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Inula graveolens induces selective cytotoxicity in glioblastoma and chronic leukemia cells

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SUMMARY

OBJECTIVE: Crude oil extracts, components of extracts, and ethanolic extracts of *Inula graveolens* possess various pharmacological activities on various cancer cells including antioxidative and antiproliferative effects. Aqueous extract of this species has not been investigated on the liquid malignancies and solid tumors with a high incidence of treatment refractoriness and poor survival outcomes such as glioblastoma and leukemia. Hence, the present study aimed to evaluate the cytotoxic efficiency of *I. graveolens* aqueous extracts on human glioblastoma multiforme and chronic myelogenous leukemia cell lines in comparison to non-cancerous primary rat cerebral cortex and human peripheral blood mononuclear cells.

METHODS: The cells were treated with the extracts of *I. graveolens* (125–1000 µg/mL) for 48 h, the cellular viability was identified using 3'-(4,5dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay, and lactate dehydrogenase release was measured to determine the cytotoxic potential. Total oxidant status and apurinic/apyrimidinic endodeoxyribonuclease 1 assays were used to determine the oxidative status of cells and DNA damage, respectively.

RESULTS: *I. graveolens* showed selective cytotoxicity toward human glioblastoma multiforme and chronic myelogenous leukemia cell lines and exhibited a higher antiproliferative effect against cancer cells in comparison to non-cancerous cells. Moreover, it significantly reduced the apurinic/apyrimidinic endodeoxyribonuclease 1 levels on both cancer cell lines as compared with their control cells without changing the levels of an oxidative stress marker.

CONCLUSION: The extracts of *I. graveolens* have anti-cancer potential on human glioblastoma multiforme and chronic myelogenous leukemia cell lines without causing oxidative stress.

KEYWORDS: Glioblastoma. Myeloid leukemia. Inula. Cell viability.

INTRODUCTION

Inula graveolens (*Dittrichia graveolens*) is an annual plant that is sticky, strongly aromatic, and widely distributed in the Mediterranean area[1]. The essential oil and extracts of *I. graveolens* possess significant antioxidant, antiproliferative, and antibacterial activities, which have been demonstrated by pharmacological studies[2-4]. Previous phytochemical investigations of this plant have revealed the presence of oxygenated monoterpenes with high contents of bornyl acetate, borneol, and τ-cadinol[5]. Besides, chlorogenic acid, quinic acid, hyperoside, protocatechuic acid, and quercetin are the major phenolic compounds of *I. graveolens*. The significant antioxidant capacity of *I. graveolens* has been thought to be related to the high

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abundance of phenolic compounds[6]. Recently, the compounds isolated from *I. graveolens* have been recorded as an important antiradical power[7]. Moreover, the methanolic extract of *I. graveolens* has been shown that the antioxidant activity helps ameliorate the oxidative stress/antioxidant status balance in elite athletes during a competition period[8]. No recent reports are available about the antiproliferative potential aqueous extract of this species the liquid malignancies and solid tumors with a high incidence of treatment refractoriness and poor survival outcomes such as glioblastoma and leukemia. Therefore, the present study was conducted to clarify the anticancer potential of aqueous extract of *I. graveolens* on human glioblastoma multiforme (U-87 MG) and chronic myelogenous leukemia (K562) cancer cells concerning non-cancerous primary rat cerebral cortex (PRCC) and peripheral blood mononuclear cells (PBMCs).

METHODS

U-87 MG (ATCC® HTB-14) and K562 (ATCC® CCL-243) cell lines were obtained from American Type Culture Collection. The PRCC culture was obtained from the Department of Medical Pharmacology of Ataturk University, Erzurum, Turkey. The PBMCs were obtained from healthy volunteers. Whole blood was collected in the heparin blood tube and transferred to a conical tube containing 4 mL of phosphate-buffered saline (PBS). The diluted blood sample was carefully added onto a new conical tube containing 8 mL of Lymphoprep solution, and the blood was layered over the Lymphoprep solution. After centrifugation, the upper layer was aspirated carefully without disturbing the mononuclear cell layer containing lymphocytes, monocytes, and platelets at the interphase. The mononuclear cell layer was put into a new tube filled with PBS and centrifuged. The supernatant was discarded for the removal of platelets. The pellet was suspended in the RPMI medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. The cells were disseminated into 24-well culture plates and cultured in 5% CO_2 and 95% moisture at 37°C for two days.

