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FULL PAPER

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Evaluation of isoindole derivatives: Antioxidant potential and cytotoxicity in the HT-29 colon cancer cells

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Abstract

Norcantharimides have an isoindole skeleton structure, and some isoindoline derivatives have positive effects on inflammatory pathologies, including cancers. The present study aims to evaluate the antioxidant and cytotoxic potential of four synthesized isoindoline derivatives (NCTD1–4). HT-29 cells exposed to 10, 50, 100, and 200 μM doses of each derivative were incubated for 24 and 48 h, respectively. The cytotoxicity of the new derivatives was analyzed using the cell growth inhibition assay and the cell membrane damage test. In vitro antioxidant activity studies showed that the derivatives have free radical-scavenging effects in a dose-dependent manner. NCTD3 and NCTD4 apparently have antioxidant effects when compared with the control group treated with dimethyl sulfoxide. Furthermore, NCTD4 inhibited the growth of the HT-29 cells due to membrane damage and exhibited a dose-dependent cytotoxic effect on colon adenocarcinoma cells. The findings suggest that NDTD4 has the highest potential for colon cancer treatment and may be interpreted as a candidate anticancer agent.

KEYWORDS

antioxidant effect, colon cancer, cytotoxicity, HT-29 cells, isoindoline derivatives

1 | INTRODUCTION

Cantharidin, which is obtained from *Mylabris*, has been found to be an anticancer agent, but it also has some toxicity on the kidney and liver. In contrast, the demethylated form of cantharidin, namely norcantharidin, has lower bioactivity and toxicity.[1] Norcantharimides have an isoindole skeleton structure and contain bicyclic imide structures (Figure 1). Two methods are generally used to synthesize norcantharimide derivatives (NCTDs). Different functional groups are bonded to the nitrogen atom in the imide ring or the cyclohexane ring. In recent years, considerable effort has been put on the synthesis of N-substituted isoindole-1,3-dione derivatives and their biological activities.[2,3] A class of N-substituted isoindole-1,3-dione derivatives is norcantharimide, which also has potential cytotoxic effects on several cancer cells.[1,2]

The anticancer activities of some NCTDs have been investigated in various cell lines, such as human hepatic carcinoma cell lines (HepG2, Hep3B, and SK-HEP-1), a bladder cancer cell line (BFTC905), human lung adenocarcinoma epithelial (A549) cells, Caucasian promyelocytic leukemia cells (HL-60), human breast adenocarcinoma cell line (MCF-7), and the SW480 colon carcinoma cell line.[4–8] McCluskey et al.[9] reported the anticancer activity of different groups bonded to the imide nitrogen in NCTDs. NCTDs are known to interact with protein phosphatases, such as serine/threonine-protein phosphatase 1 (PP1) and protein phosphates 2A (PP2A).[2,6,7,10]

We previously synthesized several norcantharimides or isoindoline-1,3-dione derivatives containing oxygen functional groups from 3-sulfolene and reported their photophysical properties.[11–14] Additionally, we recently synthesized new isoindole

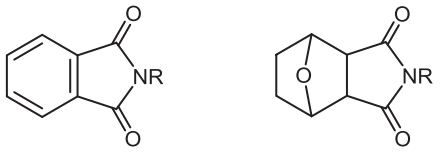


FIGURE 1 Structures of isoindoline-1,3-dione and its derivative, norcantharimide

derivatives by cleavage of ethers from tricyclic imide skeletal compounds and examined their antiproliferative properties in the MCF-7 (breast adenocarcinoma) and A549 (human alveolar basal epithelial adenocarcinoma) cell lines.[15]

Cancer is a multistage disease and reactive oxygen species are known to interact with all stages of this disease. Various substances are involved in the relationship between oxidative stress and cancer.[16] Both reactive oxygen and nitrogen species are known to be involved in carcinogenesis using two possible mechanisms as the induction of gene mutations and the disruption of signal transduction.[17] The organism has a complex protection system against harmful pro-oxidants, including enzymatic and nonenzymatic antioxidants. There are various synthetic and natural compounds that can interfere with pro-oxidant and antioxidant pathways. Targeting these systems and related pathways is of considerable importance in both cancer prevention and therapy.[17]

Based on these data, we synthesized additional isoindoline-1,3-dione derivatives in this series and examined some of the obtained compounds for antioxidant and cancer preventive activities.

2 | RESULTS AND DISCUSSION

2.1 | Chemistry

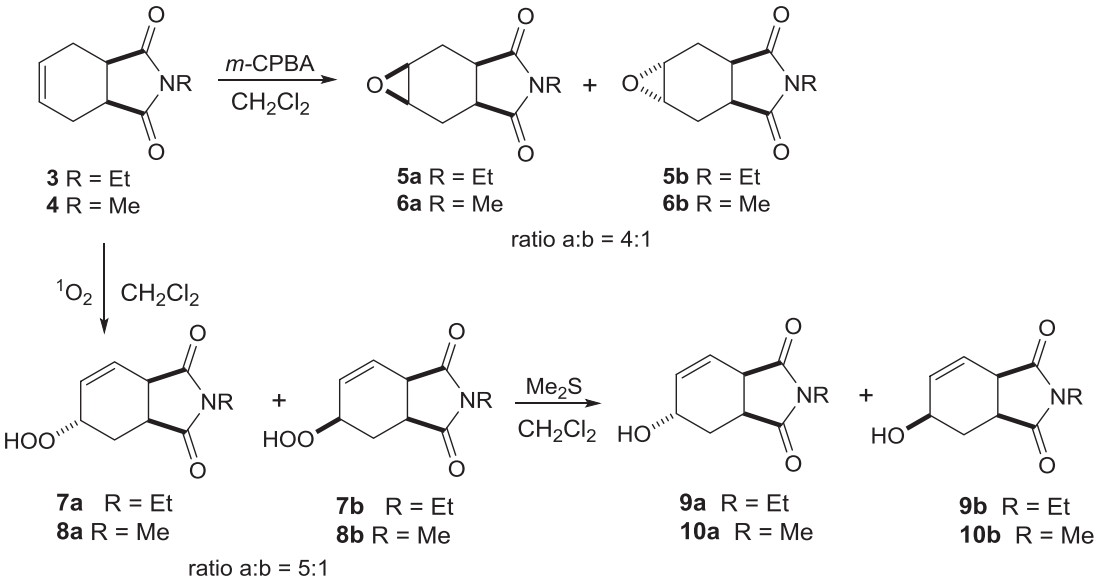
In our studies, anticancer activity was observed in compounds containing the methyl group bound to the nitrogen atom in the imide ring.[15] In light of these results, it was planned to investigate the anticancer activities of the methyl derivatives, such as **6a** and **11**. Two compounds **5a** and **9a** that contained a similar structure as **6a** and **11** were included in this study.

