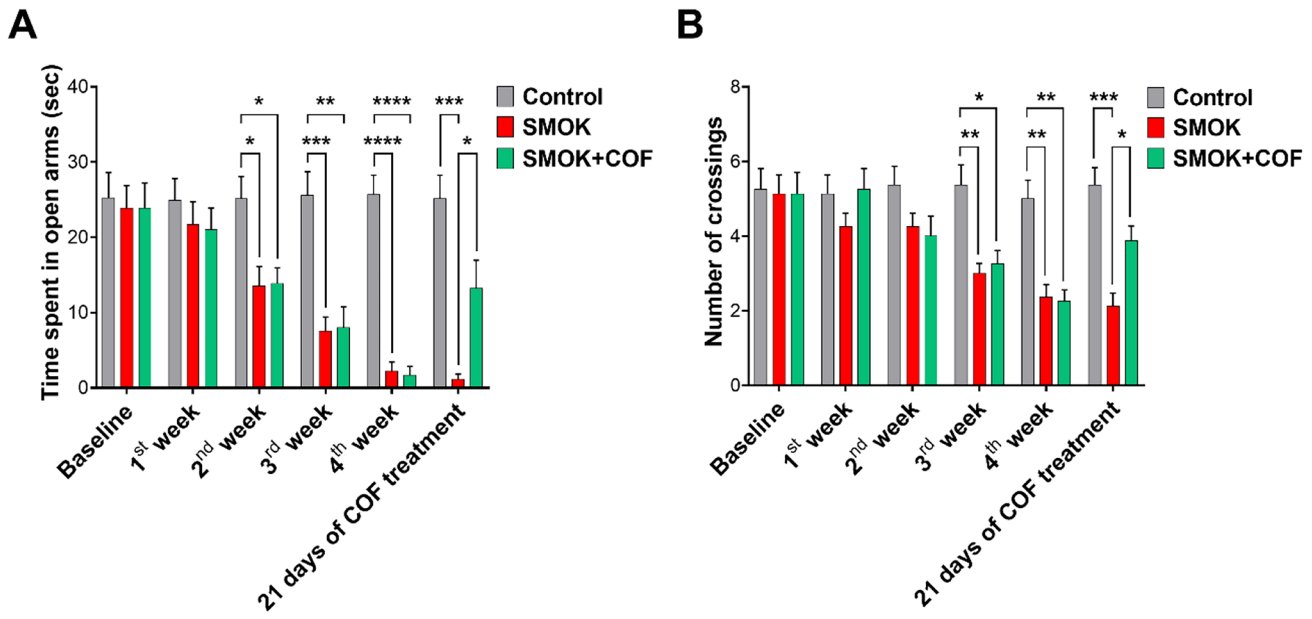

Fig. 5 Results of the elevated plus maze (EPM) behavioral test. **A** Time spent in the open arm of the EPM, and **B** number of crossings, in control, SMOK, and SMOK + COF groups. Data are presented as mean ± SEM ($n = 6$); *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$ (COF, coffee nanoassemblies; SMOK, exposed to cigarette smoke)

decrease in the time spent in the dark chamber at the 2nd and 3rd weeks of exposure in the SMOK and the SMOK + COF groups compared to the control group. While in the 4th week, there was a significant decrease in the time spent in the dark chamber for the SMOK + COF group compared to the control group. This effect was reversed by giving the rats 21 daily doses of 20 mg/kg of the coffee nanoassemblies (Fig. 4B). Consistent with the OF test, animals treated with 20 mg/kg of the coffee nanoassemblies for 21 days without cigarette smoke exposure showed no significant difference in the time spent in the light chamber and latency to enter the dark chamber in the DLB compared with the control group (SI; Fig. S2).

