Assessment of mechanism involved in the apoptotic and anti-cancer activity of Quercetin and Quercetin-loaded chitosan nanoparticles

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Abstract
In prior studies, Quercetin was revealed to exhibit anti-cancer features in a variety of cancer cell lines. However, the impact of Quercetin on neuroblastoma is unknown. This study looked into the potential cytotoxic effects of Quercetin and Quercetin-loaded chitosan nanoparticles (NPs) on the SH-SY5Y cell line. In this study, NPs containing Quercetin was prepared and characterization studies were performed. The vitality of the cells was measured using the XTT test after 24 h of treatment with various concentrations of Quercetin (0.5, 1, 2, 4, and 8 µg/mL). ELISA kits were used to detect the amounts of cleaved PARP, BCL-2, 8-Hydroxy-deoxyguanosine (8-oxo-dG), cleaved caspase 3, Bax, total oxidant status, and total antioxidant status in the cells. The results of the chitosan NPs characterization investigation revealed that the particle size, encapsulation effectiveness, and drug release profile of NPs were all appropriate for cell culture studies. Quercetin and Quercetin-loaded chitosan NPs significantly reduced cell viability in SH-SY5Y cells at different concentrations (**p < 0.05). 2 µg/mL Quercetin and Quercetin-loaded chitosan NPs significantly enhanced the levels of 8-oxo-dG, cleaved caspase 3, Bax, cleaved PARP, and total oxidant in ELISA testing. However, treatment with 2 µg/mL of Quercetin and Quercetin-loaded chitosan NPs did not affect the amount of BCL-2 protein. Overall, Quercetin and Quercetin-loaded chitosan NPs caused significant cytotoxicity in SH-SY5Y cells via producing oxidative stress, DNA damage, and eventually apoptosis.

Keywords Quercetin · Quercetin-loaded chitosan NPs · SH-SY5Y cell · DNA damage · ELISA · Apoptosis

Introduction
Cancer is a significant health problem and is the second leading cause of death. Cancer is both a disease and includes a variety of illnesses with every system and organ growing a various set of ailments [1, 2]. About one-third of cancer deaths are related to smoking, lifestyle, or dietetic practices. In addition, several types of cancers are avoidable by modifying unhealthy lifestyle habits [3, 4].

Surgery, radiation therapy, chemotherapy, immunotherapy, targeted therapy, and hormone therapy are some of the traditional therapeutic approaches and equipment used in cancer treatment [5, 6]. These approaches are frequently associated with adverse effects and a significant risk of recurrence. Neuropathies, bone marrow suppression, skin, and gastrointestinal issues, and hair loss are some of the most prevalent side effects. The fact that traditional treatment methods used in cancer treatment have the stated side effects has paved the way for researching new treatment methods [7, 8]. Nanotechnology-based drug delivery systems are promising in the treatment of cancer and pioneering these studies [9, 10]. Nanoparticles delivery systems provide significant benefits, with biocompatibility, multi-functional encapsulation of active substances, high bioavailability, easy tumor targeting, sustained blood circulation time, active and passive targeting, and reduced or eliminated side effects [11, 12].

Nanomedicine with anti-cancer features can provide anti-cancer efficacy mainly via increasing cytotoxic drug quantity in tumor area hence stimulating anti-cancer activity [13, 14]; decreasing its clearance and lowering side effects in the normal organs thus reducing undesired toxicity, and delivering various anti-cancer active substances within the same process [15–17].

Chitosan is a deacetylated (DA) chitin product that is widely employed in the medicinal and pharmaceutical
industries due to its biodegradability, biocompatibility, and low toxicity [18, 19]. It can also be used for medication transport, packaging, antibacterial agents, tissue engineering, antiaging agents, antibody response enhancers, and the treatment of diseases such as cancer treatment [20, 21]. It’s commonly used in nanoapplications including medicine delivery [22].

Quercetin is an antioxidant flavonoid that can be found in a variety of fruits and vegetables. It has anti-cancer qualities, according to research. Quercetin has been extensively researched as a chemopreventive drug displaying anti-inflammatory and antioxidant effects (mostly by scavenging reactive oxygen/nitrogen species) in a variety of in vitro and in vivo cancer models. Furthermore, Quercetin has been shown to inhibit cell proliferation, differentiation, and survival in specific types of cancer cells by targeting critical chemicals involved in tumor cell growth [23, 24]. Due to the anti-proliferative and antioxidant activity of Quercetin, this material was chosen in the study.