Synthesis of the target norcantharimide derivatives **5a**, **6a**, and **9a** was carried out according to the procedure depicted in Scheme 1. This procedure was previously demonstrated to be one of the most effective ways to prepare substituted isoindole or NCTDs. In these reactions, two main reactions were used as follows: epoxidation and singlet oxygen ene reaction. *N*-Ethyl- and *N*-methyl-isoindole-1,3-dione **3** and **4** were the key compounds for the synthesis of our target molecules **5a**, **6a**, **9a**, and **11**. This key compound was prepared to start from 3-sulfolene.

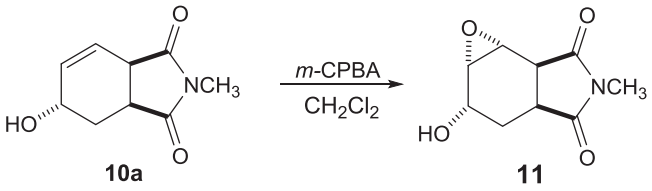
For the synthesis of *N*-Me-isoindole derivatives, the epoxidation of imide **4** with *m*-chloroperbenzoic acid (*m*-CPBA) at room temperature gave an isomeric mixture of epoxides **6a**/**6b** according to the ^{1}H nuclear magnetic resonance (NMR) spectrum of the crude product in a ratio of 4:1 (Scheme 1).

The isomeric epoxide mixture **6a**/**6b** was separated using column chromatography. The structures of **6a** and **6b** were assigned by ^{1}H and ^{13}C NMR spectra.

Additionally, the exact structure of epoxide **5a** was determined by X-ray analysis in previous studies.[13] The allylic alcohol **10a** was reacted with *m*-chloroperbenzoic acid (Scheme 2). The ^{1}H NMR spectrum of the crude product indicated that the epoxide **11** was



SCHEME 1 Synthesis of NCTD1 **5a**, NCTD2 **6a**, and NCTD3 **9a**. NCTD, norcantharimide derivative



SCHEME 2 Synthesis of NCTD4 **11**. CPBA, chloroperbenzoic acid; NCTD, norcantharimide derivative

obtained as a sole product due to the *syn*-effect of the allylic hydroxyl group.[11]

The exact structure of **11** was determined by its spectrum data compared with the literature data of a very similar (*N*-Et) system.[11] The desired compounds **5a**, **6a**, **9a**, and **11** were readily obtained in good yields. The compounds were checked using ^{1}H NMR, ^{13}C NMR, and mass spectroscopy. Subsequently, antioxidant and anticancer activities of NCTDs given in Figure 2 were performed.

2.2 | Biology

Previous studies suggested that the tumor inhibiting antioxidants, may change the intracellular redox state of the cells and enhance the cytotoxic activity.[18] The effect of different concentrations of norcantharimide derivatives (1 to 1000 μM) on antioxidant enzymes SOD and CAT are presented in Figure 3. SOD activities were found to be changed from 86% to 111% while CAT activities ranged between 63% and 99% of the control. Except for the minor effect of NCTD3 on SOD and CAT, no NCTD compound was found to follow a-dose dependent pattern. Concerning SOD activity, only NCTD3 seemed to cause a mild, dose-dependent inhibition leading up to only 85% at the highest concentration. NCTD3 also caused a similar inhibition on CAT enzyme with a small change between 96% to 86%. Although the highest inhibitory effect was recorded with 1 μM NCTD on CAT activity, high standard deviations and absence of statistical significance suggest that this may not be real effect.

In this study, the effects of NCTD exposure on HT-29 colon cancer cell viability were evaluated. The reports about the toxicity of NCTD in human colon cell lines are rare. The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay results revealed that NCTD reduced cell viability in a time-dependent manner. Cytotoxicity increased at 48-h incubation when compared with 24-h incubation for NCTD1–4. In Figure 4, it is shown that NCTD derivatives induced a decrease in cell viability in a dose-dependent manner. HT-29 cells exposed to 200 μM of NCTD for 48 h exhibited a substantial decrease in cell viability as measured by MTT reduction to formazan.

As shown in Figure 4, the cell viability significantly was diminished in the presence of 10–200 μM of NCTD, and 200 μM NCTD was found to be the most effective concentration. The cell viability decreased in 48-h incubation time compared with 24 h incubation for NCTD1 (**5a**) and NCTD4 (**11**). However, it was comparable in NCTD2 (**6a**) and NCTD3 (**9a**). The cell viability decreased 28% in NCTD1 (48 h), 26% in NCTD2 (24 h), and 21% in NCTD3 (24 h) in 200-μM dose group. The highest value of the percentage reduction of cell viability was observed in the NCTD4 (**11**) at 48-h incubation. The viability of HT-29 cells decreased as much as 42% at 200 μM NCTD4 (**11**) compared with the control, confirming that NCTD4 (**11**) cytotoxicity was dose- and time-dependent on HT-29 cells.

The release of lactate dehydrogenase (LDH) into the medium was detected in NCTD-exposed cultures. The LDH release assay results revealed that NCTD significantly increased the cytotoxicity in a concentration- and time-dependent manner. The level of extracellular LDH increased with an increase in the number of damaged cells. The maximum LDH leakage was observed at 24-h exposure at 200 μM of all tested NCTDs compared with 48-h incubation, indicating cell cytotoxicity.

In Figure 5, we show that four NCTDs' induced cytotoxicity were dose-dependent in the concentration range used. The highest cell cytotoxicity was 22% in NCTD2 (**6a**) at 200-μM dose group, 24 h incubation. For NCTDs 1, 3, and 4 (**5a**, **6a**, and **11**), cytotoxicity was around 10%. These results demonstrate that NCTDs have some cytotoxic effects in HT-29 cells, supporting the MTT results. LDH releases into the culture medium due to cell membrane damage and cell death. The increased activity of LDH is parallel to the rise in dead cell numbers.[19]

Similarly, cellular morphology is also one of the significant indicators of the cytotoxic effect of toxins. HT-29 cells exhibited altered morphology with more detached cells, as seen under a microscope. Moreover, cells exposed to NCTD were more rounded in appearance and lost their projections. Figures 6 and 7 also reflect the effects of NCTD on cell morphology of HT-29 cells after 24- and 48-h of incubation with different concentrations by trypan blue staining. Microscopic observations supported the MTT assay results and showed that the NCTD treatment decreased cell viability in a concentration-dependent manner.