In the EPM test, the anxiety-like behavior resulting from cigarette smoke withdrawal progressed over the first four weeks of exposure as indicated by the time spent in the open arms and number of crossings (Fig. 5). This effect was reversed by 21 daily doses of 20 mg/kg of the coffee nanoassemblies. For time spent in the open arms, repeated measures two-way ANOVA revealed a significant main effect of time [$F(5, 120) = 44.20$, $p < 0.0001$], treatment [$F(2, 24) = 9.763$, $p = 0.0008$], and time × treatment interaction [$F(10, 120) = 14.72$, $p < 0.0001$]. Bonferroni's multiple comparisons test showed a significant decrease in time spent in open arm at the end of the 2nd, 3rd, and 4th weeks of exposure in the SMOK and SMOK + COF groups relative to the control group. After 21 days of treatment, the SMOK group exhibited a significant difference in time spent in open arms compared with the SMOK + COF and control groups (Fig. 5A).

A similar pattern of effects was seen for the number of crossings. Repeated measures two-way ANOVA revealed a significant main effect of time [$F(5, 105) = 14.68$, $p < 0.0001$], treatment [$F(2, 21) = 7.122$, $p = 0.0044$], and time × treatment interaction [$F(10, 105) = 4.888$, $p < 0.0001$]. Bonferroni's multiple comparisons test confirmed a significant difference between the SMOK and SMOK + COF groups compared to the control group starting from the 3rd and 4th weeks of exposure. This effect was reversed after administering 21 daily doses of the coffee nanoassemblies. At the same time, there was a significant difference in the number of crossings in the NIC group compared to the SMOK + COF and control groups (Fig. 5B). In addition, daily treatment with 20 mg/kg of the coffee nanoassemblies for 21 days without cigarette exposure showed no significant difference in the time spent in open arms and number of crossings in the EPM relative to the Control group (SI; Fig. S3).

Effect of whole-body cigarette smoke exposure and coffee nanoassemblies treatment on glt-1, xct, and nf-κb mRNA expression in the PFC brain region

As shown in Fig. 6A, whole-body cigarette smoke exposure for 41 days had no effect on relative *glt-1* mRNA expression in the PFC among the different treatment groups. This pattern of effect was confirmed by one-way ANOVA revealing no significant main effect of Treatment on *glt-1* mRNA relative expression in the PFC [$F(3, 16) = 1.537$, ns].