In this assay, quercetin was encased in nano-sized particles of chitosan, a biologically friendly and bio-degradable cationic polymer. In XTT and bioactivity experiments, the effects of quercetin and quercetin nanoparticles were investigated on NIH 3T3 and SH-SY5Y cell lines. In this study, the human ELISA kits of 8-Hydroxy-Desoxyguanosine (8-oxo-dG), cleaved caspase 3, BCL-2, cleaved PARP and Bax were used to assess the amounts of cleaved caspase 3, cleaved PARP, 8-oxo-dG, BCL-2 and Bax in Quercetin and Quercetin-loaded NPs treated and untreated SH-SY5Y cells. In addition, necessary characterization studies on nanoparticles were performed in terms of the suitability and usability of quercetin-loaded nanoparticles in XTT and bioactivity studies.

**Materials and methods**

**Cell culture studies**

In the cytotoxic activity study, SH-SY5Y (neuroblastoma) cell line (ATCC CRL 2266) and NIH 3T3 (fibroblast) cell line (ATCC CRL-1658) were obtained from American Type Culture Collection. Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum (FBS), and Quercetin were purchased from Merck Millipore. Chitosan (400 kDa, DD 92), phosphate buffer saline (PBS), and polyvinyl pyrrolidone (PVP) were obtained from Sigma-Aldrich. Penicillin–Streptomycin–l-glutamine solution was purchased from Sigma-Aldrich. XTT reagent (Roche Diagnostic) was utilized in cytotoxic activity studies. SH-SY5Y and NIH 3T3 cells were seeded in DMEM containing FBS (10%), penicillin (100 IU/mL), l-glutamine (1%), and streptomycin (10 mg/mL). Well plates including cells were incubated in an incubator at 5% CO₂ and 37 °C. The cytotoxic activity studies were performed when cells reached at least 80% confluence [25]. Cell viability was then calculated using following equation:

\[
\text{Cell viability(\%)} = \frac{\text{Absorbance of sample}}{\text{Absorbance of control}} \times 100
\]  

**Preparation of chitosan NPs**

Ionic gelation method was used to prepare NPs including Quercetin. The determined amount of chitosan was dissolved in acetic acid (0.5% v/v) at 1000 rpm under magnetic stirring. To obtain high encapsulation and loading capacity of NPs, the pH value of the chitosan solution should be between 4 and 5. The pH value of the chitosan solution was adjusted to 4.4 using 5 M sodium hydroxide solution [26, 27]. PVP was dissolved in sterile deionized water at a determined concentration (0.25% w/v). PVP solution including Quercetin dropped into chitosan solution (0.5% w/v). NP suspension was centrifuged at 10,000 rpm for 30 min. The supernatant was removed and 1 mL sample was separated from the supernatant to calculate the encapsulation efficiency (EE) and loading capacity (LC) of the NPs. Then, the pellet was washed with deionized water. Deionized water (30 mL) was added to the pellet and centrifuged at 10,000 rpm for 15 min. This process was repeated twice. Then NPs were lyophilized and stored at +4 °C.

**EE and LC studies of NPs**

Ultraviolet–visible (UV–vis) spectrophotometer was used to measure the EE % and LC % of Quercetin in NPs [28]. By reading the absorbance of Quercetin at various concentrations at a wavelength of 380 nm, the standard calibration curve of Quercetin was established and a spectral line equation was obtained. The amount of Quercetin in the supernatant was calculated from the line equation. The following Eqs. (2 and 3) were used to determine the encapsulation efficiency and loading capacity of the NPs:

\[
\text{EE(\%)} = \frac{(m_0 - m_s)}{m_0} \times 100
\]

\[
\text{LC(\%)} = \frac{(m_0 - m_s)}{w_{\text{np}}} \times 100
\]

where \(m_0\) is the initial mass of Quercetin and \(m_s\) mass of Quercetin in the supernatant and \(w_{\text{np}} = \text{total weight of Quercetin of NPs}\) [29, 30]. All measurements were performed in triplicate and were reported as mean ± SEM (\(n = 3\)).
Measurement of particle size and zeta (ζ) potential

The size and ζ potential measurements of NPs were evaluated via a Zetasizer Nano ZS instrument. In this study, NPs were suspended in PBS (pH 7.4) and measured.