The aerial parts of *I. graveolens* were infused in distilled water at 98°C of temperature for 15 min and filtered. After sterilization, the infusion was diluted with a culture medium. Cell lines were grown in DMEM supplemented with 1% penicillin-streptomycin solution, 2 mM L-glutamine, and 10% heat-inactivated FBS. Cells were grown to 80% confluency, and 10,000 cells/well were seeded into 96-well plates in triplicate. After 24 h of incubation, the medium was replaced with a fresh medium containing various concentrations (125, 250, 500, and 1000 µg/mL) of *I. graveolens* according to our preliminary study.

After 48 h of treatments, the medium was removed and replaced with 150 µL of 3'-(4,5dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) stock solution (Sigma Chemical Company, St. Louis, USA). The plates were placed in an incubator at 37°C for 4 h and then MTT was replaced with 150 µL of dimethyl sulfoxide. The absorbance was measured at 570 nm in a microplate reader. Lactate dehydrogenase (LDH) released from damaged cells was quantified by using a kit (Cayman Chemical Company, USA). The rate of NADH reduction directly proportional to LDH activity was measured as an increase in the absorbance at 490 nm.

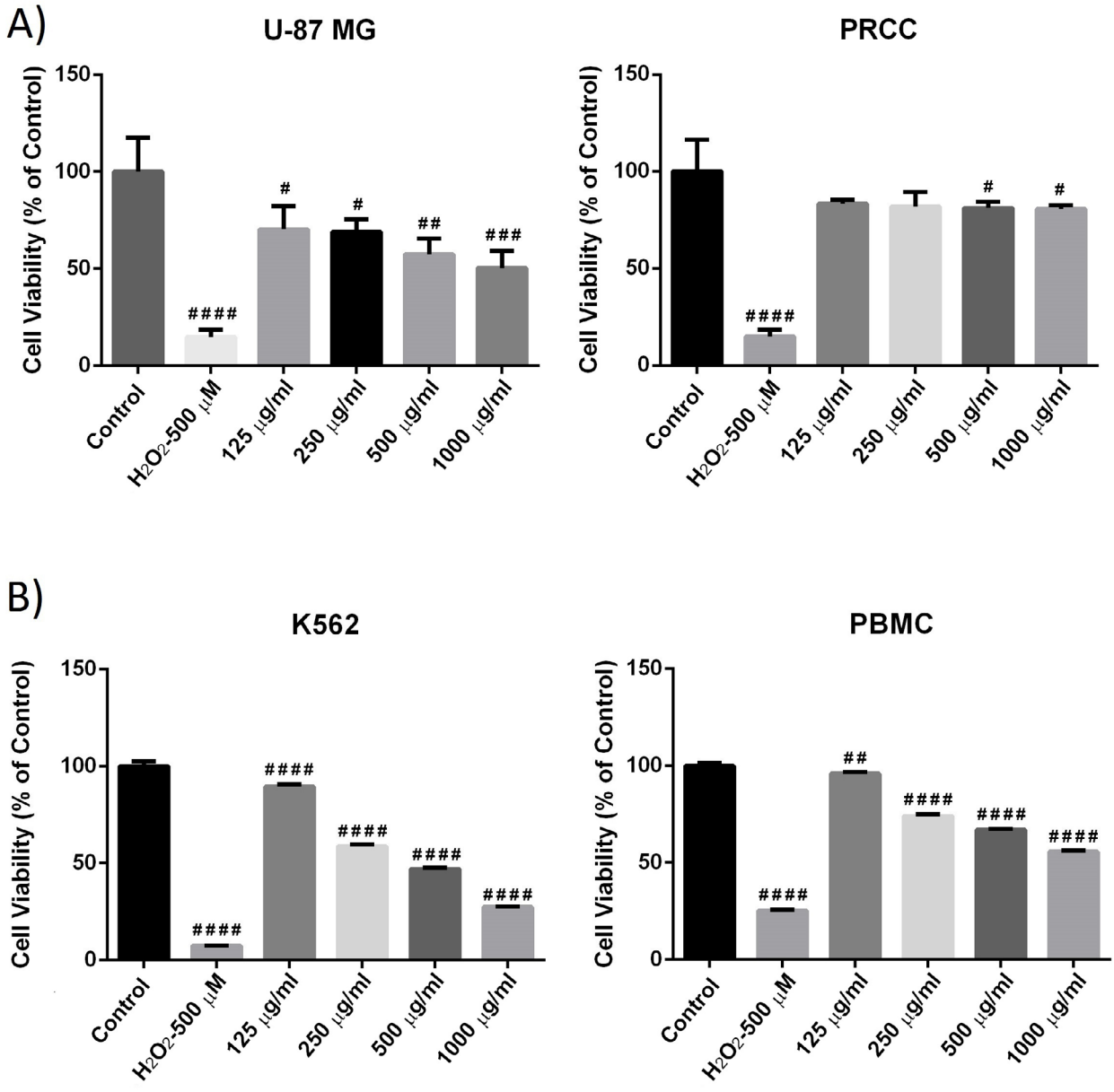
The total oxidant status (TOS) of cultured cells was measured using a commercial Rel Assay Diagnostics® Kit (Turkey). The amount of TOS in culture media was measured by the density of color change at 530 nm.