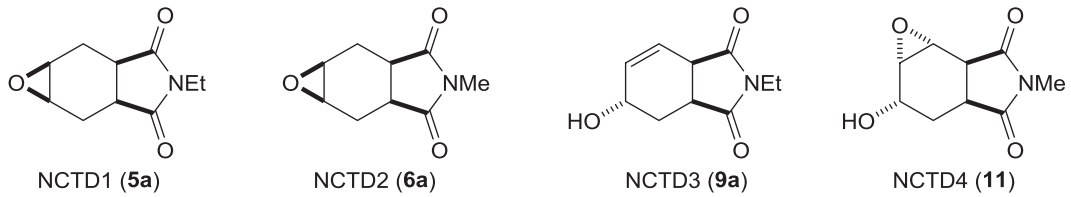


FIGURE 2 Structure of the examined antioxidant and anticancer activities of norcantharimide derivatives (NCTD1–4)

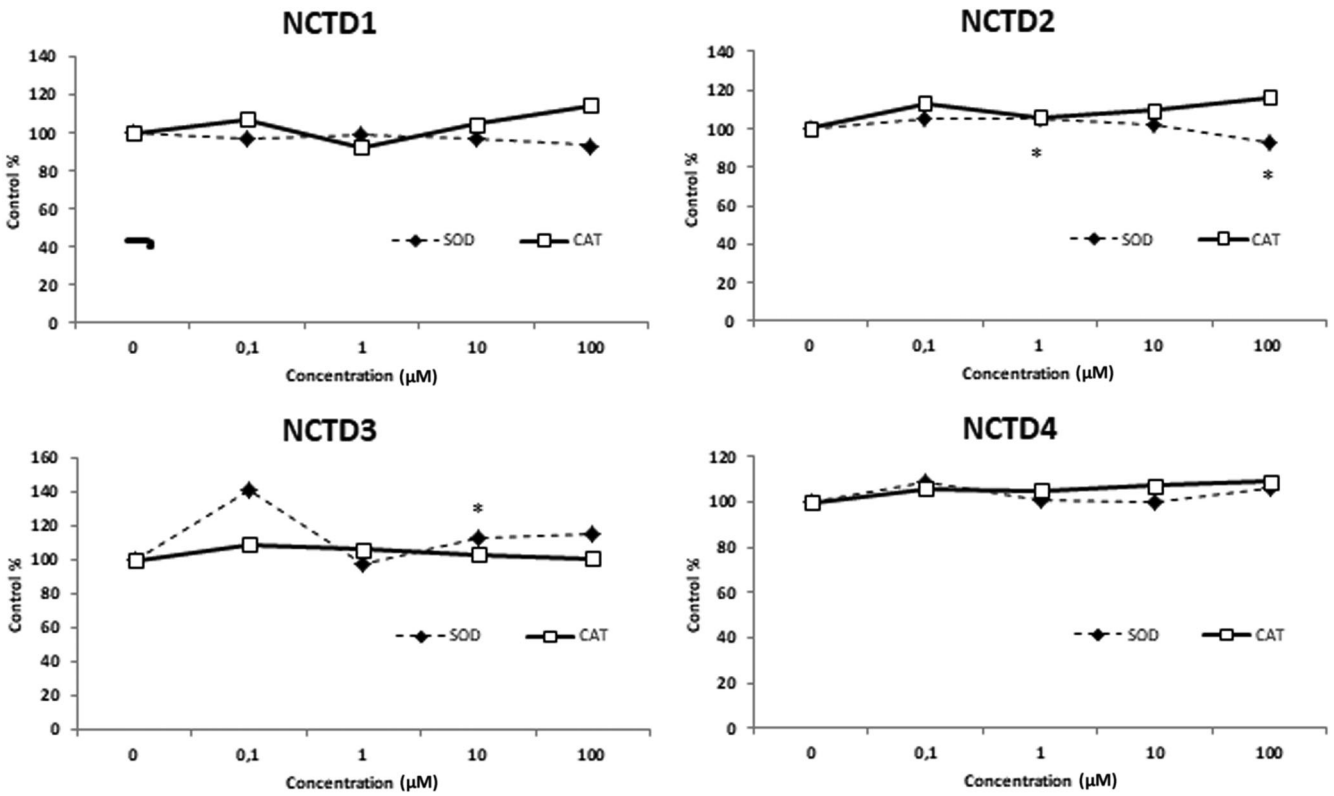


FIGURE 3 The effect of the four NCTDs on SOD and CAT enzymes at different concentrations of 1, 10, 100, and 1000 μM. % activity compared to positive control

3 | CONCLUSION

Isoindoles and norcantharimides are considered candidate anticancer compounds. Therefore, several norcantharimide analogs have been synthesized to decrease their toxic effects and enhance their anticancer effects. In this study, the cytotoxicity of four different NCTDs was evaluated with several tests based on different principles. The cytotoxic potential of the synthesized compounds was evaluated in HT-29 cell cultures using the MTT and LDH assays, and the results of both tests were consistent with each other.

Cancer is a multistage disease and reactive oxygen species are known to interact at all stages of this disease. They may induce gene mutations and/or disrupt signal transduction. Superoxide dismutase (SOD) and catalase (CAT) are the two main enzymes of the antioxidant system in an organism. SOD oxygen radical ($O_2^{\bullet -}$) can be split into hydrogen peroxide (H_2O_2) and water. Although H_2O_2 is less reactive compared with other reactive oxygen species, it may lead to the generation of hydroxyl radical ($OH^{\bullet}$). Catalase is responsible for converting H_2O_2 to carbon dioxide and water.[20] Hence, the activity of these two enzymes is essential to prevent oxidative stress, especially at the very beginning.

Although the studied compounds have been found to induce minor changes in SOD and CAT enzymes, NCTDs having different functional groups may affect the antioxidant enzymes and oxidative status of the cell. Further evaluation of NCTDs with different functional groups on different radicals, antioxidant enzymes, and oxidative status in the cell can add another perspective to the current data on NCTDs.

This preliminary study suggests that NCTD4 (**11**) can be the potential agent for additional analysis in colon cancer therapy. Our results suggest that the investigated NCTDs do not exert their effects through the antioxidant enzymes SOD and CAT. Further detailed investigations on the mechanism of the promising NCTDs should be performed.

4 | EXPERIMENTAL

4.1 | Chemistry

4.1.1 | General

All reagents used were commercially available unless otherwise specified and all solvents were distilled before use. Melting points were measured using Gallenkamp melting point devices. Infrared (IR) spectra: PerkinElmer Spectrum One FT-IR spectrometer. ^1H and ^{13}C NMR spectra: Varian 400 and Bruker 400 spectrometers. Elemental analysis results were obtained on a Leco CHNS-932 instrument.

