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ORIGINAL RESEARCH

Ethyl acetate extracts of *Nepenthes ventricosa x sibuyanensis* leaves cause growth inhibition against oral cancer cells via oxidative stress

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Introduction: The genus *Nepenthes* of the pitcher plants contains several natural and hybrid species that are commonly used in herbal medicine in several countries, but its possible use in cancer applications remains unknown as yet.

Methods: In this study, we investigated the antioral cancer properties using ethyl acetate extracts of the *Nepenthes* hybrid (*Nepenthes ventricosa* x *sibuyanensis*), namely EANS. The bioactivity was detected by a MTS-based cell proliferation assay and flow cytometric or Western blot analysis for apoptosis, oxidative stress, and DNA damage.

Results: Treatment for 24 hrs of EANS inhibited all three types of oral cancer cells that were tested (Ca9-22, CAL 27, and SCC9), with just a small difference to normal oral cells (HGF-1). This antiproliferation was inhibited by pretreatments with the reactive oxygen species (ROS) scavenger *N*-acetylcysteine (NAC), and the apoptosis inhibitor (Z-VAD). EANS treatment increased the subG1 population and it also dose- and time-dependently induced annexin V- and pancaspase-detected apoptosis as well as cleaved caspases 3 and 9 overexpressions in the oral cancer cells (Ca9-22). After EANS treatment of Ca9-22 cells, intracellular ROS and mitochondrial superoxide (MitoSOX) were overexpressed and mitochondrial membrane potential (MMP) was disrupted. Moreover, DNA damages such as γH2AX and 8-oxo-2′-deoxyguanosine (8-oxodG) were increased after EANS treatment to Ca9-22 cells. The EANS-induced effects (namely, oxidative stress, apoptosis, and DNA damage) were suppressed by ROS scavenger.

Conclusion: Our findings demonstrated that EANS inhibits ROS-mediated proliferation against oral cancer cells.

Keywords: *Nepenthes*, oral cancer, oxidative stress, apoptosis, DNA damage

Introduction

Oral cancer is one of the main threats to public health worldwide, especially for patients with the habits of alcohol drinking, betel quid-chewing, or smoking.[1] In general, oral cancer patients have 50% of five-year survival.[2] The traditional therapy for oral cancer is surgery with or without chemo- or radiotherapy,[2] which occasionally associates with side effects.[3] Therefore, there is a challenge to use alternative or supplementary therapy for cancer treatment without side effects.

Recently, chemoprevention effects of natural products against oral cancer cells have been emphasized.[4–8] Many natural product-derived bioactive compounds[9–12] have shown anticancer potential because of their high cytotoxicity to cancer cells and low toxicity to normal cells. Moreover, cancer progression is known to involve

multiple signaling pathways and different drugs may have differential targets. Therefore, it is important and helpful to identify more natural product candidates for oral cancer therapy.

Nepenthes plants are tropical plants with pitcher-shaped leaves that trap animal victims for the nutrient provision of these carnivorous plants. The genus contains a number of original species, and natural or manmade hybrids that increase its diversity to more than 170 species.[13] *Nepenthes* plants have a wide range of habitats located in the tropical belt of Australia, Madagascar, Papua New Guinea, the Seychelles, and Southeast Asia. Several representatives have been used for herbal medicine in several countries. For example, *N. mirabilis* is famous for its treatments against cough, jaundice, fever, hypertension,[14] and inflammation.[15] Cell model studies showed that *Nepenthes* plant extracts based on several solvents suppressed the growth of certain bacteria[16] and fungi.[17] However, the anticancer effect of *Nepenthes* plants remains unclear.

Nepenthes plants are rich in antioxidant components. For example, *N. mirabilis* and *N. gracilis* were reported to contain flavonoids[18] and phenolic compounds,[19] respectively. Methanolic extracts of *N. bicalcarata* leaves also displayed high antioxidant properties.[20] Antioxidants have a potential for oral cancer prevention.[21] Hence, possible anticancer effects of *Nepenthes* plants warrant in-depth investigation. Moreover, the ethyl acetate extraction for *Nepenthes* plants is rarely investigated. Therefore, we focused on evaluating the antioral cancer effect of *Nepenthes* plants. Using ethyl acetate extract of *N. ventricosa* x *sibuyanensis* (EANS), the changes of cell viability, apoptosis, oxidative stress, and DNA damage were investigated using oral cancer cells.

Materials and methods

Plant materials, ethyl acetate extract, and drug inhibitors

Species identification and sample collection of *Nepenthes* species (*N. ventricosa x sibuyanensis*) was performed by Mr. Jui-Hsuan Kuo in the Dr. Celica Koo Botanic Conservation Center (KBCC), Taiwan, in October, 2014. The voucher sample (K45532) was air-dried and deposited in the Graduate Institute of Natural Products, Kaohsiung Medical University. *N. ventricosa* x *sibuyanensis* twigs and leaves (210 g) were soaked in methanol (1 L) to provide crude extract. Subsequently, this was partitioned between water and EtOAc. Finally, the EtOAc layer, namely EANS, was harvested (96 mg) and stored at 4°C. All treatments with or without EANS had the same concentration of dimethyl sulfoxide (DMSO) (Sigma-Aldrich; St. Louis, MO, USA) as a carrier of the active compounds.

In subsequent experiments with EANS, several kinds of inhibitors were pretreated as follows: Free radical scavenger *N*-acetylcysteine (NAC) (2 mM, 1 hr) (Sigma),[22] pan-caspase inhibitor Z-VAD-FMK (Z-VAD) (20 μM, 2 hrs) (Selleckchem. com; Houston, TX, USA),[11] and mitochondrial superoxide inhibitor Mito TEMPO (20 μM, 1 hr) (Cayman Chemical, Ann Arbor, Michigan, USA).[23]

Cell culture and cell viability

Oral cancer (Ca9-22, SCC9, and CAL 27) and normal oral (HGF-1) cells were used as described previously.[24] All cell lines were purchased from American Type Culture Collection (ATCC) and the Japanese Collection of Research Bioresources (JCRB) Cell Bank. Cells were incubated (37°C, 5% CO_2) in a humidified atmosphere and maintained with Dulbecco's Modified Eagle Medium (DMEM)/Nutrient Mixture F-12 (Gibco, Grand Island, NY, USA) at 3:2 (oral cancer cells) and 4:1 (HGF-1 cells) ratio with 10% fetal bovine serum and common antibiotics (Gibco). Cell viability is measured by mitochondrial-activity-based cell proliferation MTS assay (Promega Corporation, Madison, WI) as described previously.[25]

Cell cycle phases

Cellular DNA content was measured by 7-aminoactinmycin D (7AAD) (Biotium, Inc., Hayward, CA, USA) staining and is commonly used for cell cycle phase analysis as previously described.[26] After 75% ethanol fixation, cells were stained with 7AAD (1 μg/mL in phosphate-buffered saline (PBS), 30 mins) for Accuri™ C6 flow cytometry (Becton-Dickinson, Mansfield, MA, USA). The G1, S, and G2/M populations added up to 100% where the sub-G1 population was calculated separately as described before.[27]

Annexin V/7AAD apoptosis assay

Apoptosis was analyzed by the phosphatidylserine-based annexin V kit (Strong Biotech Corporation, Taipei, Taiwan) as described previously.[28] Cells were double-stained with annexin V-labeled with FITC (10 μg/mL)

and 7AAD (1 μg/mL) for 30 mins. Finally, cells were analyzed by Accuri™ C6 flow cytometry.