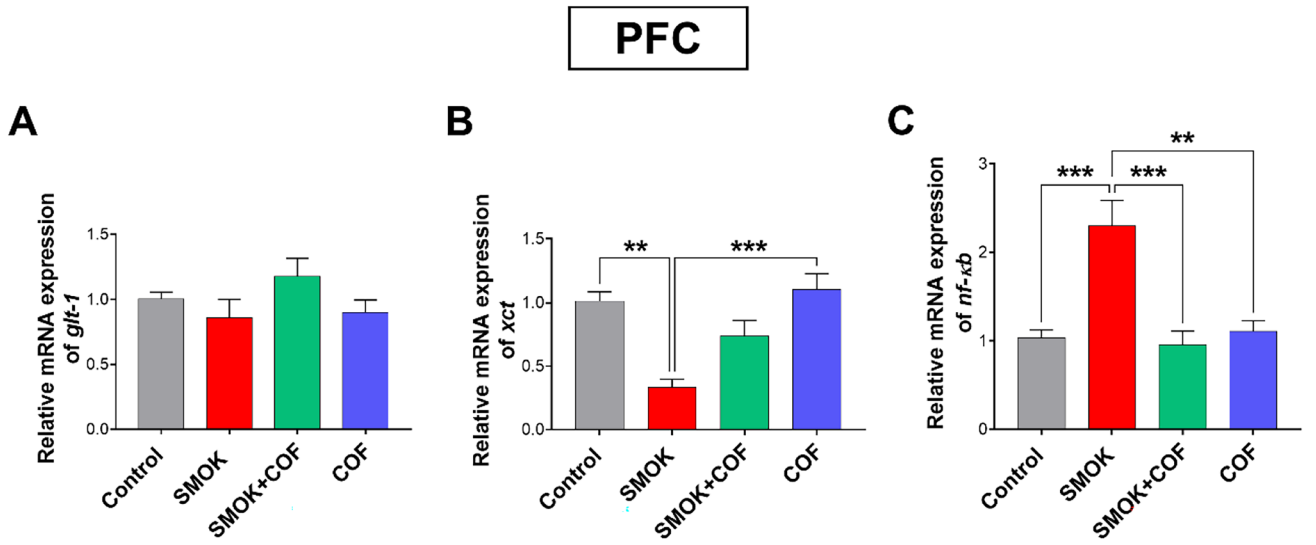

Fig. 6 Relative mRNA expression in the prefrontal cortex (PFC) brain region of **A** glutamate transporter-1 (*glt-1*), **B** cystine/glutamate antiporter (*xct*), and **C** nuclear factor-kappa B (*nf-κb*), after cigarette smoke exposure and coffee nanoassemblies treatment. Data are presented as mean ± SEM ($n = 5$); $^{**}p < 0.01$, and $^{***}p < 0.001$ (COF, coffee nanoassemblies; SMOK, exposed to cigarette smoke)

Meanwhile, a significant decrease in *xct* mRNA expression in the PFC was observed in the SMOK group compared to the control and COF groups after whole-body cigarette smoke exposure for 41 days. This pattern of effect was confirmed by one-way ANOVA revealing a significant effect of treatment on the *xct* mRNA expression levels in the PFC [$F(3, 16) = 11.85$, $p = 0.0002$; Fig. 6B]. Moreover, a significant upregulation in *nf-κb* mRNA expression levels in the PFC was revealed in the SMOK group compared to the control, SMOK + COF, and COF groups after whole-body cigarette smoke exposure for 41 days. This effect was confirmed by one-way ANOVA revealing a significant effect of treatment [$F(3, 16) = 12.64$, $p = 0.0002$; Fig. 6C].

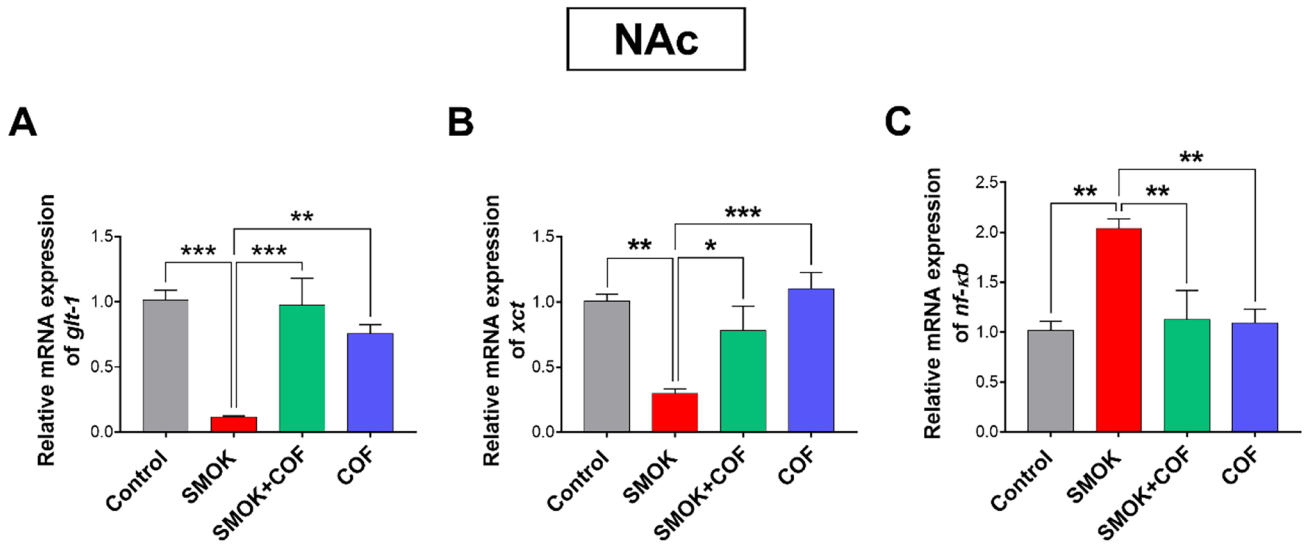

Fig. 7 Relative mRNA expression in the nucleus accumbens (NAc) brain region of **A** glutamate transporter-1 (*glt-1*), **B** cystine/glutamate antiporter (*xct*), and **C** nuclear factor-kappa B (*nf-κb*), after cigarette smoke exposure and coffee nanoassemblies treatment. Data are presented as mean ± SEM ($n = 5$); $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ (COF, coffee nanoassemblies; SMOK, exposed to cigarette smoke)