The InChI keys of the investigated compounds are provided as Supporting Information Data.

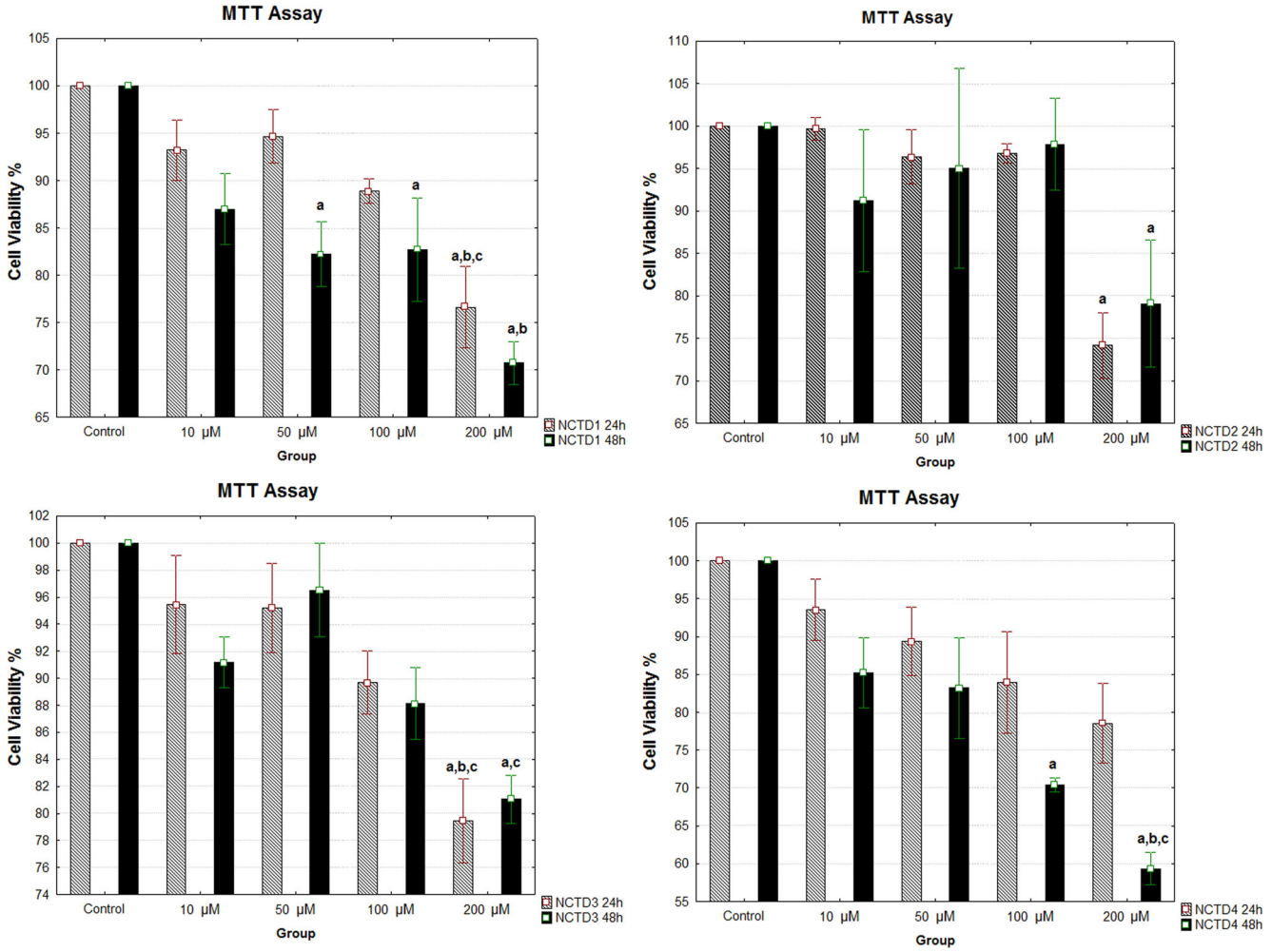


FIGURE 4 MTT assay results after 10, 50, 100, and 200 µM NCTD1–4 exposure for 24 and 48 h of incubation in HT-29 cells. The results (mean ± *SE*) of three independent experiments are shown as % of the control. MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; NCTD, norcantharimide derivative; *SE*, standard error. [a]Statistically significant from control group, [b]statistically significant from 10-µMNCTD group, [c]statistically significant from 50-µM NCTD group, ($p \leq .05$)

4.1.2 | Synthesis of (1a*R*,2a*S*,5a*R*,6a*S*)-4-ethylhexahydro-3*H*-oxireno[2,3-*f*]isoindole-3,5(4*H*)-dione (NCTD1, 5a)

The imide **3** (2.38 g, 13.3 mmol), 70–80% *m*-CPBA (3.62 g, 20 mmol), and excess $NaHCO_3$ were magnetically stirred in CH_2Cl_2 (120 ml) for 6 h. The resulting slurry was transferred to a separation funnel and treated with 15% aqueous $Na_2S_2O_3$ (50 ml) to eliminate unreacted peracid. The solution was then washed successively with 10% aqueous $NaHCO_3$ (50 ml) and brine, dried ($MgSO_4$), and concentrated to give a crude product (91% yields). The isomeric products were separated by column chromatography (EtOAc–hexanes [first isomer, 15:85; second isomer, 40:60]) then crystallized (CH_2Cl_2–hexanes). *syn*-**5a**, colorless crystals; mp: 75–76°C. ^{1}H NMR (400 MHz, $CDCl_3$): 3.45 (q, J = 7.1 Hz, 2H, CH_2), 3.19 (m, 2H, 2 × CH), 2.85 (m, 2H, CH_2), 2.54 (m, 2 H, 2 × CH), 1.83 (m, 2H, CH_2), 1.07 (t, J = 7.1, Hz, 3H, CH_3). ^{13}C NMR (100 MHz, $CHCl_3$): 179.6, 49.3, 35.4, 33.7, 23.2, 13.1. Anal. calcd. for $C_{10}H_{13}NO_3$: C, 61.53; H, 6.71; N, 7.18. Found: C, 61.91; H, 7.16; N, 6.81.