Apurinic/apyrimidinic endodeoxyribonuclease 1 (APE1) level was examined from cell culture supernatants (ab207616, Abcam, UK). The absorbance (OD) was read at 450 nm, and the concentration of the target in the samples was calculated by constructing a standard curve prepared from standard samples.

To compare the statistical significance of differences between the obtained results of the negative control and treated groups of *I. graveolens* aqueous part, statistical analysis was performed using a one-way analysis of variance (ANOVA) test followed by GraphPad Prism 6.0 statistics software (GraphPad, La Jolla, CA, USA). Tukey's test was used as a *post hoc*. Comparisons among the groups were represented as the mean±standard deviation (SD). A $p<0.05$ was considered statistically significant.

RESULTS

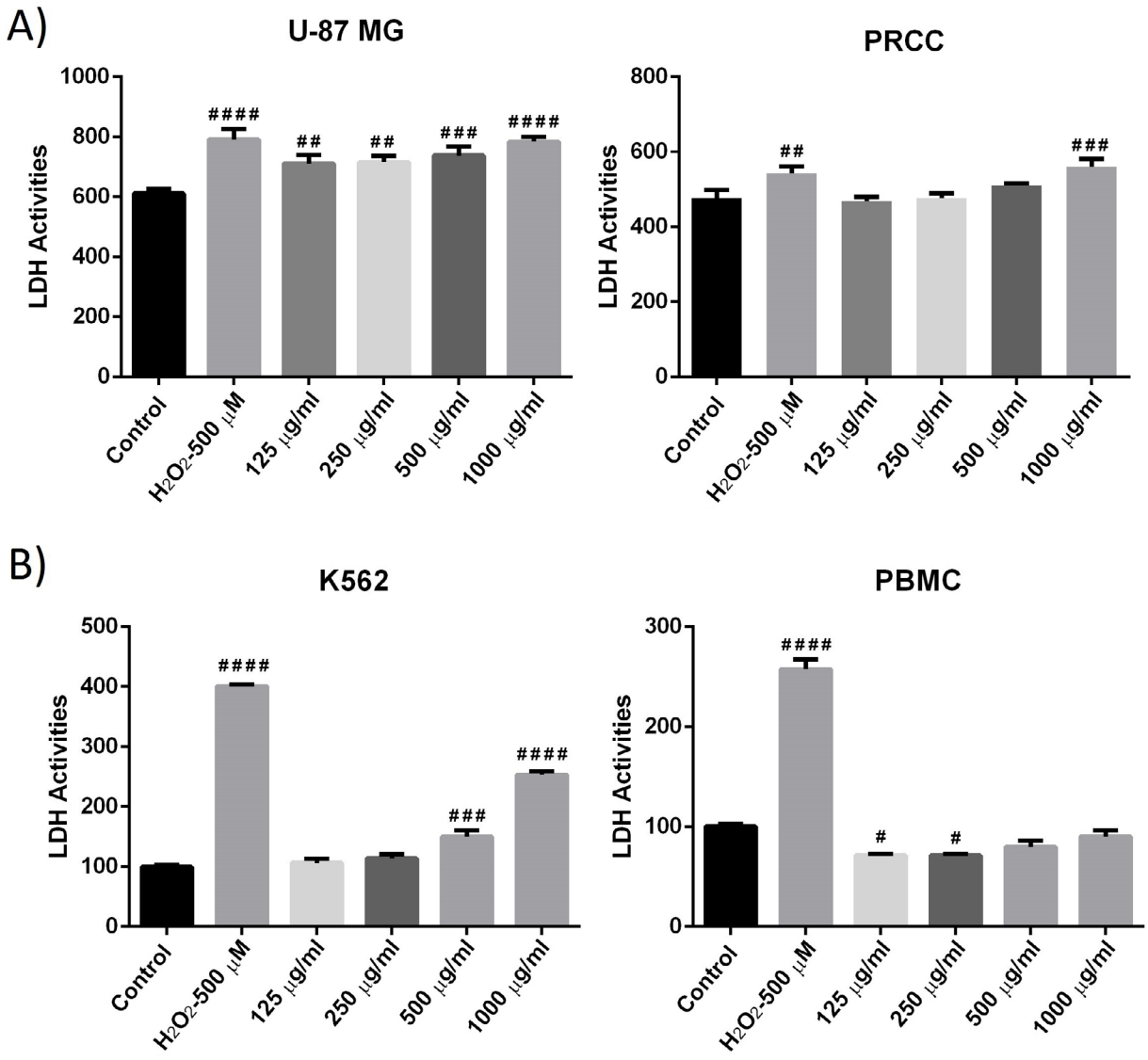
The cytotoxicity of the aqueous extracts of *I. graveolens* against human brain and leukemic cancer cells and non-cancerous cells was detected by MTT and LDH release assay. *I. graveolens* elicited a significant decrease in cell viability in both U-87 MG and K562 cell lines, which occurred in a dose-dependent manner. The extracts at the concentrations of 500 and 1000 µg/mL depicted considerable antiproliferative activity on U-87 MG cell lines ($p<0.01$, reduction of 43% and $p<0.001$, reduction of 50%, respectively). However, more than 80% of PRCC remained viable after treatment with 500 and 1000 µg/mL of the extract ($p<0.05$), indicating that *I. graveolens* exhibited low cytotoxicity against PRCCs. Furthermore, the exposure to *I. graveolens* at the doses of 500 and 1000 µg/mL resulted in the 53% ($p<0.0001$) and 73% ($p<0.0001$) decrease in cell viability on K562 cells, but it is less toxic against healthy blood cells (Figure 1B). The same doses of the extract resulted in 33% ($p<0.0001$) and 44% ($p<0.0001$) decrease in cell viability on PBMCs. Treatment with 1000 µg/mL *I. graveolens*



U-87 MG: Human glioblastoma multiforme; PRCC: primary rat cerebral cortex; K562: Chronic myelogenous leukemia; PBMC: peripheral blood mononuclear cells. #p<0.05, ##p<0.01, ###p<0.001, ####p<0.0001 indicate statistically significant differences when compared to nontreated cells or cells treated with other concentrations.