Caspase activity assays: pancaspase flow cytometry and Western blotting

Pancaspase activity (caspases-1, 3–9) was analyzed by a generic caspase activity assay kit (Abcam, Cambridge, UK) using flow cytometry as previously described.[29] After treatment with 0.5X TF2-VAD-FMK for 2 hrs, cells were washed and resuspended for Accuri™ C6 flow cytometry.

Protein lysates (45 μg) were performed with 8% SDS-PAGE and transferred to a PVDF membrane for 5% nonfat milk blocking overnight. Apoptosis antibodies (diluted 1:1,000) were chosen for Western blotting, including: cleaved forms of caspase 3 (c-cas 3) and caspase 9 (c-cas 9) from the Apoptosis Antibody Sampler Kit and cleaved caspase 8 (c-cas 8) (1C12) (Cell Signaling Technology, Inc., Danvers, MA, USA). Loading control was mAb-β-actin (Sigma-Aldrich). WesternBright™ enhanced chemiluminescence (ECL) horseradish peroxidase (HRP) kit (Advansta, Menlo Park, CA, USA) was used for detecting HRP activity to secondary antibody.

Cellular ROS and mitochondrial superoxide (MitoSOX) assays

The reactive oxygen species (ROS)-reacting chemical 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) (Sigma-Aldrich, St. Louis, MO, USA) was oxidized by ROS and became fluorescent for analysis by flow cytometry.[30,31] After treatment with DCFH-DA (10 μM, 37°C, 30 mins), cells were analyzed by flow cytometry (Accuri™ C6). Similarly, superoxide-reacting dye MitoSOX Red (Molecular Probes, Invitrogen, Eugene, OR, USA) was specifically oxidized with mitochondrial superoxide[32] and became a fluorescent chemical for flow cytometry.[33] After treatment with MitoSOX™ Red (5 μM, 37°C, 30 mins), cells were analyzed by flow cytometry (Accuri™ C6).

Mitochondrial membrane potential (MMP) assay

A membrane-potential-sensitive cyanine dye $DiOC_2(3)$ (Invitrogen, San Diego, CA, USA) was used for MMP analysis as previously described.[34] After treatment with $DiOC_2$ (3) (50 nM, 30 mins), cells were analyzed by flow cytometry (Accuri™ C6).

γH2AX DNA damage assay

Flow cytometry indications using the DNA double-strand break marker (γH2AX) was described in an earlier study.[9] After treatment with primary antibody p-Histone H2A.X (Ser 139) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) (1:50 dilution, 4°C, 1 hr), the secondary antibody was further reacted for 30 mins for Accuri™ C6 flow cytometry.

8-Oxo-2′-deoxyguanosine (8-oxodG) assay

For flow cytometric detection, the antibody from fluorometric OxyDNA kit (EMD Millipore, Darmstadt, Germany) was added to recognize 8-oxodG as described previously.[35] After fixation and washing, cells were immersed in 10X diluted FITC-labeled antibody for 1

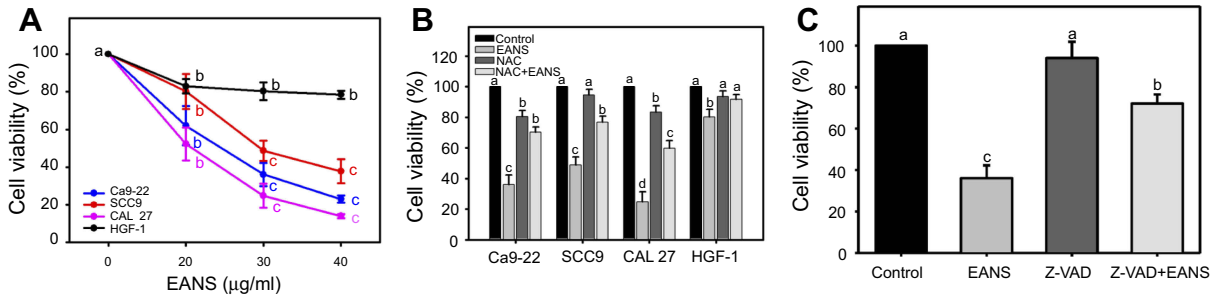

Figure 1 Effect of ethyl acetate extract of *N. ventricosa* x *sibuyanensis* (EANS) on viability of oral cancer and normal oral cells. (**A**) Viabilities of EANS-treated cells. Oral cancer cells (CAL 27, Ca9-22, and SCC9) and oral normal cells (HGF-1) were treated with 0 (untreated control), 20, 30, and 40 μg/mL of EANS for 24 hrs. $p<0.01$~0.0001. (**B**) Viabilities of *N*-acetylcysteine (NAC)-pretreated and EANS-treated cells. Cells were pretreated with or without NAC (2 mM, 1 hr) and posttreated with EANS (30 μg/mL, 24 hrs), ie, NAC+EANS vs EANS. $p<0.05$~0.0001. (**C**) Viabilities of pan-caspase inhibitor Z-VAD-FMK (Z-VAD)-pretreated and EANS-treated Ca9-22 cells. Cells were pretreated with Z-VAD (20 μM, 2 hrs) and posttreated with EANS (30 μg/mL, 24 hrs), ie, Z-VAD+EANS. $p<0.01$~0.0001. Different treatments of the same cell lines were compared with each other. Treatments without the same labels (a–d) indicate the significant difference. From the example of Figure 1C, control (**A**) is differed significantly to EANS (**C**) and Z-VAD+EANS (**B**) in Ca9-22 cells, ie, "a" without the same small letters to "c" or "b". Similarly, Z-VAD (**A**) is differed significantly to Z-VAD+EANS (**B**) without the same small letters. In contrast, control (**A**) is nonsignificant to Z-VAD (**A**) because they have the same letter "a". Data, mean ± SD (n=3).

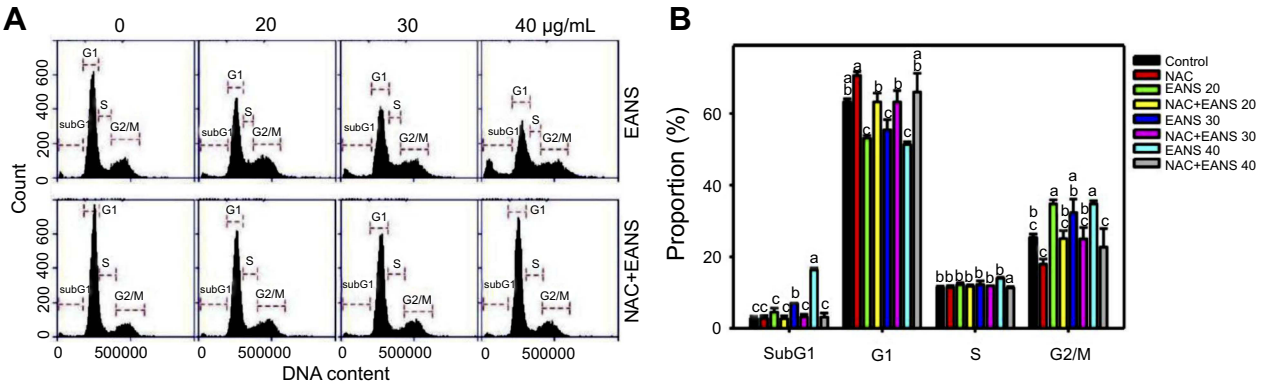

Figure 2 Effect of ethyl acetate extract of *N. ventricosa* x *sibuyanensis* (EANS) on cell cycle distributions of oral cancer cells. (**A**) DNA histograms of different concentrations of EANS treatments in oral cancer cells. Ca9-22 cells were pretreated with or without *N*-acetylcysteine (NAC) (2 mM, 1 hr) and posttreated with EANS for 24 hrs (0 (untreated control), 20, 30, and 40 μg/mL, namely EANS 20, 30, and 40), ie, NAC+EANS vs EANS. (**B**) Statistics of cell cycle distribution in Figure 2A. Different treatments of the same cell cycle phases were compared with each other. Treatments without the same labels (a to c) indicate the significant difference. For the example of G1 population, control (ab) is differed significantly to EANS 20 (**C**) in Ca9-22 cells, ie, "ab" without the same small letters to "c". In contrast, control (ab) is nonsignificant to NAC (**A**) or NAC+EANS 20 (**B**) because they have the same letter "a" or "b". p<0.05~0.0001. Data, mean ± SD (n=3).