In vitro release study of Quercetin-loaded NPs

In vitro release of Quercetin from chitosan NPs in PBS (pH 7.4) were performed according to methods with slight modifications of the release assay [30, 31]. Initially, a determined amount of Quercetin-loaded NPs was dispersed in 2 mL of buffer solution and maintained in a shaking water bath at 25°C. At predetermined time intervals, samples were centrifuged at 10,000 rpm for 10 min at 25°C. 800 μl of the supernatant was withdrawn for analysis, and was replaced with an equivalent volume of fresh buffer to maintain the total volume. The amount of released Quercetin at a specific time was determined using UV–vis spectrophotometer.

Cell viability assay

Cytotoxic activity of Quercetin and Quercetin-loaded NP were evaluated using the XTT assay on the SH-SY5Y and NIH 3T3 cells. Initially, cells were seeded in 96-well plates with DMEM (100 μL, 10% FBS) and incubated overnight [25, 32]. After that, the various concentrations (0.5, 1, 2, 4, and 8 μg/mL) of Quercetin and Quercetin-loaded NP were added to cells for 24 h. The samples were homogenized using pipetting and vortexing in DMEM and added to each well. Then, plates including cells and samples were incubated for 24 h. Following this period, wells were washed with the PBS (200 μL). Colorless DMEM (100 μL) and XTT (50 μL) reagents were added to each well and the cells were incubated for 4 h. A microplate ELISA reader was used to measure the absorbance of XTT-formazan at 450 nm. Cell viability and IC50 values on SH-SY5Y and NIH 3T3 cell lines of Quercetin and Quercetin-loaded NP were calculated.

The measurement of cleaved PARP, cleaved caspase 3, BCL-2, and Bax amounts

The human ELISA kits of 8-Hydroxy-Deoxyguanosine (8-oxo-dG) (BT Lab, catalog #E1436HU), cleaved caspase 3 (BT Lab, catalog # E6970HU), BCL-2 (BT Lab, catalog #E1832HU), cleaved PARP (BT Lab, catalog #E6971HU) and Bax (BT Lab, catalog #E1825HU) were used to assess the amounts of cleaved caspase 3, cleaved PARP, 8-oxo-dG, BCL-2 and Bax in Quercetin and Quercetin-loaded NPs treated and untreated SH-SY5Y cells. Initially, SH-SY5Y cells were inserted with DMEM into a 6-well plate and treated with 2 μg/mL Quercetin and Quercetin-loaded NP for 24 h. SH-SY5Y cells were treated with Quercetin and NP including Quercetin and those that were not gathered and diluted in PBS. Then they were frozen and thawed in triplicate. After that, the quantities of cleaved PARP, cleaved caspase 3, 8-oxo-dG, BCL-2, and Bax in cell lysates were examined in accordance with the manufacturer’s directions. Bradford protein assay kit (Merck Millipore, Darmstadt, Germany) was used to calculate the total protein quantities in Quercetin and Quercetin-loaded NP treated and untreated SH-SY5Y cells.

Total antioxidant status (TAS) and Total oxidant status (TOS) assessment in Quercetin and Quercetin-loaded NP treated and untreated SH-SY5Y cells

The total antioxidant status assay kit (Rel Assay Diagnostics, Turkey) and total oxidant status assay kit (Rel Assay Diagnostics, Turkey) were used to evaluate TAS and TOS quantities in Quercetin and Quercetin-loaded NP treated and untreated SH-SY5Y cells, respectively [33]. SH-SY5Y cells were treated with 2 μg/mL Quercetin and Quercetin-loaded NP for 24 h. For TAS and TOS, the data were expressed as mmol Trolox Equiv./L and mol H2O2 Equiv./L, respectively.

Statistical analysis

The findings were evaluated using one-way ANOVA and repeated measures ANOVA followed by a Tukey post hoc test (SPSS 14.0 for Windows) for multiple comparisons between groups. All results are presented as a mean ± SEM. The significance level was determined as **p < 0.05, ***p < 0.01 and *p < 0.1.