Effect of whole-body cigarette smoke exposure and coffee nanoassemblies treatment on glt-1, xct, and nf-κb mRNA expression levels in the NAc brain region

As shown in Fig. 7A, whole-body cigarette smoke exposure for 41 days caused a significant decrease in relative *glt-1* mRNA expression in the NAc of the SMOK group compared to the control, SMOK + COF, and COF groups. This pattern of effect was confirmed by one-way ANOVA revealing a significant main effect of treatment [F (3, 16) = 13.21, $p = 0.0001$]. Additionally, a significant decrease in *xct* mRNA expression in the NAc was observed in the SMOK group compared to the control, SMOK + COF, and COF groups after whole-body cigarette smoke exposure for 41 days. This pattern of effect was confirmed by one-way ANOVA revealing a significant effect of treatment [F (3, 16) = 9.454, $p = 0.0008$; Fig. 7B]. Moreover, whole-body cigarette smoke exposure for 41 days resulted in a significant upregulation in *nf-κb* mRNA expression in the NAc region of the SMOK group compared to the control, SMOK + COF, and COF groups. This effect was confirmed by one-way ANOVA revealing a significant effect of treatment [F (3, 16) = 7.579, $p = 0.0022$; Fig. 7C].

Effect of whole-body cigarette smoke exposure and coffee nanoassemblies treatment on GLT-1, xCT, and NF-κB protein expression in the PFC brain region

The results of the gene expression analysis were complemented by analyzing protein expression levels of GLT-1, xCT, and NF-κB in the PFC. Consistent with the results in Fig. 6A, whole-body cigarette smoke exposure for 41 days had no effect on GLT-1 levels in the PFC among the different treatment groups. This pattern of effect was confirmed by one-way ANOVA revealing no significant main effect of treatment [F (3, 8) = 1.355, ns; Fig. 8A]. However, a significant decrease in xCT levels in the PFC was observed in the SMOK group compared to the control and COF groups after 2 h/day of whole-body cigarette smoke exposure for 41 days. This pattern of effect was confirmed by one-way ANOVA revealing a significant effect of treatment [F (3, 8) = 6.493, $p = 0.0155$; Fig. 8B]. Moreover, a significant upregulation in NF-κB levels in the PFC was exhibited by the SMOK group compared to the control, SMOK + COF, and COF groups after whole-body cigarette smoke exposure for 41 days. This effect was confirmed by one-way ANOVA revealing a significant effect of treatment [F (3, 8) = 21.16, $p = 0.0004$; Fig. 8C].