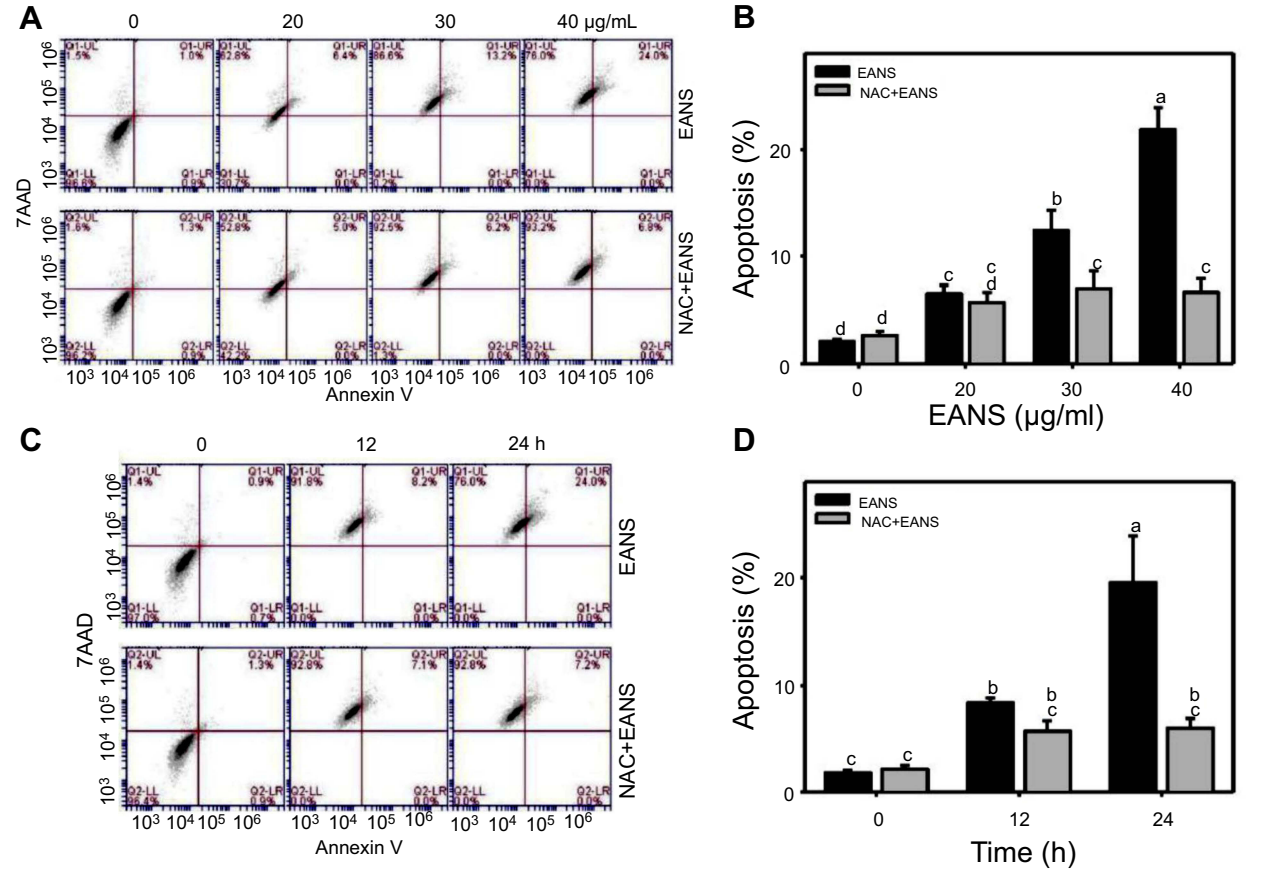

Figure 3 Effect of ethyl acetate extract of *N. ventricosa* x *sibuyanensis* (EANS) on annexin V-based apoptosis of oral cancer cells. (**A**) Annexin V/7AAD dot-plot graphs of different concentrations of EANS treatments in oral cancer cells. Ca9-22 cells were pretreated with or without *N*-acetylcysteine (NAC) (2 mM, 1 hr) and posttreated with EANS (0 (untreated control), 20, 30, and 40 μg/mL, 24 hrs), ie, NAC+EANS vs EANS. Populations of annexin V (+)/7AAD (+) and annexin V (+)/7AAD (–) were classified to apoptosis. (**B**) Statistics of apoptosis change in Figure 3A. Different treatments were compared with each other. Treatments without the same labels (a–d) indicate the significant difference. p<0.05~0.0001. (**C**) Annexin V/7AAD dot-plot graphs of time course of EANS treatments in oral cancer cells. Ca9-22 cells were pretreated with or without NAC and posttreated with EANS (40 μg/mL, 0, 12, and 24 hrs). (**D**) Statistics of apoptosis change in Figure 3C. Treatments without the same labels (a to c) indicate the significant difference. p<0.05~0.0001. Data, mean ± SD (n=3).