Results

EE and LC of NP including quercetin

EE rate shows quantity of Quercetin coated in chitosan NPs. The rate of the encapsulated Quercetin into chitosan NP was calculated and the results were shown in Table 1. Results indicated that, EE value of NPs including Quercetin was calculated at 74.52 ± 0.03%. In addition, LC of NPs was found as 8.87 ± 0.03%. According to the results, it can be concluded that both EE and LC values of NPs including Quercetin was suitable for the cell culture and bioactivity studies.

Characterization of chitosan NPs

Particle size, ζ potential, polydispersity index (PDI) of NPs were assessed, and the results are indicated in Table 2. The size of the NP was measured as 212.18 ± 1.40 nm. The ζ
The potential value of the NP was measured as 3.12 ± 0.03 mV. In addition, PDI value of NP was measured 0.234 ± 0.05. Each measurement was repeated three times. According to the results, it can be concluded that NP was homogeneous features without any aggregate.

**In vitro release kinetics study result of Quercetin-loaded NP**

The release profile of Quercetin from NP was investigated at 37 °C over a period of 240 h (Fig. 1). In this study, 0.1 M PBS was used in accordance with physiological conditions. The release results of Quercetin were shown a controlled release characterized by a fast initial release (50%) during the first 24 h, followed by a continuous and slower release (75%) till 120 h. Almost all of the Quercetin (99.58%) in the NP was released in 240 h. This type of continuous and slow release has been experienced in a study for acetylsalicylic acid (34). Diffusion and molecular matrix degradation of the Quercetin play an important role in the release of the Quercetin from the NP. Since the size of Quercetin is lower than the particle, Quercetin can easily diffuse from the surface or pores of the NP [28, 34].

**Assessment of cytotoxic activity results**

To calculate the IC_{50} values of Quercetin and NP including Quercetin at determined concentrations were treated to SH-SY5Y and NIH 3T3 cell lines. According to the results of the XTT study (Fig. 2) both Quercetin and the Quercetin-loaded NP significantly suppressed the SH-SY5Y cell viability depending on the increase in concentration. When the only Quercetin and the Quercetin-loaded NP were administered with SH-SY5Y cells at 0.5 µg/mL concentration, the cell viability rates were calculated as 69.08 ± 0.53% and 63.08 ± 0.51%, respectively. Quercetin and the Quercetin-loaded NP were treated with SH-SY5Y cells at the highest concentration (16 µg/mL), and the cell viability rates were calculated as 21.97 ± 0.29% and 15.97 ± 0.27%, respectively. According to the results of the cytotoxic activity study performed on SH-SY5Y cells, Quercetin-containing NP showed more effective (*p < 0.1 and **p < 0.05, Fig. 2) anti-proliferative activity at all concentrations of Quercetin and Quercetin-containing NP applied to the cells. According to these data IC_{50} values of Quercetin and NP including Quercetin were calculated. IC_{50} values of Quercetin and NP including Quercetin were 2.08 ± 0.03 µg/mL and 1.67 ± 0.02 µg/mL on SH-SY5Y cell line. The results indicate that the
Quercetin-loaded NP has a lower IC50 value and more cytotoxic activity in SH-SY5Y cells compared to the only Quercetin.

According to the concentration-dependent XTT assay results in the NIH 3T3 cell line (Fig. 3), the Quercetin and the NP including Quercetin were treated with NIH 3T3 cells at 0.5 µg/mL concentration, the cell viability was calculated as 90.08 ± 0.53% and 95.58 ± 0.47%, respectively. Quercetin and the Quercetin-loaded NP were administered with NIH 3T3 cells at 4 µg/mL concentration, and the cell viability was calculated as 86.30 ± 0.11% and 90.86 ± 0.08%, respectively. Quercetin and the Quercetin-loaded NP were treated with NIH 3T3 cells at the greatest concentration (16 µg/mL), the cell viability was calculated as 80.97 ± 0.29% and 86.10 ± 0.33%, respectively. According to the results IC50 values of the Quercetin and NP containing the Quercetin at the determined concentrations could not be calculated.