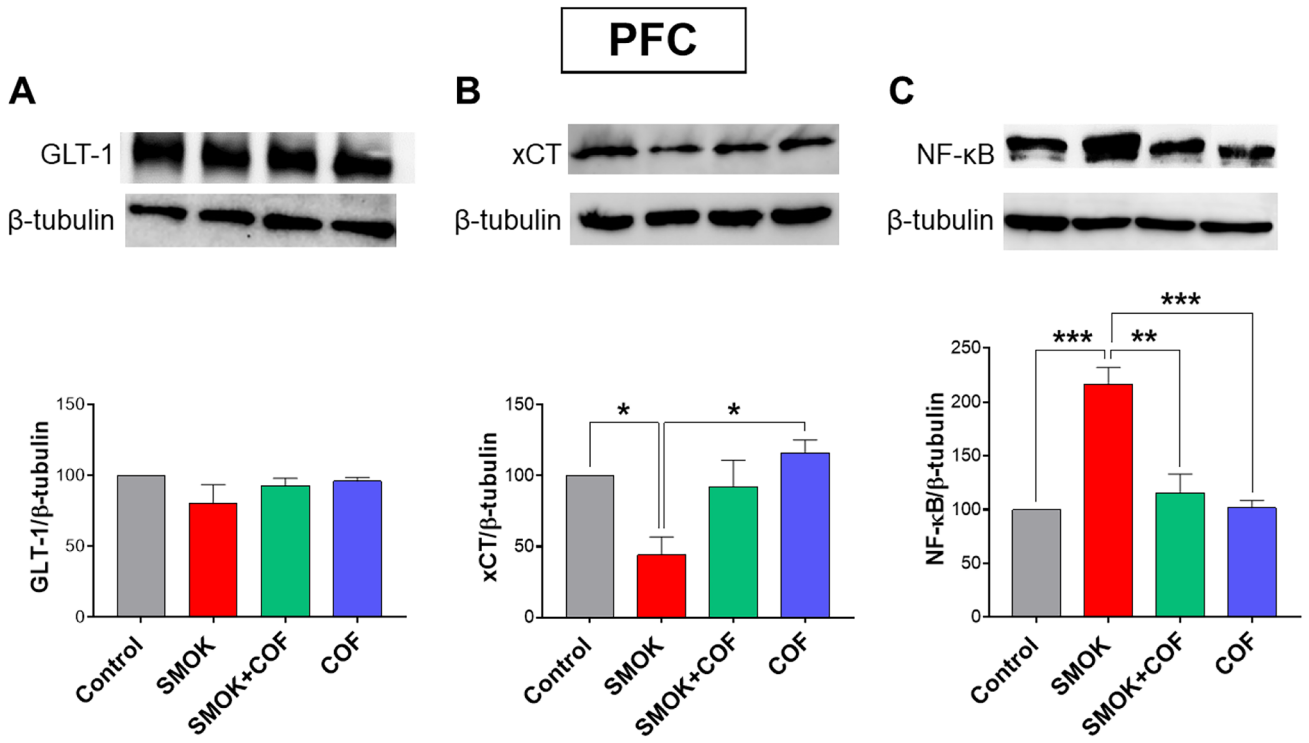

Fig. 8 Protein expression levels in the prefrontal cortex (PFC) brain region of **A** glutamate transporter-1 (GLT-1), **B** cystine/glutamate antiporter (xCT), and **C** nuclear factor-kappa B (NF-κB), following cigarette smoke exposure and coffee nanoassemblies treatment. Data are presented as mean ± SEM ($n = 5$); *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ (COF, coffee nanoassemblies; SMOK, exposed to cigarette smoke)

Effect of whole-body cigarette smoke exposure and coffee bean extract treatment on GLT-1, xCT, and NF-κB protein expression in the NAc brain region

Protein expression analysis confirmed the results of the gene expression analysis of *glt-1*, *xct*, *and nf-κb* in the NAc. As shown in Fig. 9A, whole-body cigarette smoke exposure at 2 h/day for 41 days caused a significant decrease in relative GLT-1 levels in the NAc of the SMOK group compared to the control, SMOK + COF, and COF groups ($p < 0.0001$). This pattern of effect was further confirmed by one-way ANOVA revealing a significant main effect of treatment [F (3, 8) = 61.77, $p < 0.0001$]. In addition, a significant decrease in xCT expression in the NAc was observed in the SMOK group compared to the control, SMOK + COF, and COF groups after whole-body cigarette smoke exposure for 41 days. This pattern of effect was confirmed by one-way ANOVA revealing a significant effect of treatment [F (3, 8) = 10.59, $p = 0.0037$; Fig. 9B]. Moreover, a significant upregulation in NF-κB levels in the NAc was revealed in the SMOK group compared to the control, SMOK + COF, and COF groups after 2 h/day whole-body cigarette smoke exposure for 41 days. This effect was confirmed by one-way ANOVA revealing a significant effect of treatment [F (3, 8) = 18.18, $p = 0.0006$; Fig. 9C].

Discussion

Plant polyphenols have received considerable attention over the past few years as precursors for various functional materials including nanoparticles (NPs) [33]. Plant polyphenol–derived NPs are typically synthesized by taking advantage of the propensity of these molecules to undergo oxidative coupling reactions under various conditions that control NP self-assembly behavior. Examples include NPs from quercetin [34–37], tea polyphenols [38], tannic acid [39], and grape seed extracts [40], which were developed for various biomedical applications including drug delivery and amelioration of oxidative stress. This led us to investigate our recently reported nanoassemblies derived from coffee bean extracts as potential modulators of anxiety-like behavior linked to cigarette smoking withdrawal.