4.1.3 | Synthesis of 4-methylhexahydro-3*H*-oxireno-[2,3-*f*]isoindole-3,5(4*H*)-dione (NCTD2, 6a)

The imide **4** (1.0 g, 5.52 mmol), 70–80% *m*-CPBA (2.0 g, 11.04 mmol), and excess $NaHCO_3$ were refluxed in CH_2Cl_2 (120 ml) for 72 h. The resulting slurry was transferred to a separation funnel and treated with 15% aqueous $Na_2S_2O_3$ (50 ml) to eliminate unreacted peracid. The solution was then washed successively with 10% aqueous $NaHCO_3$ (50 ml) and brine, dried ($MgSO_4$), and concentrated to give a crude product. The isomeric products were separated by column chromatography with EtOAc–hexanes (first isomer, 15:85; second isomer, 40:60). (*syn*)-4-Methylhexahydro-3*H*-oxireno[2,3-*f*]isoindole-3,5(4*H*)-dione (**6a**; 80% yields). Colorless crystals; mp: 129–130°C. ^{1}H NMR (400 MHz, $CDCl_3$): 3.16 (m, 2H, 2 × CH), 2.99 (s, 3H, CH_3), 2.76 (m, 1H, CH), 2.75 (m, 2H, CH_2), 2.70 (m, 1H, CH), 2.18 (dm, A part of AB system, J = 15.4 Hz, 1H, CH), 2.14 (dm, A part of AB system, J = 15.4 Hz, 1H, CH). ^{13}C NMR (100 MHz, $CDCl_3$): δ 180.77, 77.56, 77.25, 76.93, 50.80, 35.58, 25.53, 22.52. High-resolution mass

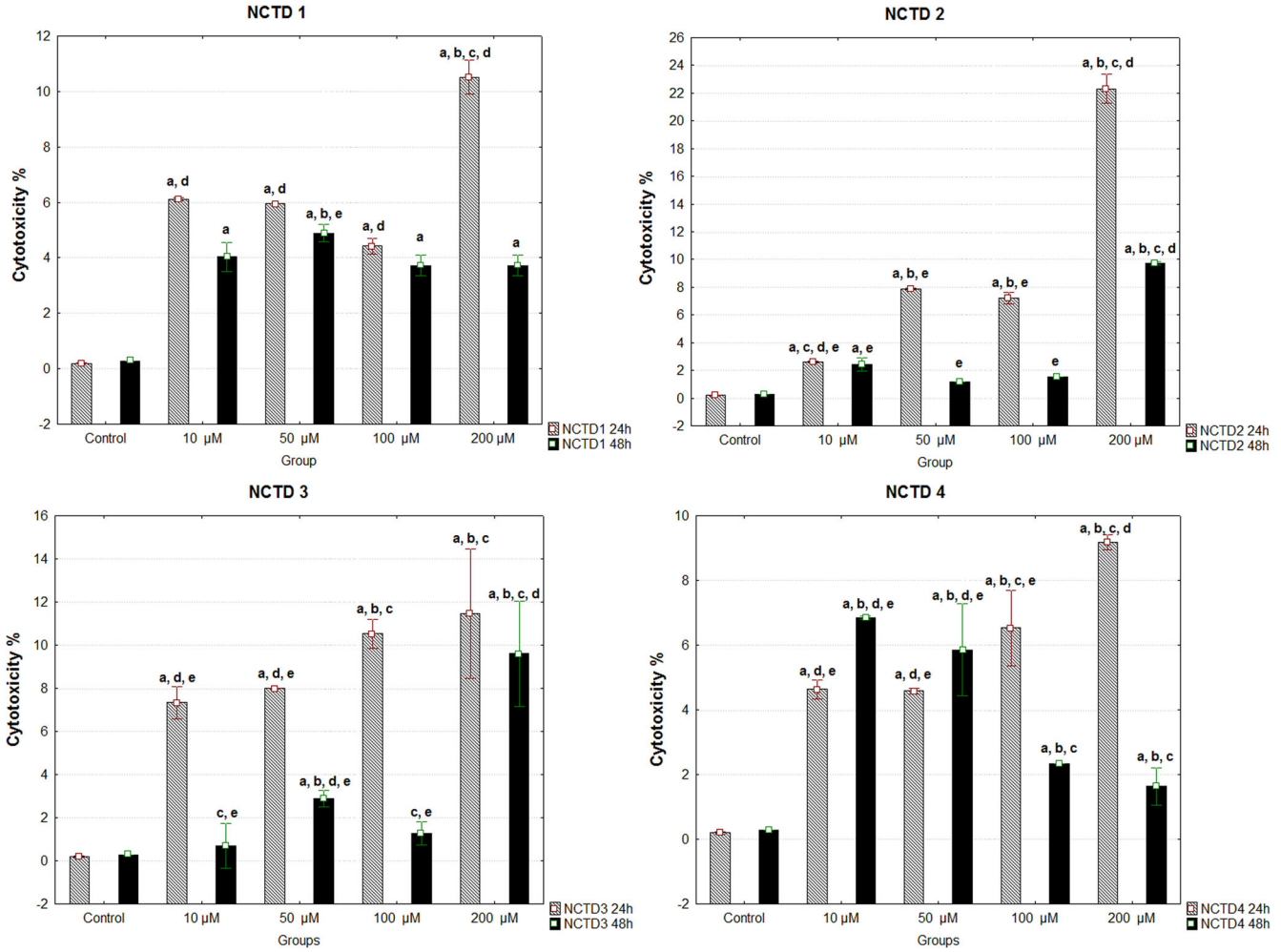


FIGURE 5 Influence of NCTD1–4 exposure on the LDH leakage in HT-29 cells after 24 and 48 h of incubation. The results (mean ± *SE*) of three independent experiments are shown as % of the control. LDH, lactate dehydrogenase; NCTD, norcantharimide derivative; *SE*, standard error. [a]Statistically significant from control group, [b]statistically significant from 10-µM NCTD group, [c]statistically significant from 50-µM NCTD group, significant from 200-µM NCTD group, ($p \leq .05$)

spectroscopy (HRMS): (electrospray ionization [ESI]/$[M+H]^+$) *m/z* found: 182.0766, calcd for: *m/z*: 181.0739.

^{13}C NMR (100 MHz, $CDCl_3$): 178.7, 176.2, 129.1, 126.2, 75.5, 41.3, 36.3, 34.0, 25.5, 13.1. Anal. calcd. for $C_{10}H_{13}NO_4$: C, 56.86; H, 6.20; N, 6.63. Found: C, 56.92; H, 6.38; N, 6.61.