The effect of Quercetin and Quercetin-loaded NPs on Bax, cleaved caspase 3, BCL-2, cleaved PARP, and 8-oxo-dG quantities in SH-SY5Y cells

ELISA was used to assess the expression of proteins associated with apoptosis in SH-SY5Y cells, such as Bax, cleaved caspase 3, BCL-2, cleaved PARP, and 8-oxo-dG. The treatment with Quercetin and Quercetin-loaded NP (2 µg/mL) for 24 h significantly increased Bax quantity (**p < 0.01; Fig. 4A). According to the results, Bax quantities of control, Quercetin and Quercetin-loaded NP was measured as 21.90 ± 0.29 ng/mg protein, 34.94 ± 0.36 ng/mg protein, and 40.16 ± 0.54 ng/mg protein, respectively. According to the results, cleaved caspase 3 quantities of control, Quercetin and Quercetin-loaded NP was measured as 651.90 ± 4.40 pg/mg protein, 867.37 ± 5.82 pg/mg protein, and 925.32 ± 5.06 pg/mg protein, respectively (Fig. 4B). According to the results, BCL-2 quantities of control, Quercetin and Quercetin-loaded NP was measured as 54.22 ± 0.38 ng/mg protein, 53.22 ± 0.28 ng/mg protein, and 51.15 ± 0.60 ng/mg protein, respectively (Fig. 4C).

Cleaved PARP quantities of control, Quercetin and Quercetin-loaded NP was measured as 848.49 ± 2.54 pg/mg, 986.14 ± 5.32 pg/mg protein, and 1038.28 ± 5.24 pg/mg protein, respectively (Fig. 5A). DNA damage effects of Quercetin and Quercetin-loaded NP was evaluated via performing to 8-oxo-dG expression assay. 8-oxo-dG quantities of control, Quercetin and Quercetin-loaded NP was measured as 46.63 ± 0.37 ng/mg, 68.74 ± 0.45 ng/mg protein, and 74.92 ± 0.56 ng/mg protein, respectively (Fig. 5B). These results showed that Quercetin and Quercetin-loaded NP significantly increased the quantity of cleaved caspase 3, cleaved PARP, and 8-oxo-dG in SH-SY5Y cells. However, Quercetin and Quercetin-loaded NP had no effect on BCL-2 quantity (p > 0.05).
The effect of Quercetin and Quercetin-loaded NPs on TAS and TOS quantities in SH-SY5Y cells

The effect of Quercetin and Quercetin-loaded NP on TAS and TOS quantities in SH-SY5Y cells was investigated using a TAS and TOS assay kit. Both Quercetin and Quercetin-loaded NP had no effect on TAS quantity ($p > 0.05$). In addition, the Quercetin and Quercetin-loaded NP increased in TOS quantity in SH-SY5Y cells ($p < 0.05$). TAS value of control, Quercetin and Quercetin-loaded NP was measured as $0.666 \pm 0.018 \mu g/mL$, $0.647 \pm 0.016 \mu g/mL$, and $0.682 \pm 0.011 \mu g/mL$, respectively. Additionally,
TOS value of control, Quercetin and Quercetin-loaded NP was measured as 36.946 ± 0.281 μg/mL, 45.336 ± 0.550 μg/mL, and 52.276 ± 0.589 μg/mL, respectively (Fig. 6).

Discussion

Drug resistance, recurrence, and metastasis have hampered neuroblastoma treatment in many developing and developed countries around the world, despite the use of sophisticated chemotherapy and radiotherapy in cancer treatment. Antioxidant, anti-inflammatory, and anti-cancer characteristics are among the bioactive qualities of quercetin. On SH-SY5Y cells, Quercetin and Quercetin-loaded NP demonstrated a concentration-dependent cytotoxic impact. After 24 h, it dramatically reduced SH-SY5Y cell reproduction in a concentration-dependent manner, with an IC50 values of Quercetin and NP of 2.08 ± 0.03 µg/mL and 1.67 ± 0.02 µg/mL on SH-SY5Y cell line. In particular, NP containing Quercetin showed a significantly more potent cytotoxic effect on SH-SY5Y cells compared to Quercetin and the control group. In this context, the cytotoxic effects of Quercetin and Quercetin-loaded NP on healthy NIH 3T3 cells were investigated, and the results revealed that Quercetin and NP had no significant cytotoxic effects on NIH 3T3 cells.