This study revealed that exposure to cigarette smoke for 2 h per day, for five consecutive days followed by two days with no cigarette exposure per week, for 41 days, caused withdrawal-induced anxiety in female rats. Importantly, treatment with the antioxidant-rich nanoassemblies from dark-roasted coffee beans significantly attenuated this effect. Exploring the underlying molecular effects, we showed that cigarette smoke exposure affected glutamate homeostasis in the brain by decreasing the expression levels of GLT-1 and xCT and

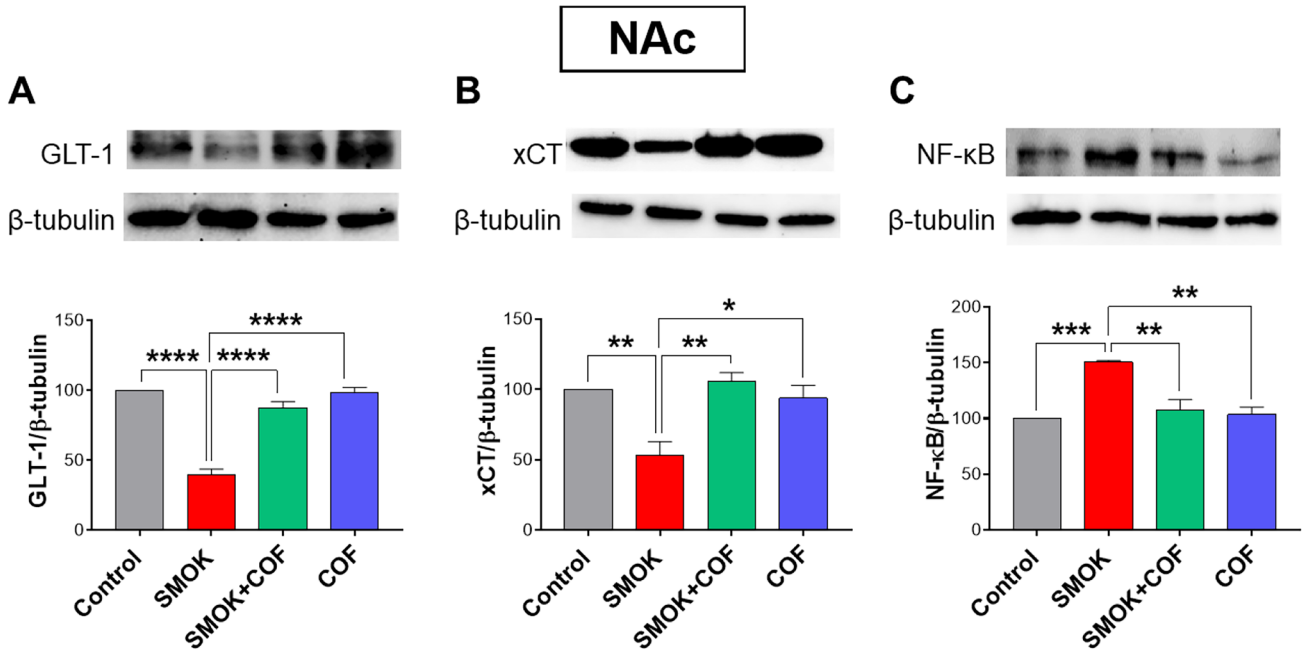

Fig. 9 Protein expression levels in the nucleus accumbens (NAc) brain region of **A** glutamate transporter-1 (GLT-1), **B** cystine/glutamate antiporter (xCT), and **C** nuclear factor-kappa B (NF-κB), following cigarette smoke exposure and coffee nanoassemblies treatment. Data are presented as mean ± SEM ($n = 5$); *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.001$ (COF, coffee nanoassemblies; SMOK, exposed to cigarette smoke)

upregulating NF-κB in subregions of the mesocorticolimbic pathways, namely, the PFC (for xCT and NF-κB) and NAc (for all three markers). These effects were normalized after daily treatment with the coffee nanoassemblies for 21 days.