4.1.4 | Synthesis of *anti*-2-ethyl-5-hydroperoxy-3a,4,5,7a-tetrahydro-1*H*-isoindole-1,3(2*H*)-dione (7a)

Tetraphenylporphyrin (20 mg) was added to a stirred solution of imide **3** (0.985 g, 5.5 mmol) in CH_2Cl_2 (150 ml). The mixture was irradiated with a tungsten–halogen projection lamp (500 W), while oxygen was passed through the solution and the mixture was stirred at room temperature. The solvent was then evaporated at 30°C under reduced pressure. The major product **7a** was separated using fractional crystallization in CH_2Cl_2–hexanes. Colorless crystals; mp: 101–102°C. ^{1}H NMR (400 MHz, $CDCl_3$): 8.3 (s, 1H, OOH), 6.1 (m, 2H, 2 × CH), 4.46 (m, 1H, CH), 3.53 (q, J = 7 Hz, 2H, CH_2), 3.46 (dm, J = 8 Hz, A part of AB system, 1H, CH), 3.23 (q, J = 8 Hz, B part of AB system, 1H, CH), 2.18 (t, J = 6 Hz, 2H, CH_2), 1.14 (t, J = 7 Hz, 3H, CH_3).

4.1.5 | Synthesis of 5-hydroperoxy-2-methyl-3a,4,5,7a-tetrahydro-1*H*-isoindole-1,3(2*H*)-dione (8a)

Tetraphenylporphyrin (20 mg) was added to a stirred solution of imide **4** (2-(methyl)-3a,4,7,7a-tetrahydro-1*H*-isoindol-1,3(2*H*)-dione; 0.907 g, 5.5 mmol) in CH_2Cl_2 (100 ml). The mixture was irradiated with a tungsten–halogen projection lamp (500 W), while oxygen was passed through the solution and the mixture was stirred at room temperature. The solvent was then evaporated at 30°C under reduced pressure. The major product **8a** was separated by fractional crystallization in CH_2Cl_2–hexanes (44% yields). Colorless crystals; mp: 117–118°C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.68 (m, 1H, OOH), 6.12 (m, 2H, 2 × CH), 4.46 (m, 1H, CH), 3.47 (dd, J = 8.1, 1.5 Hz, 1H,

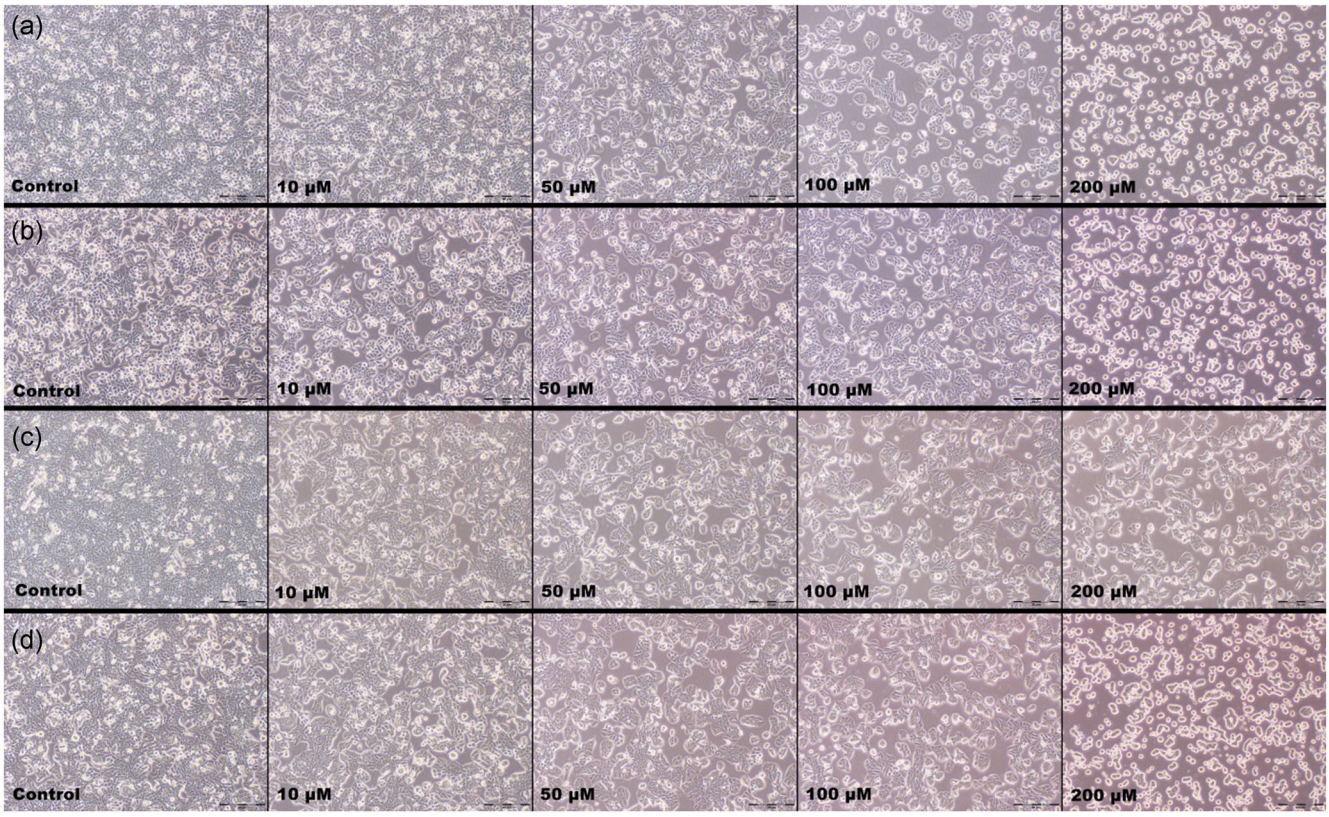


FIGURE 6 Trypan blue staining of HT-29 cells exposed to various concentrations of NCTDs after 24 h (a) NCTD1 (**5a**), (b) NCTD2 (**6a**), (c) NCTD3 (**9a**), and (d) NCTD4 (**11**). NCTD, norcantharimide derivative

CH), 3.46 (dq, J = 6.6, 14.3 Hz, 1H, CH), 2.97 (m, 3H, CH_3), 2.19 (m, 3H, 3 × CH). ^{13}C NMR (100 MHz, $CHCl_3$): δ 179.2, 176.6, 129.1, 126.0, 75.4, 41.3, 36.3, 25.5, 25.2. IR (KBr, cm^{-1}): 3,374, 2,946, 1,774, 1,692, 1,440, 1,381, 1,290. Anal. calcd. for $C_9H_{11}NO_4$: C, 54.82; H, 5.62; N, 7.10. Found: C, 55.01; H, 5.16; N, 7.14.