Figure 1. Cytotoxic effects of the *I. graveolens* extract against cell lines: (A) Human glioblastoma multiforme and primary rat cerebral cortex and (B) Chronic myelogenous leukemia and peripheral blood mononuclear cells after 48 h of incubation. The cellular viability was determined as the percentage of absorbance of *I. graveolens* treated cultures compared with those of untreated control cells, used as a negative control. Cells treated with 500 µm H_2O_2 were used as a positive control. Values are expressed as mean±standard deviation of three independent replicates.

resulted in more than 50% viable PBMCs and PRCCs. Moreover, *I. graveolens* led to a remarkable increase in the LDH level of U-87 MG cells, while concentrations below 1000 µg/mL did not cause any change in PRCCs ($p<0.001$). The extract at 500 and 1000 µg/mL LDH release significantly increased in K562 cells ($p<0.001$ and $p<0.0001$, respectively), while at the same concentrations, it did not cause any change in the LDH release of PBMCs (Figure 2). Thus, brain cancer and leukemic cells were more sensitive to *I. graveolens* than normal cells, implying that *I. graveolens* could damage the membrane



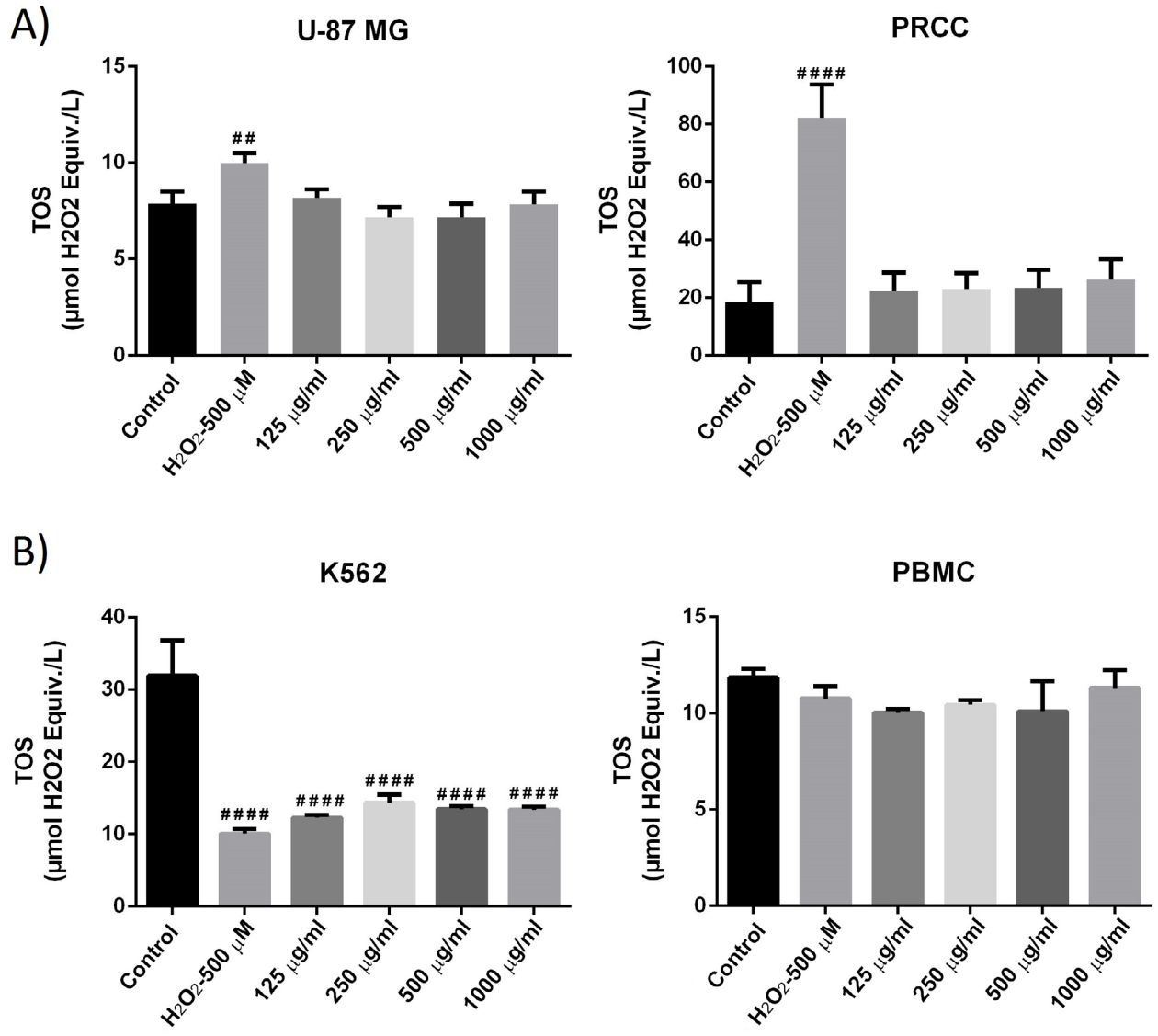
U-87 MG: human glioblastoma multiforme; PRCC: primary rat cerebral cortex; K562: chronic myelogenous leukemia; PBMC: peripheral blood mononuclear cells.