A previous study has reported that adolescent female rats are more likely to self-administer nicotine than adolescent male rats, and nicotine intake is higher in females [41]. However, not all studies reported sex-dependent differences [42, 43]. The majority of research does report sex-dependent variations in nicotine reactions, demonstrating that females are more susceptible. It is important to note that a prior study found that nicotine had a more rewarding effect on teenage female rats than on teenage male rats [44]. Additionally, female rats exhibit more significant behavioral sensitization to nicotine than male rats [45]. Therefore, we decided to employ female rats in this study.

Ample data have revealed that nicotine or cigarette smoking withdrawal induce anxiogenic effects [23, 24]. In fact, somatic symptoms in male rats exposed to high-nicotine cigarette smoke gradually increased over time, demonstrating that the degree of dependence grew over time [24]. Numerous assays are used to measure anxiety-like behavior in rodents including, EPM, OF, and DLB [46]. These tests are based on rodents' innate propensity to avoid open spaces and/or surroundings with strong lighting. The animal can decide where it will spend its time when it is placed directly into the test equipment (i.e., forced exposure), such as close to the open field's walls, inside the plus-maze's closed arms, or inside the dark compartment of the light/dark apparatus. Alternately, it might investigate regions of the device that could be riskier (such as the open field's center, the plus-maze's open arms, or the light compartment). Anxiolytics inhibit behaviorally selective avoidance of the risk locations, while anxiogens induce the opposite behavioral hallmark of increased avoidance [47].

Our results showed that anxiety-like behavior increased in cigarette smoke-exposed rats as evidenced by the decreased time spent in the open arm and open arm entries measured in the EPM, as well as the distance traveled and time spent in the center zone measured in the OF [25]. This finding is similar to one that was made in an open field "conflict" test conducted on adolescents with nicotine exposure [26]. Other studies have also shown that adult rats' short-term nicotine withdrawal caused an anxiety reaction. For instance, in adult rats, repeated nicotine injections followed by a brief period of abstinence (24 h) augmented anxiety-like behaviors in the EPM [48, 49].

Data have connected anxiety-like behavior to alterations in synaptic activity brought on by glutamatergic marker dysregulation [50]. Moreover, a growing number of studies have shown that glutamate neurotransmission is essential for the etiology of anxiety disorders [51, 52]. Importantly, glutamate neurotransmission has been linked to anxiety in various animal models [53]. According to prior studies, GLT-1 downregulation is a consistent neuroadaptation mechanism that has been observed across drug classes, and GLT-1 expression restoration is associated with a decrease in cue-induced reinstatement of cocaine craving [35]. A number of investigations have also demonstrated that xCT can alter extracellular glutamate concentrations [36, 37]. Several studies have reported that xCT is essential for relapse in nicotine addiction and seeking behavior [38]. These findings imply that xCT might be useful in the management of nicotine dependence. In the PFC of rats fed nicotine orally or by mini-pumps for 21 days [39] or in mice exposed intermittently to nicotine-containing e-cigarette vapor [40], there were no reported changes in GLT-1 or xCT [54]. On the other hand, GLT-1 and xCT protein expression were found to be downregulated in the NAc of nicotine-self-administering rats in earlier studies, while restoration of GLT-1 expression was associated with a decrease in nicotine self-administration [36, 37]. GLT-1 protein expression was also dramatically downregulated in the NAc following cue-induced nicotine reinstatement [38]. Additionally, chronic obstructive pulmonary disease (COPD) patients and smokers showed greater levels of NF-κB activation and expression in their bronchial biopsies, which was linked to decreased airflow [42]. Following exposure to cigarette smoke, an animal model showed increased NF-κB expression in lung tissue as well [26]. In fact, previous studies from our lab have also shown that rats subjected to waterpipe tobacco smoke have increased NF-κB expression [39].

Numerous dietary polyphenols have been shown to have anxiolytic and antidepressant-like characteristics, in both animal and human studies [55–57]. Importantly, previous studies have reported that chlorogenic acid, a polyphenol commonly found in coffee beans [58], decreased anxiety-related behaviors and improved motor function in rodents [59, 60]. Parallel results were found in the current study where the polyphenol-rich coffee nanoassemblies ameliorated anxiety-like behavior observed in cigarette smoke-exposed rats.