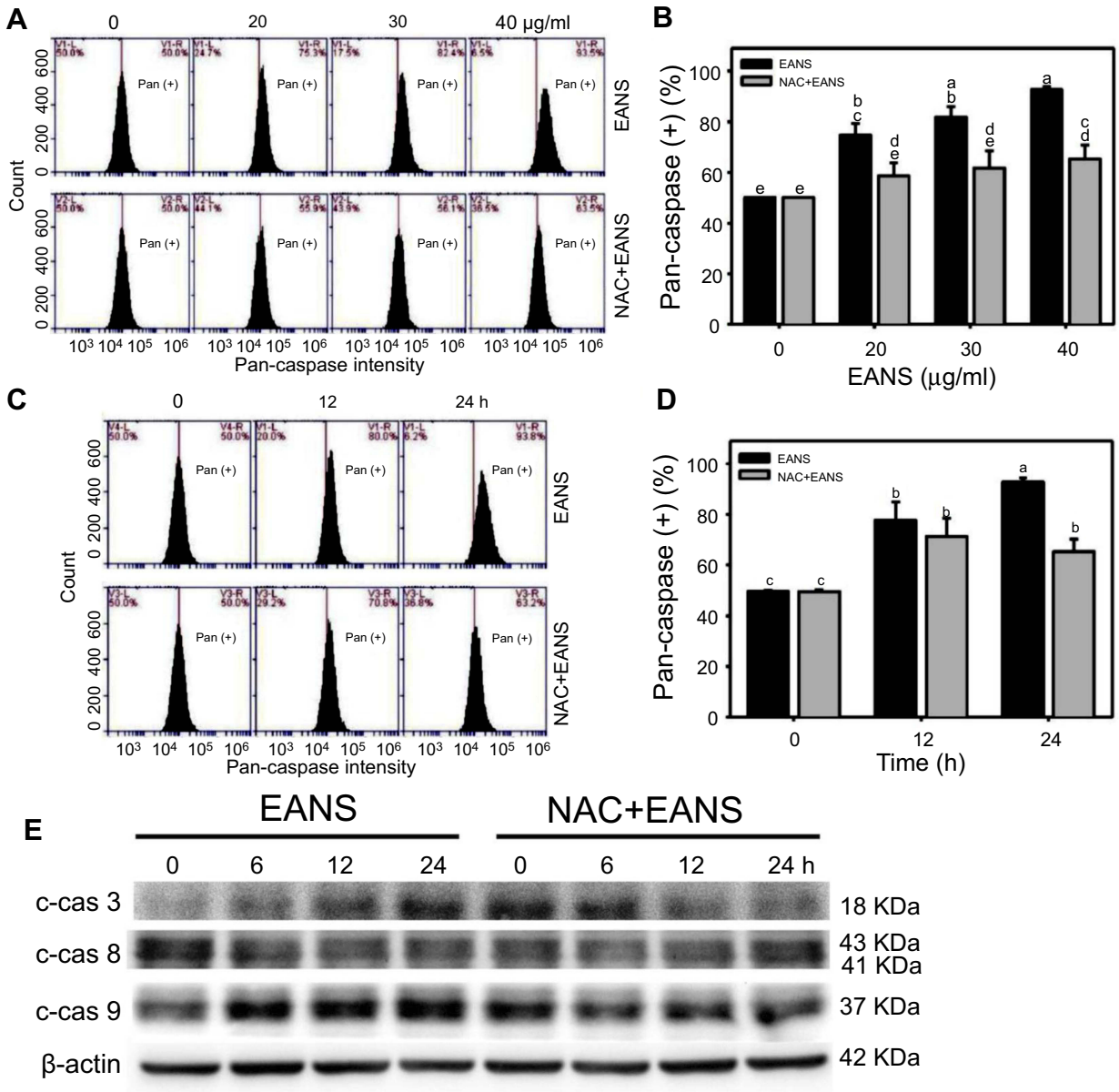

Figure 4 Effect of ethyl acetate extract of *N. ventricosa* x *sibuyanensis* (EANS) on caspases-based apoptosis of oral cancer cells. (**A**) Pancaspase graphs of different concentrations of EANS treatments in oral cancer cells. Ca9-22 cells were pretreated with or without *N*-acetylcysteine (NAC) (2 mM, 1 hr) and posttreated with EANS (0 (untreated control), 20, 30, and 40 μg/mL, 24 hrs), ie, NAC+EANS vs EANS. Pancaspase-positive population is marked as Pan (+). (**B**) Statistics of apoptosis change in Figure 4A. Different treatments were compared with each other. Treatments without the same labels (a–e) indicate the significant difference. $p<0.05$~0.0001. (**C**) Pancaspase graphs of time course of EANS treatments in oral cancer cells. Ca9-22 cells were pretreated with or without NAC and posttreated with EANS (40 μg/mL, 0, 12, and 24 hrs). (**D**) Statistics of apoptosis change in Figure 4C. Treatments without the same labels (a–c) indicate the significant difference. $p<0.05$~0.0001. Data, mean ± SD (n=3). (**E**) Western blotting for apoptosis signaling protein expressions. Ca9-22 cells were pretreated with or without NAC and posttreated with EANS (40 μg/mL, 0, 12, and 24 hrs). Cleaved forms of caspase 3, caspase 8, and caspase 9 are abbreviated as c-cas 3, c-cas 9, and c-cas 8.

hr and were subsequently resuspended in PBS buffer for Accuri™ C6 flow cytometry.

Statistical analysis

Significant differences between multiple comparisons were determined by one-way analysis of variance (ANOVA) and the Tukey HSD test (JMP12; SAS Institute, Cary, NC, USA). Data analysis was performed in triplicate and presented as mean ± SD. Different groups without the same small letters indicate significant differences.

Results

Effect of EANS on cell viability

In this study, HGF-1 cells were used as a normal control compared to three oral cancer cell lines, ie, Ca9-22, CAL 27, and SCC9. In MTS assay, EANS dose-dependently