Figure 2. Effects of the *I. graveolens* extract on lactate dehydrogenase release in cell lines (A) Human glioblastoma multiforme and primary rat cerebral cortex and (B) Chronic myelogenous leukemia and peripheral blood mononuclear. Values are expressed as mean±standard deviation of three independent replicates. $^{\#}p<0.05$, $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$, $^{\#\#\#\#}p<0.0001$ indicate statistically significant differences when compared to non-treated cells or cells treated with other concentrations.

integrity and resulting in dose-dependent induction of cellular toxicity.

Whether the extract from *I. graveolens* induced oxidative stress on the cells was tested by measuring the TOS level. The extracts at all concentrations did not cause any change in the TOS level of U-87 MG cells, while significantly reduced the TOS level of K562 cells. On the other hand, the extract at all concentrations tested did not cause any change in the TOS level on both non-cancerous cells (Figure 3).

APE1 activity was measured to investigate the DNA repair status of *I. graveolens* on cancer and non-cancerous cells. The level of APE1 significantly decreased in a dose-dependent manner on *I. graveolens*-treated U-87 MG cells compared with the control ($p<0.05$ and $p<0.01$). Also, the level of APE1 decreased significantly at all concentrations of the extract on PRCC ($p<0.001$). The level of APE1 in H_2O_2-treated K562 cells was also enhanced compared with the control ($p<0.001$). On the other hand, the level of APE1 decreased at all concentrations of the extract ($p<0.01$). The extracts at concentrations above