4.1.6 | Synthesis of (3a*S*,5*S*,7a*R*)-2-ethyl-5-hydroxy-3a,4,5,7a-tetrahydro-1*H*-isoindole-1,3(2*H*)-dione (NCTD3, 9a)

A solution of peroxide **7a** (300 mg, 1.42 mmol) in CH_2Cl_2 (25 ml) was added to a magnetically stirred slurry of Me_2S (176 mg, 2.84 mmol) in CH_2Cl_2 (25 ml) at room temperature. After the addition was complete (~10 min), the mixture was stirred for 6 h. The solvent was then removed by rotary evaporation, and the residue was extracted with CH_2Cl_2 (3 × 30 ml). The extracts were dried (Na_2SO_4) and the solution was concentrated to give a residue that was crystallized (CH_2Cl_2–hexanes) to give **9a** (72% yields). Colorless crystals; mp: 93–94°C. ^{1}H NMR (400 MHz, $CDCl_3$): 6.00 (dtd, J = 10.1, 2.2, 0.7 Hz, A part of AB system, 1H, CH), 5.85 (ddd, J = 10.1, 4.2, 1.8 Hz, B part of AB system, 1H, CH), 4.11 (m, 1H, OH), 3.49 (q, J = 7.3 Hz, 2H, CH_2), 3.44 (tt, J = 6.4, 1.8 Hz, 1H, CH), 3.18 (dt, J = 5.5, 1.1 Hz, 1H, CH), 2.43 (dtd, A part of AB system, J = 13, 4.9, 0.7 Hz, 1H, CH), 1.73 (ddd, B part of AB system, J = 13, 9.2, 6.2 Hz, 1H, CH), 1.09 (t, J = 7.3 Hz, 3H, CH_3). ^{13}C NMR (100 MHz, $CDCl_3$): 178.8, 176.8, 135.2, 122.9, 62.6, 41.1, 37.0, 34.0, 30.2, 13.1. Anal. calcd. for $C_{10}H_{13}NO_3$: C, 61.53; H, 6.71; N, 7.18. Found: C, 61.53; H, 6.78; N, 7.15.

4.1.7 | Synthesis of 5-hydroxy-2-methyl-3a,4,5,7a-tetrahydro-1*H*-isoindole-1,3(2*H*)-dione (10a)

A solution of peroxide **8a** (280 mg, 1.42 mmol) in CH_2Cl_2 (25 ml) was added to magnetically stirred slurry of Me_2S (176 mg, 2.84 mmol) in CH_2Cl_2 (25 ml) at room temperature. After the addition was complete (~10 min), the mixture was stirred for 6 h. The solvent was then removed by rotary evaporation, and the residue was extracted with CH_2Cl_2 (3 × 30 ml). The extracts were dried (Na_2SO_4) and the solution was concentrated to give a residue that was crystallized (CH_2Cl_2–hexanes) to give **10a** (82% yields). Colorless crystals; mp 95–96°C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 6.02 (dtd, J = 10.1, 2.2, 0.7 Hz, A part of AB system, 1H, CH), 5.85 (ddd, J = 10.1, 4.0, 1.8 Hz, B part of AB system, 1H, CH), 4.13 (s, 1H, OH), 3.45 (tt, J = 6.2, 2.1 Hz, 1H, CH), 3.20 (dt, J = 13.5, 5.5 Hz, 1H, CH), 2.94 (s, 3H, CH_3), 2.48 (s, 1H, CH), 2.39 (dtd, J = 13.2, 4.7, 0.7 Hz, A part of AB system, 1H, CH), 1.73 (ddd, J = 13.2, 8.8, 5.8 Hz, B part of AB system, 1H, CH). ^{13}C NMR (100 MHz, $CDCl_3$): δ 179.1, 176.9, 135.2, 122.8, 62.6, 41.1, 37.1, 37.0, 30.1, 25.1. IR (KBr, cm^{-1}):

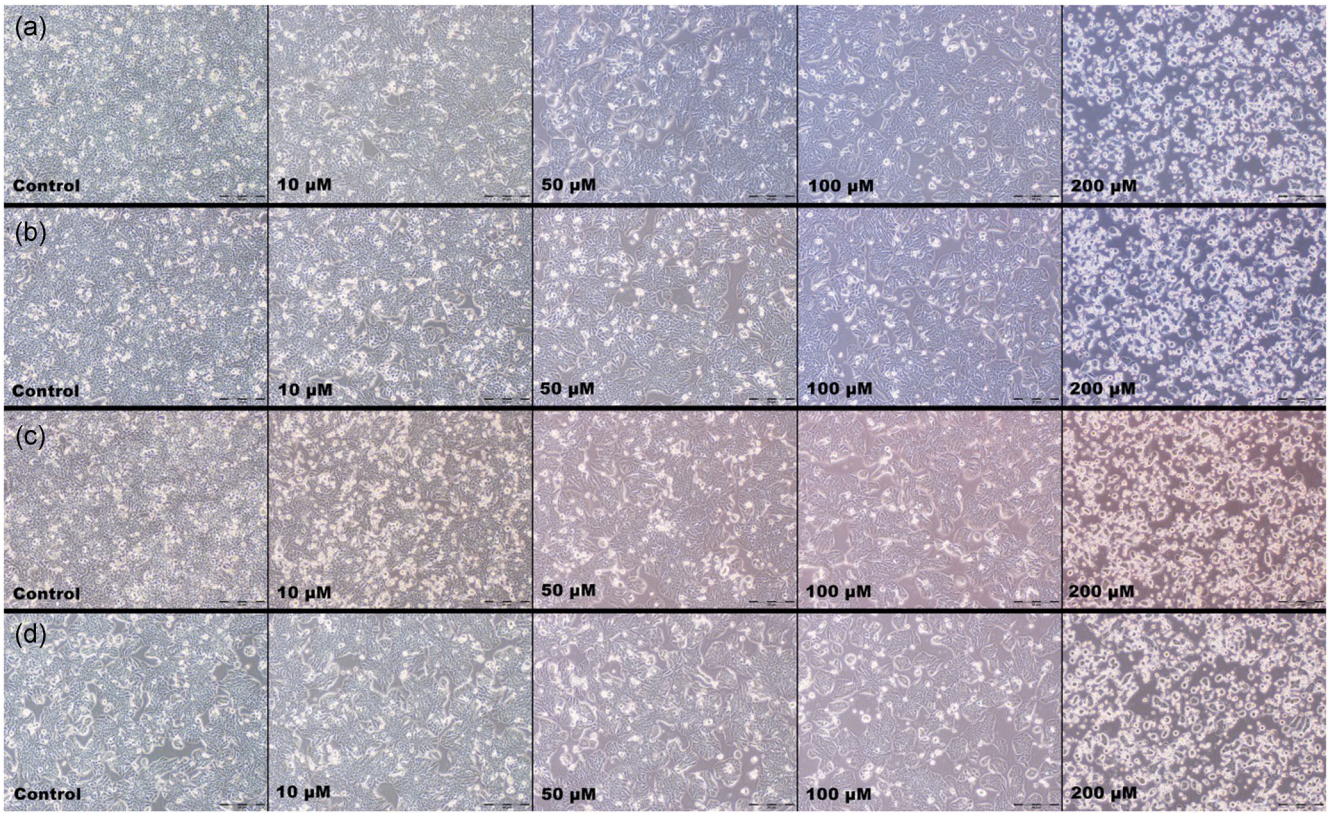


FIGURE 7 Trypan blue staining of HT-29 cells exposed to various concentrations of NCTDs after 48 h. (a) NCTD1 (**5a**), (b) NCTD2 (**6a**), (c) NCTD3 (**9a**), and (d) NCTD4 (**11**). NCTD, norcantharimide derivative