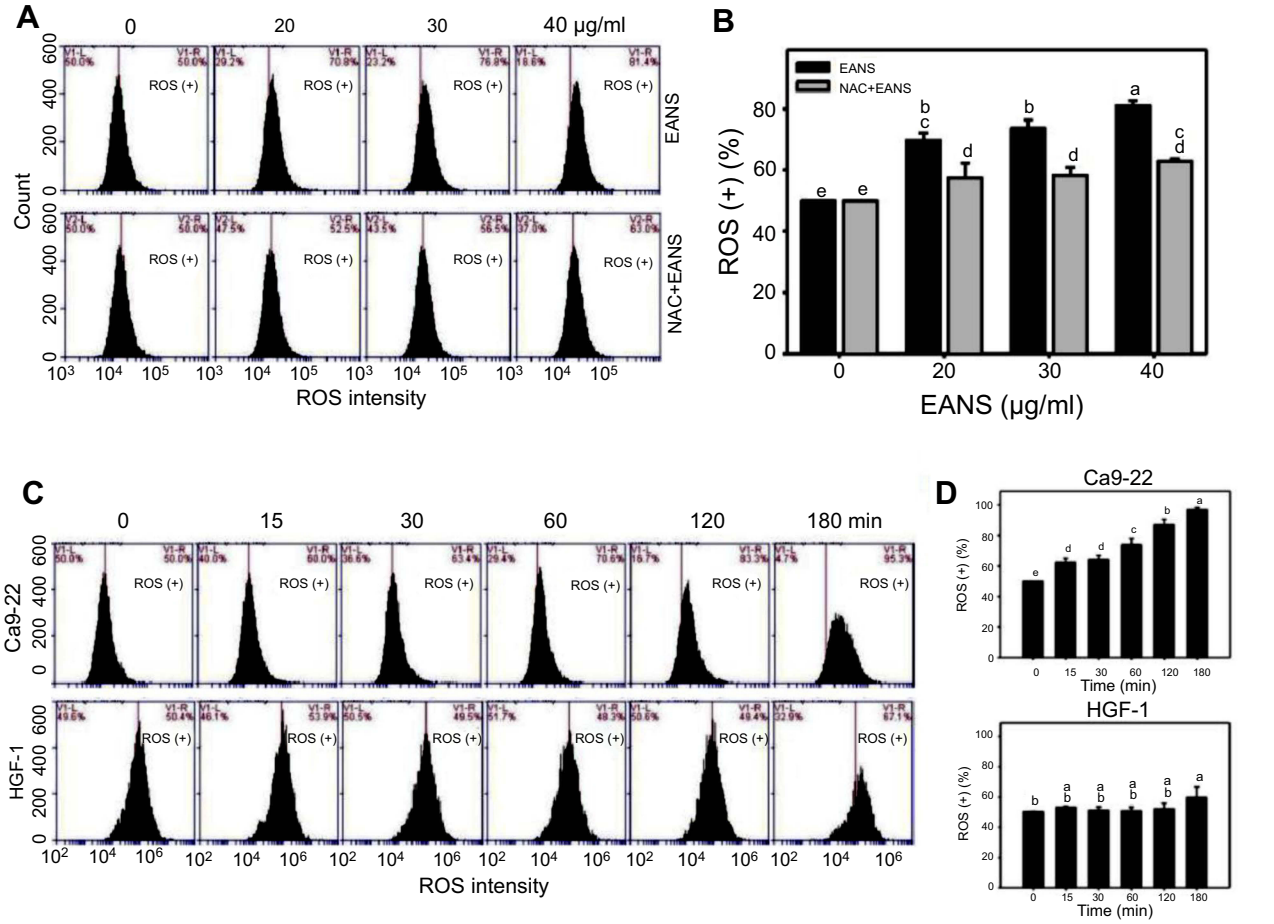

Figure 5 Effect of ethyl acetate extract of *N. ventricosa* x *sibuyanensis* (EANS) on reactive oxygen species (ROS) generation of oral cancer cells. (**A**) ROS graphs of different concentrations of EANS treatments in oral cancer cells. Ca9-22 cells were pretreated with or without *N*-acetylcysteine (NAC) (2 mM, 1 hr) and posttreated with EANS (0 (untreated control), 20, 30, and 40 μg/mL, 3 hrs), ie, NAC+EANS vs EANS. ROS-positive population is marked as ROS (+). (**B**) Statistics of ROS change in Figure 5A. Different treatments were compared with each other. Treatments without the same labels (a–e) indicate the significant difference. p<0.05~0.0001. (**C**) ROS graphs of time course of EANS treatments in oral cancer and normal oral cells. Oral cancer cells (Ca9-22) and normal oral cells (HGF-1) were pretreated with or without NAC and posttreated with EANS (40 μg/mL, 0, 15, 30, 60, 120, and 180 mins). (**D**) Statistics of ROS change in Figure 5C. Treatments without the same labels (a–e) indicate the significant difference. p<0.05~0.0001. Data, mean ± SD (n=3).

reduced the viability (%) of these oral cancer cells (Figure 1A). However, EANS-treated normal oral cells (HGF-1) showed less cytotoxic effect (~80% viability) compared to oral cancer cells.

The EANS-inhibiting viabilities of those test cells were significantly suppressed by NAC pretreatment (p<0.05~0.0001) (Figure 1B). In the example of Ca9-22 cells, the EANS-inhibiting viabilities of oral cancer cells were suppressed by a pancaspase inhibitor Z-VAD pretreatment (Figure 1C).

Effect of EANS on cell cycle distributions of oral cancer cells

Proliferation, cell cycle progression, and apoptosis may interact between each other.[36] Therefore, we investigated whether EANS may influence cell cycle progression. DNA histograms of different concentrations of EANS treatments in oral cancer cells were shown in Figure 2A. Incubation of Ca9-22 cells for 24 hrs with EANS dose-dependently increased the sub-G1 and G2/M populations, and decreased the G1 population, but it showed little change in the S population (Figure 2B). Furthermore, those EANS-affected cell cycle disturbances of Ca9-22 cells were suppressed by NAC.

Effect of EANS on annexin V-based apoptosis of oral cancer cells

To confirm whether the subG1 accumulation of EANS on Ca9-22 cells was related to apoptosis, we detected apoptosis using phosphatidylserine exposure-dependent annexin V/7AAD flow cytometry. Annexin V/7AAD dot-plot graphs of different concentrations and time course of EANS treatments in Ca9-22 cells were shown in

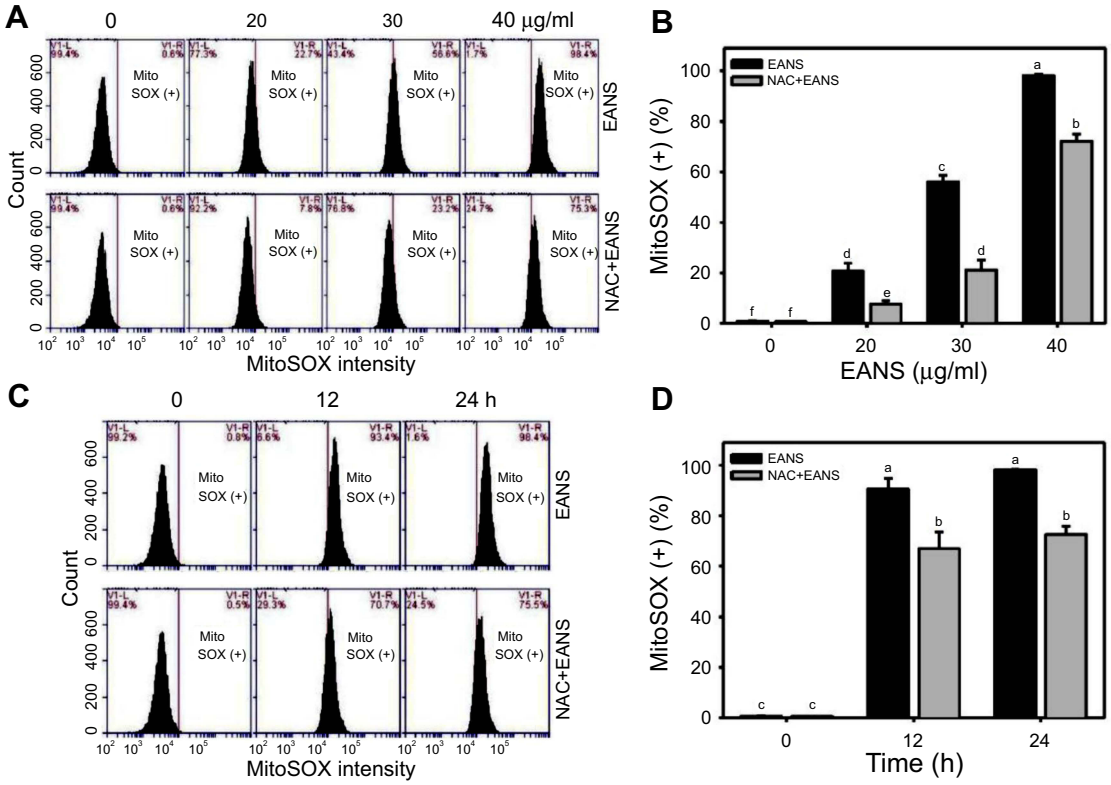

Figure 6 Effect of ethyl acetate extract of *N. ventricosa* x *sibuyanensis* (EANS) on mitochondrial superoxide (MitoSOX) generation of oral cancer cells. (**A**) MitoSOX graphs of different concentrations of EANS treatments in oral cancer cells. Ca9-22 cells were pretreated with or without *N*-acetylcysteine (NAC) (2 mM, 1 hr) and posttreated with EANS (0 (untreated control), 20, 30, and 40 μg/mL, 24 hrs), ie, NAC+EANS vs EANS. MitoSOX-positive population is marked as MitoSOX (+). (**B**) Statistics of MitoSOX change in Figure 6A. Different treatments were compared with each other. Treatments without the same labels (a–f) indicate the significant difference. $p<0.05$~0.0001. (**C**) MitoSOX graphs of time course of EANS treatments in oral cancer cells. Ca9-22 cells were pretreated with or without NAC and posttreated with EANS (40 μg/mL, 0, 12, and 24 hrs). (**D**) Statistics of MitoSOX change in Figure 6C. Treatments without the same labels (a–c) indicate the significant difference. $p<0.001$~0.0001. Data, mean ± SD (n=3).

Figure 3A and C. EANS increased apoptosis in terms of annexin V (+) % in Ca9-22 cells in a dose- and time-dependent manner (Figure 3B and D). Furthermore, those EANS-induced apoptosis expressions of Ca9-22 cells were suppressed by NAC.