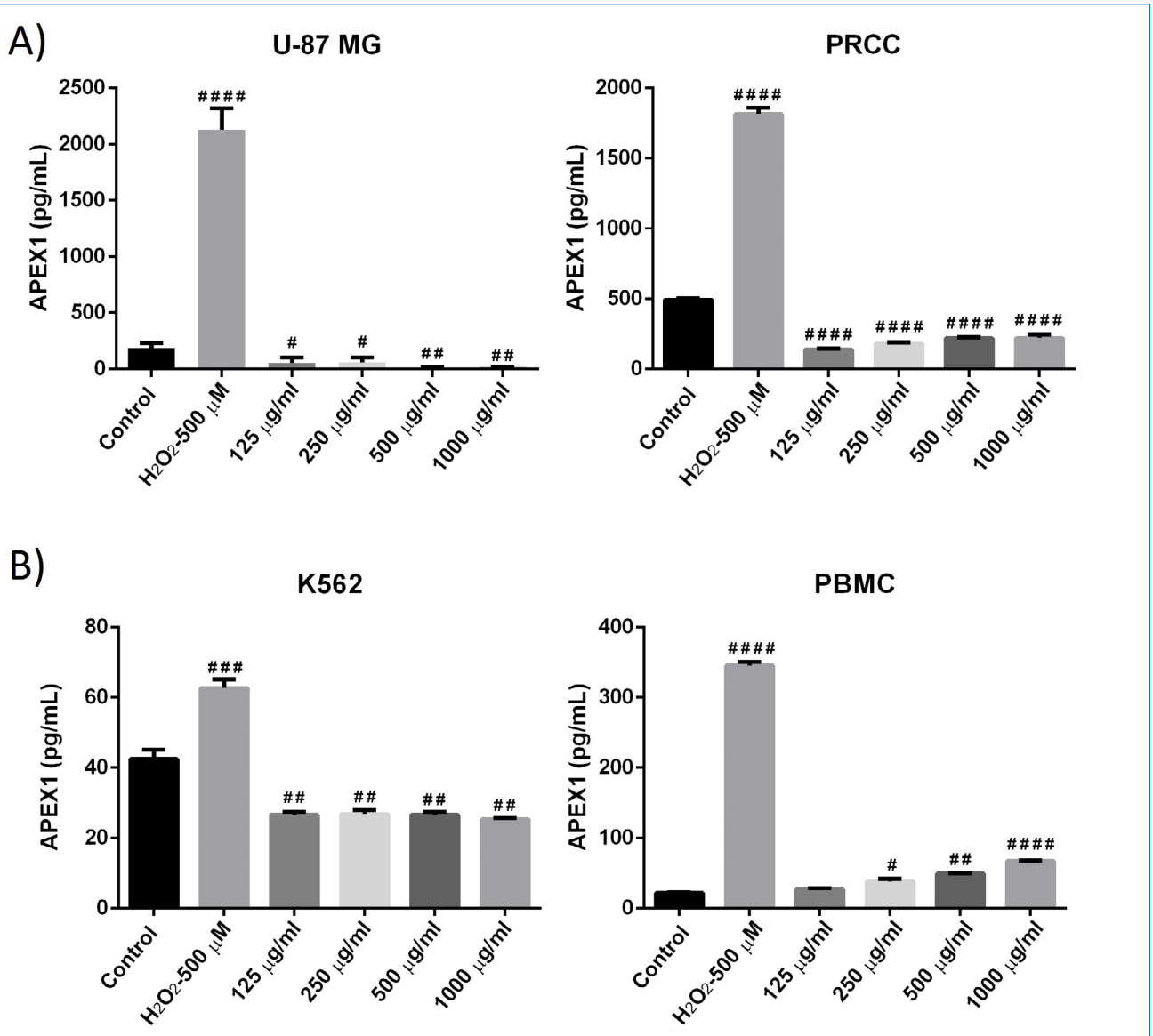
U-87 MG: Human glioblastoma multiforme; PRCC: primary rat cerebral cortex; K562: Chronic myelogenous leukemia; PBMC: peripheral blood mononuclear cells. ##p<0.01, ####p<0.0001 indicate statistically significant differences when compared to nontreated cells or cells treated with other concentrations.

Figure 3. Effects of the *I. graveolens* extract on TOS level in cell lines: (A) human glioblastoma multiforme and primary rat cerebral cortex and (B) Chronic myelogenous leukemia and peripheral blood mononuclear. Values are expressed as mean±standard deviation of three independent replicates.

125 µg/mL led to a dose-dependent increase at the APE1 level in PBMCs (Figure 4).

DISCUSSION

Many of the plants and their products provide numerous opportunities because of their unique properties including easy availability, easy biodegradability, easy handling, low cost, safety for mankind and the environment, greater acceptance among users, and minimum side effects[9]. Plant-derived compounds have been previously shown to suppress tumor onset, development, and progression and may diminish the disease and treatment-related side effects[10]. Similar biological and pharmacological activities of *Inula* species have been demonstrated in previous studies. The anticancer activities of *I. graveolens* were just reported only crude oil extracts, their components, and ethanolic extracts not aqueous parts[4]. Thus, this study aimed to evaluate the antiproliferative potential of aqueous extracts



U-87 MG: Human glioblastoma multiforme; PRCC: primary rat cerebral cortex; K562: Chronic myelogenous leukemia; PBMC: peripheral blood mononuclear cells. #p<0.05, ##p<0.01, ###p<0.001, ####p<0.0001 indicate statistically significant differences when compared to nontreated cells or cells treated with other concentrations.

Figure 4. Effects of the *I. graveolens* extract on apurinic/apyrimidinic endodeoxyribonuclease 1 level in cell lines: (A) Human glioblastoma multiforme and primary rat cerebral cortex and (B) Chronic myelogenous leukemia and peripheral blood mononuclear. Values are expressed as mean±standard deviation of three independent replicates.

of *I. graveolens* on the aggressive solid tumor U-87 MG, liquid malignancy K562 cells, and non-cancerous cells.