Several biological characteristics of polyphenols, such as their antioxidant [61], anti-inflammatory [62, 63], and neuroprotective [64] effects, have motivated the scientific community to investigate them as important modulators of biological functions. Multiple studies into the mechanism of action of polyphenols have established that they reduce the expression of proinflammatory mediators including tumor necrosis factor-alpha (TNF-α), interleukin-1 (IL-1), and inducible nitric oxide synthase (iNOS), thereby protecting against neuroinflammation [65, 66]. According to a study done on 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-intoxicated mice, polyphenols, particularly chlorogenic acid, protected against dopaminergic neuronal degeneration by downregulating the NF-κB-mediated neuroinflammatory pathway in

Parkinson's disease [65]. Another study revealed that chlorogenic acid could reverse the upregulation of NF-κB protein expression and protect hippocampus injury induced by cerebral ischemia [67]. Similarly, the polyphenolic extract from Tarocco (*Citrus sinensis* L. Osbeck) had the ability to reduce inflammation in part due to its ability to prevent NF-κB nuclear translocation and subsequent upregulation of immune markers [68].

Collectively, these reports demonstrate that the anti-inflammatory activity of polyphenols is largely attributed to their effect on the inflammatory mediator NF-κB. This is in accordance with our results, which showed that treatment with the coffee nanoassemblies attenuated the upregulation of NF-κB in mesocorticolimbic brain subregions caused by cigarette smoke exposure. The fact that the coffee nanoassemblies could be conveniently synthesized by spontaneous oxidation of the coffee bean extracts imparts an additional advantage where these biocompatible and easy-to-synthesize nanomaterials can be used as carriers for other active molecules in the treatment of inflammatory disorders for potential synergistic effects. Furthermore, no previous study has linked glutamate transporter levels with polyphenols. We have shown here, for the first time, that treatment with the antioxidant-rich coffee nanoassemblies can modulate dysregulation in glutamate transporters, including GLT-1 and xCT, caused by whole-body cigarette smoke exposure. It is worth noting that sex-related differences could also play a role in anxiety-like behavior exhibited by the female rats. In addition, it would be interesting to investigate nanoassemblies from other coffee types and polyphenols and compare them with unprocessed coffee extracts containing caffeine for their potential role in modulating anxiety-like behavior following cigarette smoking exposure.

Conclusion

This study confirmed a causal link between smoking withdrawal–induced anxiety and altered glutamate homeostasis in mesocorticolimbic subregions of the brain of female rats exposed to cigarette smoke. Treating cigarette smoke–exposed animals daily with antioxidant-rich nanoassemblies extracted from roasted coffee beans for 21 days modulated dysregulation in glutamate transporters GLT-1 and xCT in the PFC and NAc brain regions caused by whole-body cigarette smoke exposure. Additionally, the coffee nanoassemblies treatment attenuated the upregulation of NF-κB in the same brain regions. These molecular effects translated into normalization of anxiety-like behavior in rats during periods of smoking withdrawal as confirmed by OF, DLB, and EPM behavioral tests. Our findings strongly support the beneficial role of natural antioxidants such as coffee bean polyphenols in reducing neuroinflammation and the accompanying anxiety-like behavior caused by cigarette smoke exposure and withdrawal. Not only does this have implications for anxiety-linked behavioral disorders, but also other neurological conditions linked to neuroinflammation.

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Author contribution All authors contributed to the study conception and design. Material preparation, data collection, and analysis were performed by Alaa M. Hammad, Lujain Alzaghari, and Malek Alfaraj. The first draft of the manuscript was written by Alaa M. Hammad, Lujain F. Alzaghari, and Suhair Sunoqrot. All authors read and approved the final manuscript.

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Data availability All data generated or analyzed during this study are included in this published article and its supplementary information files.

Declarations

Ethics approval The housing and treatments of animals used in this study were in accordance with the Declaration of Helsinki. The study protocol was approved by the Animal Use and Care Committee of Al-Zaytoonah University of Jordan (decision no. 26/12/2019–2020).

Consent to participate Not applicable.

Consent for publication Not applicable.

Competing interests The authors declare no competing interests.

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