Effect of EANS on caspases-based apoptosis of oral cancer cells

To further confirm apoptosis-inducing ability of EANS on Ca9-22 cells, we detected caspase activation using flow cytometry. Pancaspase graphs of different concentrations and time course of EANS treatments in Ca9-22 cells were shown in Figure 4A and C. EANS increased apoptosis in terms of pancaspase (+) % in Ca9-22 cells in a dose- and time-dependent manner (Figure 4B and D). Furthermore, those EANS-induced pancaspase (apoptosis) activations of Ca9-22 cells were suppressed by NAC.

Since the applied pancaspase kit was developed to generically detect the pancaspase activity (caspases (cas)-1, 3, 4, 5, 6, 7, 8, 9),[29] it was necessary to further examine the involvement of caspase signaling for apoptosis. Therefore, we detected several members of caspase signaling by Western blotting. After EANS treatment to Ca9-22 cells, the cleaved forms of caspases such as c-cas 3 and c-cas 9 expressions were increased but c-cas 8 was undetectable (Figure 4E), suggesting that EANS mainly induced intrinsic apoptosis in Ca9-22 cells. Furthermore, those EANS-induced expressions of apoptosis signaling proteins of Ca9-22 cells were suppressed by NAC.

Effect of EANS on ROS generation of oral cancer cells

Excess ROS may induce DNA damage and apoptosis.[37] Therefore, we investigated whether EANS may influence intracellular ROS generation. ROS graphs of different concentrations of EANS treatments in Ca9-22 cells were shown in Figure 5A. EANS treatment to Ca9-22 cells enhanced the ROS generation in a dose-dependent manner

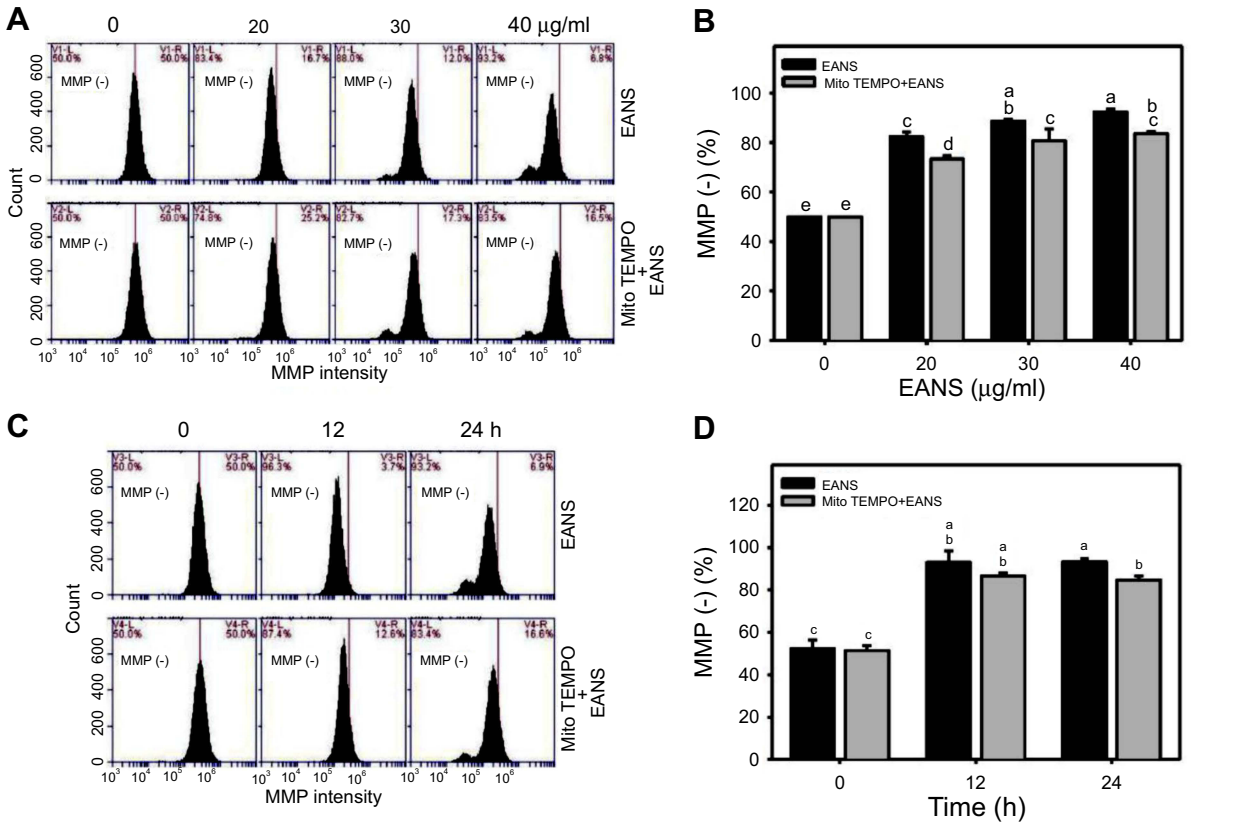

Figure 7 Effect of ethyl acetate extract of *N. ventricosa* x *sibuyanensis* (EANS) on mitochondrial membrane potential (MMP) of oral cancer cells. (**A**) MMP graphs of different concentrations of EANS treatments in oral cancer cells. Ca9-22 cells were pretreated with or without mitochondrial superoxide inhibitor (Mito TEMPO) (20 μM, 1 hr) and posttreated with EANS (0 (untreated control), 20, 30, and 40 μg/mL, 24 hrs), ie, Mito TEMPO+EANS vs EANS. MMP-negative population is marked as MMP (–). (**B**) Statistics of MMP change in Figure 7A. Different treatments were compared with each other. Treatments without the same labels (a–e) indicate the significant difference. $p<0.05$~0.0001. (**C**) MMP graphs of time course of EANS treatments in oral cancer cells. Ca9-22 cells were pretreated with or without Mito TEMPO (20 μM, 1 hr) and posttreated with EANS (40 μg/mL, 0, 12, and 24 hrs). (**D**) Statistics of MMP change in Figure 7C. Treatments without the same labels (a–c) indicate the significant difference. $p<0.05$~0.0001. Data, mean ± SD (n=3).

(Figure 5B). Furthermore, those EANS-induced ROS generation of Ca9-22 cells were suppressed by NAC.

A time course of ROS development after EANS treatments in oral cancer cells (Ca9-22) and normal oral cells (HGF-1) is shown in Figure 5C. As shown in Figure 5D, EANS dramatically induces ROS generation of Ca9-22 cells in a time-dependent manner within 3 hrs. In contrast, HGF-1 cells maintain basal level of ROS after the same treatment by EANS.

Effect of EANS on the MitoSOX generation of oral cancer cells

Oxidative stress is mainly produced in mitochondria,[38] especially for mitochondrial superoxide. Therefore, we examined MitoSOX upon EANS treatment. MitoSOX graphs of different concentrations and time course of EANS treatments in Ca9-22 cells are shown in Figure 6A and C. EANS increases MitoSOX (+) % in Ca9-22 cells in a dose- and time-dependent manner (Figure 6B and D). Furthermore, those EANS-induced MitoSOX generations of Ca9-22 cells were suppressed by NAC.

Effect of EANS on MMP of oral cancer cells

Oxidative stress generated from mitochondria may regulate apoptotic signaling.[39] Therefore, we examined MMP upon EANS treatment. MMP graphs of different concentrations and time course of EANS-treated Ca9-22 cells are shown in Figure 7A and C. EANS increased MMP (–) % in Ca9-22 cells in a dose- and time-dependent manner (Figure 7B and D). Furthermore, those EANS-induced MMP destructions of Ca9-22 cells are suppressed by the pretreatment of MitoSOX-specific inhibitor Mito TEMPO.