Our results clearly showed that extracts of *I. graveolens* exhibited cytotoxic effects toward U-87 MG and K562 cells in a dose-dependent manner. The highest (1000 µg/mL) dose of the extract exerted a highly cytotoxic effect by suppressing the viability of U-87 MG cells 50% without any harmful effects on normal cells. The results of this study are consistent with the previous studies on *I. graveolens*. The crude oil and some pure volatile compounds of *I. graveolens* showed antiproliferative activity on the breast cancer cells[11]. In another study, bornyl acetate and essential oil from *I. graveolens* caused the growth inhibition of HeLa, HT29, A549, MCF7, and FL (human amnion) cells. The essential oil and cisplatin have also exhibited the same inhibitory effect on FL normal cells[12]. *I. graveolens* significantly increased the LDH release in U-87 MG and K562 cells at higher doses. However, the extracts did not affect LDH release on healthy PRCC and PBMCs, except the increased LDH level in PRCC at 1000 µg/mL dose only. It has

been reported that the induction of LDH release in glioma cells had antiproliferative effects. Also, unsaturated fatty acids are high in glia and glioma cell membranes, their peroxidation damages the double-layer structure of the membrane, and this negatively affects important cellular events[13]. Lipid peroxidation-induced cell membrane damages may be associated with the enhanced oxidative stress. TOS is used to determine the oxidative damage and the total response created by antioxidant systems against oxidative stress[14]. *I. graveolens* extracts did not show any statistically significant effect on TOS levels in U-87 MG, PRCC, and PBMC cell lines, while TOS levels decreased in K562 cells. Reactive oxygen species (ROS) plays an important role in the onset and progression of cancer, and although medium ROS levels cause tumor development, excessive levels of ROS suppress tumor progression[15]. The similarity between TOS levels of control and extract-treated glioblastoma cells may be associated with the suppression of excessive oxidant levels. Thus, high oxidative stress is known to cause brain tumor development by damaging cell components[16]. Furthermore, the controversial role of oxidative stress in the suppression of tumor progression may be associated with non-specific oxidative damage to biomolecules in leukemic cells[17]. The results of this study revealed that *I. graveolens* at high concentrations led to apoptosis without causing oxidative stress on cancer cells.

Furthermore, a positive correlation was found between oxidative stress values and APE1 activity on K562 and U-87 MG cells. Oxidative stress in gliomas was shown to increase APE1 activity and, as a result, promote resistance to chemotherapy drugs[18]. In this study, *I. graveolens* significantly reduced the APE1 levels on U-87 MG compared with the control, without increasing oxidative stress. On the other hand, unrepaired abasic sites strongly block DNA replication. Accordingly, the blocking of DNA repair increases cell death in cancer[19]. Thus, the antisense suppression of APE1 as a way of decreasing base excision repair activity enhances the cytotoxicity of chemotherapy in glioma cells[20]. The inverse relationship between cell death and APE1 expression has been established in several studies[21]. It was also demonstrated that glioma cell lines with high APE1 activity also have high radioactive resistance[22]. The decreasing radiation resistance on the cancer cell is more effective in the treatment of glioblastoma that is mandatory to radiotherapy with chemotherapy[23]. Thus, APE1 inhibitors may be a promising new target factor for the treatment strategy of brain cancer[24]. The results of this study revealed that increasing the effectiveness of chemoradiotherapy via inhibition of APE1 provides an advantage for glioblastoma therapy.

CONCLUSIONS

The results of this study first represented that the aqueous extract of *I. graveolens* possesses antiproliferative and cytotoxic activity and also dysfunction in DNA repair mechanism without inducing oxidative stress against human glioblastoma cancer and human leukemic cells *in vitro*. Further comprehensive studies are necessary to identify the active compounds from *I. graveolens* and also to investigate the action mechanism of these compounds on various cancer types.

AUTHORS' CONTRIBUTIONS

KK: Conceptualization, Data curation, Formal Analysis, Writing – original draft, Writing – review & editing. **FA**: Data curation, Formal Analysis, Investigation, Writing – review & editing. **NSO:** Data curation, Formal Analysis, Investigation, Writing – review & editing. **FG:** Project administration. **AT:** Data curation. **OOA:** Data curation. **OC:** Data curation. **GYD:** Data curation.

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