Apoptosis is widely known to play a function in tumor cellular development and to influence the outcome of pharmacological therapies [35]. To confirm the apoptotic effect of Quercetin on SH-SY5Y cells, the levels of cleaved PARP, cleaved caspase 3, Bax, and BCL-2 were evaluated by ELISA. The onset of cell apoptosis, which is a key focus of cancer treatment [36], is one of the defense mechanisms against tumor growth and development. The BCL-2 protein family contains both pro-apoptotic and anti-apoptotic proteins, and the ratio of pro-apoptotic-to-anti-apoptotic proteins is commonly utilized to determine cell fate.

The apoptosome complex is formed when the apoptosis-inducing protein Bax disrupts the mitochondrial membrane, releasing cytochrome c. Apoptosis is triggered by this complex, which activates effector caspases. BCL-2, on the other hand, preserves membrane stability, suppresses cytochrome c release, and prevents apoptosis [37].

Caspases are a family of enzymes that are critical apoptosis effectors, and their activation is a key feature of the process. PARP is also an important component of DNA repair pathways, particularly in the repair of base excisions, and its cleavage or suppression induces cell death by exploiting a DNA repair deficiency [38].

In this study 2 μg/mL Quercetin and Quercetin-loaded NP significantly increased Bax, cleaved caspase 3, and PARP protein expressions, but no potential effect on anti-apoptotic BCL-2 expression. When compared to Quercetin alone and the control group, Quercetin-loaded NP samples dramatically enhanced Bax, cleaved caspase 3, and PARP amount.

Fig. 6 Quercetin and Quercetin-loaded NP at dose of 2 μg/mL didn’t cause a significant change in the TAS level A but increased the TOS level B of SH-SY5Y cells significantly. Results are represented as mean ± SEM.
To test if the cytotoxic impact of Quercetin and Quercetin-loaded NP samples is connected with DNA damage, the 8-oxo-dG ELISA technique was used to investigate DNA fragmentation in SH-SY5Y cells after 24 h of Quercetin and Quercetin-loaded NP samples administration. 8-oxo-dG is a well-known DNA oxidative damage indicator [38–40]. The amount of 8-oxo-dG in SH-SY5Y cells was significantly increased after treatment with Quercetin and Quercetin-loaded NP at 2 g/mL, suggesting that Quercetin and Quercetin-loaded NP had cytotoxic and apoptotic effects.

The majority of anti-cancer drugs act by creating oxidative stress in tumor cells, which is regarded to be the root of most macromolecular changes in the cell. The total oxidant status is one of the numerous measures used to estimate oxidative stress and is thus frequently used to evaluate the overall oxidative condition of cells. The overall antioxidant status of cells is also assessed using the total antioxidant status.

We wanted to see whether Quercetin and Quercetin-loaded NP could cause cytotoxicity by increasing total oxidant status levels. Exposure to Quercetin and Quercetin-loaded NP for 24 h raised total oxidant status levels in comparison to untreated cells, but there was no significant difference in total antioxidant status levels. According to the results treatment with Quercetin and Quercetin-loaded NP increased total oxidant status while having no effect on total antioxidant status, indicating that oxidative stress was produced in the Quercetin and Quercetin-loaded NP treated SH-SY5Y cells. Furthermore, the intrinsic apoptotic pathway can be triggered by disruption to the mitochondrial membrane and an excessive rise in reactive oxygen species [40, 41].

Conclusions

Quercetin and Quercetin-loaded NP significantly reduced SH-SY5Y cell reproduction in a concentration-dependent manner while generating no cytotoxicity in NIH 3T3 cells. The expression of pro-apoptotic Bax, cleaved caspase 3, and cleaved PARP proteins was significantly increased by Quercetin and Quercetin-loaded NP. In SH-SY5Y cells, Quercetin and Quercetin-loaded NP dramatically increased 8-oxo-dG levels, suggesting that the cytotoxic action of Quercetin and Quercetin-loaded NP is linked to DNA damage. The cytotoxic effects of Quercetin and Quercetin-loaded NP were supported by an increase in TOS. Because of its encouraging results in cancer cell suppression and lack of toxicity in healthy cells, Quercetin and Quercetin-loaded NP have the potential to be a new and effective anti-cancer approach.

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Author contributions MD performed experiments and analyzed data. MD supervised the entire project and designed the experiments. MD wrote the paper and read and approved the final manuscript.

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Data availability All the data generated or analyzed during this study are included in this manuscript.

Declarations

Conflict of interest The author declare that they have no conflict of interest.

Ethical approval Not applicable.

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