Effect of EANS on γH2AX-based DNA damage of oral cancer cells

To investigate the effects of EANS on inducting apoptosis in Ca9-22 cells, DNA damage in terms of γH2AX were evaluated. γH2AX graphs of different concentrations and time course of

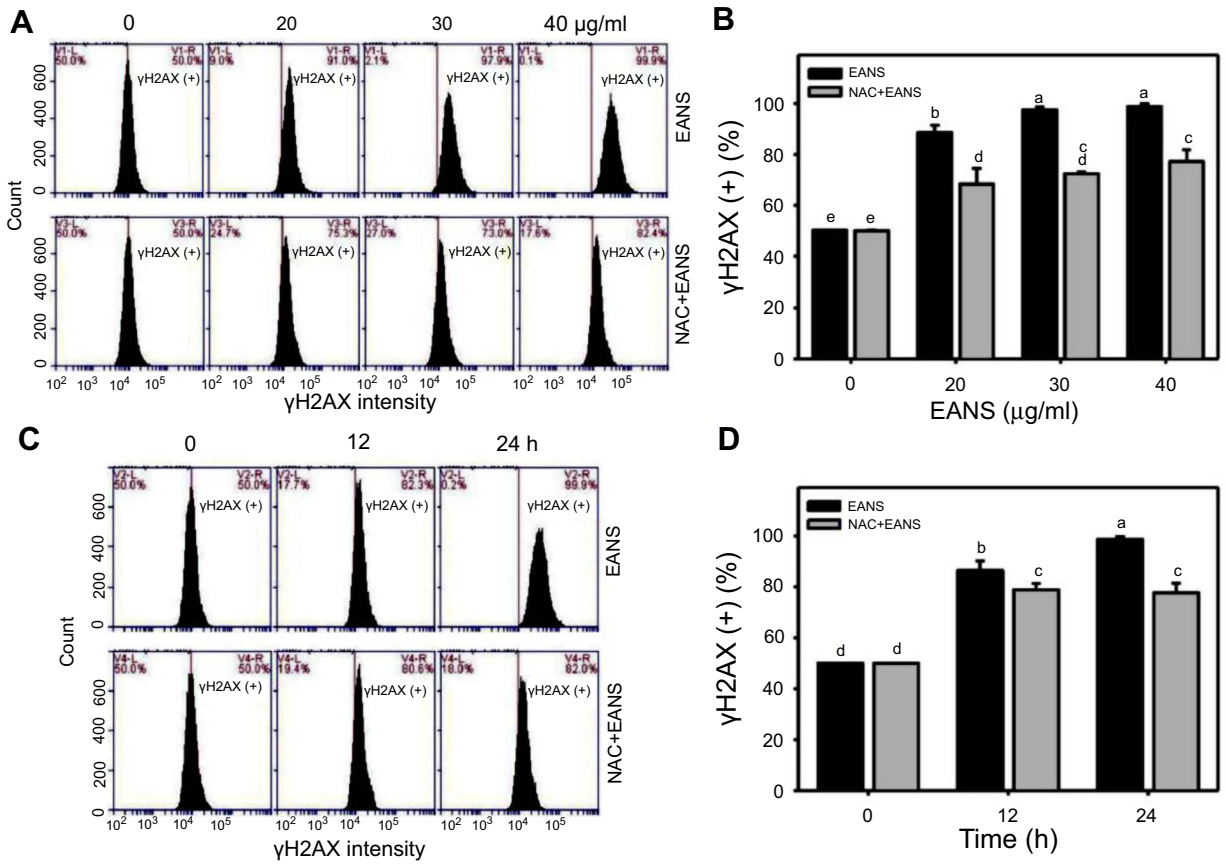

Figure 8 Effect of ethyl acetate extract of *N. ventricosa* x *sibuyanensis* (EANS) on γH2AX-based DNA damage of oral cancer cells. (**A**) γH2AX graphs of different concentrations of EANS treatments in oral cancer cells. Ca9-22 cells were pretreated with or without *N*-acetylcysteine (NAC) (2 mM, 1 hr) and posttreated with EANS (0 (untreated control), 20, 30, and 40 μg/mL, 24 hrs), ie, NAC+EANS vs EANS. γH2AX-positive population is marked as γH2AX (+). (**B**) Statistics of γH2AX change in Figure 8A. Different treatments were compared with each other. Treatments without the same labels (a–e) indicate the significant difference. $p<0.05$~0.0001. (**C**) γH2AX graphs of time course of EANS treatments in oral cancer cells. Ca9-22 cells were pretreated with or without NAC and posttreated with EANS (40 μg/mL, 0, 12, and 24 hrs). (**D**) Statistics of γH2AX change in Figure 8C. Treatments without the same labels (a–d) indicate the significant difference. $p<0.05$~0.0001 . Data, mean ± SD (n=3).

EANS treatments in Ca9-22 cells were shown in Figure 8A and C. EANS increased γH2AX (+) % in Ca9-22 cells in a dose- and time-dependent manner (Figure 8B and D). Furthermore, those EANS-induced γH2AX expressions of Ca9-22 cells were suppressed by NAC.

Effect of EANS on 8-oxodG-based DNA damage of oral cancer cells

To investigate the effects of EANS on inducting apoptosis in Ca9-22 cells, oxidative DNA damage in terms of 8-oxodG were evaluated. 8-OxodG graphs of different concentrations and time course of EANS treatments in Ca9-22 cells were shown in Figure 9A and C. EANS increased 8-oxodG (+) % in Ca9-22 cells in a dose- and time-dependent manner (Figure 9B and D). Furthermore, those EANS-induced 8-oxodG expressions were suppressed by NAC.

Discussion

Nepenthes plants are used for herbal medicine[14,15] and display diverse biological effects against bacteria[16] and fungi.[17] However, an anticancer effect of *Nepenthes* plants remains unclear. Moreover, different solvents were used to extract *Nepenthes* plant in earlier studies[20,40,41] with the exception of ethyl acetate which was used here for the first time.

In this study, we used ethyl acetate extract of *N. ventricosa* x *sibuyanensis* (EANS) to evaluate the antiproliferative effect for oral cancer cells. Incubation of oral cancer cells (Ca9-22, CAL 27, and SCC9) for 24 hrs with EANS show IC_{50} values with 25, 20, and 32 μg/mL. Incubation of normal oral cells (HGF-1) for 24 hrs with the highest test concentration of EANS (40 μg/mL) shows about 80% viability. Therefore, EANS has higher cytotoxicity against oral cancer cells than normal oral cells. The antiproliferative effect of EANS