3,450, 2,932, 1,774, 1,693, 1,439, 1,385, 1,288, 1,221, 1,166. Anal. calcd. for $C_9H_{11}NO_3$: C, 59.66; H, 6.12; N, 7.73. Found: C, 59.67; H, 5.83; N, 7.72.

4.1.8 | Synthesis of 2-hydroxy-5-methylhexahydro-4*H*-oxireno[2,3-*e*]isoindole-4,6(5*H*)-dione (NCTD4, 11)

The allyl alcohol **10a** (1.0 g, 5.52 mmol), 70–80% *m*-CPBA (2.0 g, 11.04 mmol) and excess $NaHCO_3$ were refluxed in CH_2Cl_2 (120 ml) for 72 h. The resulting slurry was transferred to a separation funnel and treated with 15% aqueous $Na_2S_2O_3$ (50 ml) to eliminate unreacted peracid. The solution was then washed successively with 10% aqueous $NaHCO_3$ (2 × 50 ml) and brine, dried ($MgSO_4$), and concentrated to give a crude product. The crude product was purified using column chromatography with EtOAc–hexanes (40:60) to give **11** (70% yields). Colorless crystals; mp: 136–137°C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 3.9 (dd, J = 11.2, 4.6 Hz, 1H, CH), 3.49 (m, 1H, CH), 3.31 (d, J = 8.8 Hz, 1H, CH), 3.28 (m, 2H, CH_2), 2.95 (s, 3H, CH_3), 2.95 (OH), 2.24 (dm, J = 12.8 Hz, A part of AB system, 1H, CH), 1.84 (m, B part of AB system 1H, CH). ^{13}C NMR (100 MHz, $CDCl_3$): δ 178.82, 176.01, 64.50, 56.19, 54.75, 42.80, 38.64, 25.49, 25.15. HRMS: (ESI/$[M+H]^+$) m/z found: 198.0733, calcd. for: 197.0688.

4.2 | Biological assays

4.2.1 | Cell culture

Human colorectal adenocarcinoma (HT-29) cells were obtained from HUKUK, Foot and Mouth Disease Institute (Ankara, Turkey). HT-29 cells were grown in Dulbecco's modified Eagle's medium (HyClone Laboratories, Inc., Logan, UT) supplemented with 10% fetal bovine serum (Biochrom, Germany) and 1% gentamicin (Sigma-Aldrich, St. Louis, MO) at 37°C in 5% CO_2 in a humidified incubator.

4.2.2 | Measurement of cell viability by MTT assay

Growth inhibition was measured using an MTT assay (Sigma-Aldrich). HT-29 cells were plated at a density of 2.7×10^4 in 96-well plates. The cultures were grown for 24 h. The medium was changed to that containing four different concentrations (10, 50, 100, and 200 μM) of each NCTD and the control media (final concentration of DMSO is ≤0.1%). After 24-h of incubation, MTT was added to each well. After incubation for 4 h, DMSO/ammoniac mixture was added and optical density at 570 nm was recorded with a microplate reader (BioTek Instruments Inc., Winooski, VT). Experiments have been triplicated and surviving cell percentage was defined as the treatment

group/control group. The mean of the control group was assumed as 100% survival.

4.2.3 | LDH leakage assay

LDH activity was measured with the LDH Cytotoxicity Assay Kit II (BioVision) according to the manufacturer's instructions.[21] The HT-29 cells were plated at a density of 6×10^5 cells/well in a plate for 24 h. After 24 h, determined concentrations of NCTD and control media were added directly to each well, and the plates were incubated at 37°C for 24 h. After incubation in the presence of NCTD and control media for 24 h, the culture medium was collected and centrifuged at 600*g* for 10 min at 4°C. The LDH activity in the culture supernatant was measured after transferring the supernatant to 96-well plates. The reaction was run in the dark for 30 min before measurement, and the absorbance was measured at 450 nm with a multiplate reader (BioTek Instruments Inc.). Each experiment was repeated at least three times. Results were expressed as a percentage of control.

4.2.4 | Trypan blue staining

The HT-29 cells were plated at a density of 3×10^5 cells/well in a plate for 24 h. After incubation, various concentrations of NCTD were added directly to each well, and the plates were incubated at 37°C for 24 h. After staining with 0.5% trypan blue, viability was evaluated in a Bürker chamber by using light microscopy. The cellular morphology was observed and photographed using an inverted microscope (Olympus, Japan) equipped with an Olympus DP73 digital camera.

4.2.5 | Erythrocyte incubation

Two hundred microlitre red blood cells (RBCs) were suspended in 2,300 μl phosphate-buffered saline (PBS), which contained 0–100 μM concentrations (0.1, 1, 10, and 100 μM) of each NCTD and DMSO as a solvent. Negative and positive controls were prepared with RBCs suspended in PBS (as negative control) and in PBS + DMSO (as positive control). All incubations were performed in duplicates in a shaking water bath for 2.5 h at 37°C.

4.2.6 | Antioxidant enzyme activities

Following the incubation, the test tubes were centrifuged, and the upper layer was discarded. RBCs were washed three times with PBS and were hemolyzed in cold water. Lysates were used for enzyme analysis after cellular debris was removed. Both antioxidant enzyme activities and protein content were determined in the erythrocyte supernatant as described before.[22–24] SOD activity was determined by calculating the decrease in pyrogallol autoxidation, while CAT activity calculation was based on the reduction rate of hydrogen peroxide. Both enzyme activities were expressed in IU/g protein.

4.3 | Statistical analysis

Data are presented as mean ± standard error of the mean. Statistical analyses were performed by Student's *t* test. Spearman's rank correlation test was used to investigate the correlations between the doses and results. $p < .05$ was considered statistically significant.

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CONFLICTS OF INTERESTS

The authors declare that there are no conflicts of interests.

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