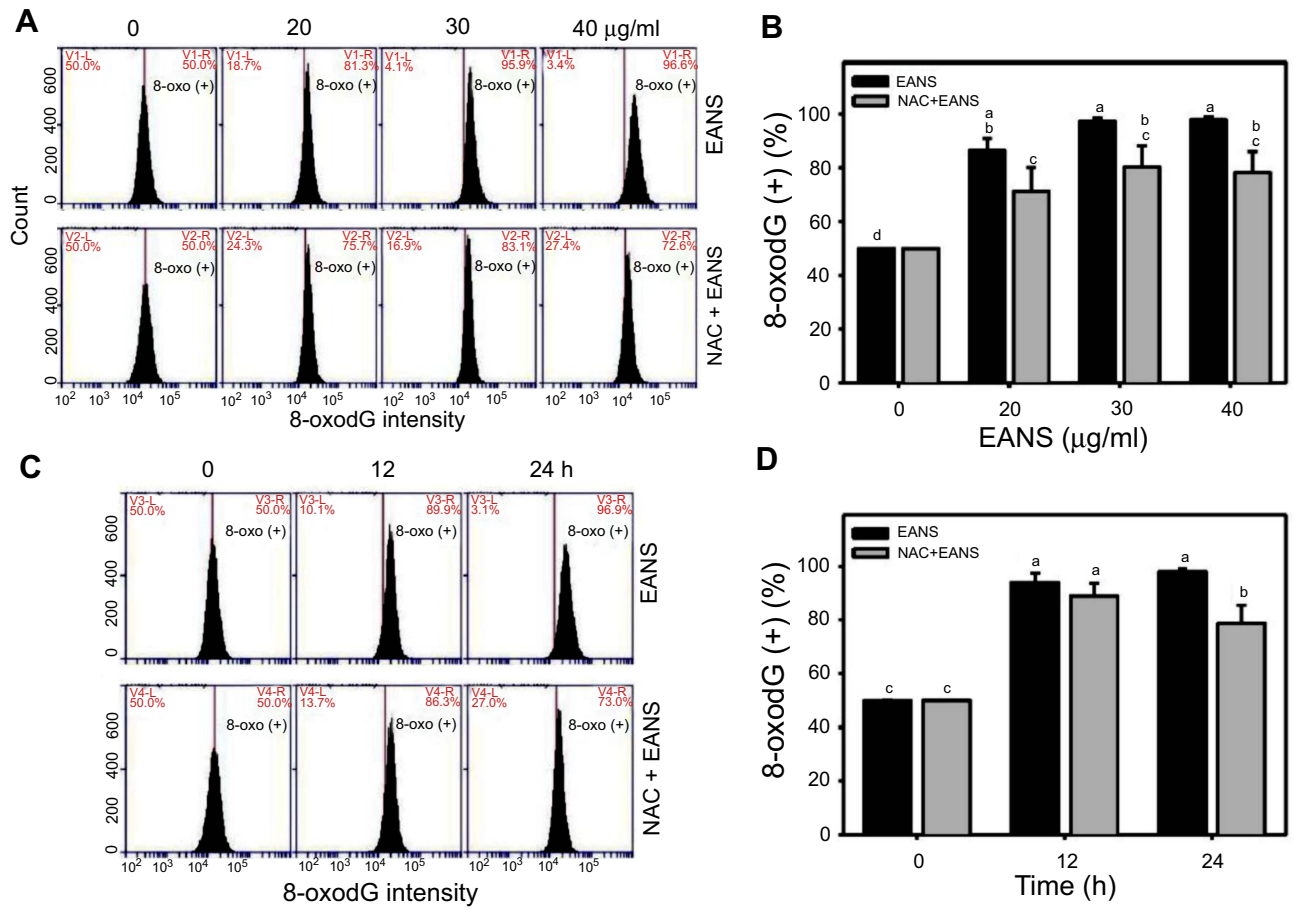

Figure 9 Effect of ethyl acetate extract of *N. ventricosa* x *sibuyanensis* (EANS) on 8-oxo-2′-deoxyguanosine (8-oxodG)-based DNA damage of oral cancer cells. (**A**) 8-oxodG graphs of different concentrations of EANS treatments in oral cancer cells. Ca9-22 cells were pretreated with or without *N*-acetylcysteine (NAC) (2 mM, 1 hr) and posttreated with EANS (0 (untreated control), 20, 30, and 40 μg/mL, 24 hrs), ie, NAC+EANS vs EANS. 8-oxodG-positive population is marked as 8-oxodG (+). (**B**) Statistics of 8-oxodG change in Figure 9A. Different treatments were compared with each other. Treatments without the same labels (a–d) indicate the significant difference. $p<0.05$~0.0001. (**C**) 8-oxodG graphs of time course of EANS treatments in oral cancer cells. Ca9-22 cells were pretreated with or without NAC and posttreated with EANS (40 μg/mL, 0, 12, and 24 hrs). (**D**) Statistics of 8-oxodG change in Figure 9C. Treatments without the same labels (a–c) indicate the significant difference. $p<0.05$~0.0001. Data, mean ± SD (n=3).

treatment against oral cancer cells was suppressed by the ROS scavenger NAC and the pancaspase inhibitor Z-VAD (Figure 1), suggesting that oxidative stress and apoptosis play vital roles in the antioral cancer effect of EANS. It should be emphasized that the concentration of EANS used in this study was based on a cell line model and may not be suitable to apply to blood or tissue levels.

Although antioxidants at normal concentration may reduce the ROS generation, antioxidants at high concentration may induce intracellular ROS generation.[42] Many antioxidant components were reported from *Nepenthes* plants.[18–20] In our test concentrations (10–40 μg/mL), 3 hrs incubation of EANS enhanced ROS generation in oral cancer cells (Ca9-22) ranging from 70% to 80% ROS (+) (Figure 5B). Moreover, 2 hrs incubation of EANS (40 μg/mL) induced 80% ROS (+) of ROS generation in Ca9-22 cells (Figure 5C). Accordingly, *Nepenthes* plants containing antioxidant constitutes may have concentration effects on ROS induction in Ca9-22 cells. In contrast, 3 hrs incubation of EANS (40 μg/mL) maintained basal level of ROS generation in normal oral cells (HGF-1) (Figure 5C), suggesting on differential ROS induction between oral cancer and normal oral cells. Since ROS may induce apoptosis[43] and DNA damage,[44] this differential ROS induction may partly explain the differential killing against oral cancer cells but less cytotoxic effects on normal oral cells.

In addition to ROS generation, EANS also induce MitoSOX generation and MMP destruction in oral cancer cells. Therefore, EANS induces oxidative stress against oral cancer cells. These inductions were inhibited by NAC or Mito TEMPO pretreatments, which further supported that oxidative stress was involved in antioral cancer effect of EANS.

Furthermore, EANS-induced subG1 accumulation (Figure 2), annexin V-detected apoptosis (Figure 3), pancaspase-detected apoptosis (Figure 4B and 4D), and caspase signaling activation (Figure 4E) was also inhibited by NAC pretreatment. Accordingly, the role of oxidative stress in these EANS-induced apoptosis-related effects was validated.

Excessive oxidative stress frequently induces several types of DNA damages such as DNA double-strand breaks (γH2AX)[44] and oxidative DNA damage (8-oxodG).[45] Consistently, we found that EANS-induced oxidative stress, in turn, induced γH2AX and 8-oxodG expressions, which were inhibited by NAC pretreatment (Figures 8 and 9). Again, the function of oxidative stress in EANS-induced ROS-mediated DNA damage was validated. However, the signaling transduction for EANS-induced oxidative stress was not examined in the present study. It was reported that oxidative stress may activate mitogen-activated protein kinase (MAPK) signaling[46] and in turn regulate apoptosis[47] and DNA damage.[48,49] A detailed investigation of the effect of MAPK of antioral cancer effects caused by EANS is warranted in the future.

Conclusion

The antioral cancer effect of *Nepenthes* plants is reported here for the first time. In this study, we demonstrated that ethyl acetate extraction for *N. ventricosa x sibuyanensis* (EANS) preferentially inhibited the proliferation of oral cancer cells but showed little effect on normal oral cells. Oxidative stress detected by intracellular ROS and mitochondrial superoxide were also induced by EANS treatment for oral cancer cells. Oxidative stress-induced apoptosis and DNA damage appeared in EANS-treated oral cancer cells. Using NAC pretreatment to oral cancer cells, we further demonstrated that the EANS-induced antiproliferation, oxidative stress-associated changes, apoptosis, and DNA damages were mediated by ROS.

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Disclosure

The authors declare that they have no conflicts of interest in regard to this work.

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