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Vanillic acid protects mortality and toxicity induced by N-ethyl-N-nitrosourea in mice; in vivo model of chronic lymphocytic leukemia

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ABSTRACT

Alkylating agents such as N-Ethyl-N-Nitrosourea (ENU) are ubiquitous within living cells and in the environment. This study designed to evaluate the chemopreventive activity of vanillic acid on ENU-induced toxicity and carcinogenesis in mice as an animal model of chronic lymphocytic leukemia (CLL). The female, Swiss albino mice were divided into three groups each with 7 mice, group I received normal saline, group II, mice received ENU at a dose of 80 mg/kg body weight i.p. to induce CLL on the 31th day of the study, and group III, the mice pretreated with vanillic acid at a dose of 20 mg/kg body weight/day, i.p. up to 30 days and received ENU. The animals were monitored for weight changes and mortality during 120 days, and then were sacrificed for isolation of lymphocytes, as target cells in CLL. Cellular parameters like reactive oxygen species (ROS) formation, malondialdehyde (MDA) production, depletion of glutathione (GSH), mitochondrial membrane potential (MMP) and lysosomal membrane integrity were studied. We found that pretreatment with vanillic acid significantly increased the survival of mice up to 57%, delay in death time (30%) and prevented weight changes after exposure to ENU. In addition, it was found that vanillic acid protected ROS formation, lipid peroxidation mitochondrial dysfunction, and lysosomal membrane destabilization in isolated lymphocytes. These data suggest that vanillic acid exhibited significant protection against ENU-induced toxicity and carcinogenicity, which might be related to the protection of the mitochondria and lysosomes and the reduction of ROS formation and oxidative stress.

1. Introduction

Alkylating agents as electrophilic chemicals attack to the nucleophilic centers of DNA in genome and mitochondria. Alkylating agents are categorized to two main groups including monofunctional (single-reactive groups) and bifunctional (double-reactive groups, prevent DNA replication are used as chemotherapeutic agents). There are several sources for exposure to alkylating agents through different occupations (industrial and medical), environment (in cosmetics, detergents, food, fuel combustion products, water and the atmosphere), cigarette smoke, and via endogenous nitrosation of secondary amines [1]. Therefore, these agents are ubiquitous within living cells and in the environment. At steady-state levels in cells, the major contributor to the total background levels of all DNA adducts are endogenous DNA alkylation adducts [2]. It has been reported that endogenous alkylating DNA adducts produce from different sources within body, for example from reacting with cellular methyl donors such as *S*-adenosylmethionine, or as byproducts of lipid peroxidation, or a common cofactor in cellular methylation reactions and or metabolic activity of gut bacteria [3]. For example, N-ethyl-N-nitrosourea (ENU) as an alkylating agents and full carcinogen has been demonstrated to be formed in the human gastrointestinal by nitrosation processes [4].

Alkylating agents after intramolecular cyclization reactions produce highly reactive electrophilic cations and alkylate nucleophilic sites in biological molecules, via transfer an alkyl group to oxygen, phosphorous, sulphur and nitrogen atoms. Alkylating agents via inter- and intra-strand cross-linking DNA and RNA inhibit all cellular reactions such as DNA replication, transcription and translation [5]. Besides DNA

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damage, alkylating agents contribute to cell death via induction of oxidative stress due to increase in reactive oxygen and nitrogen species, glutathione depletion and lipid peroxidation [6], or due to activation of the DNA repair enzyme Poly (ADP-ribose) polymerase (PARP-1) by these chemical and the depletion of NAD^+ pools [7]. Moreover, events such as mitochondrial disruption, calcium overload, inflammation [8], alteration in NF-kB/p53/p38 MAPKs signaling pathways [9] and matrix metalloproteinase-9 (MMP-9) activation [10] can also contribute to the overall toxicity of alkylating agents. DNA damage due to endogenous and exogenous alkylating agents finally lead to the deterioration of genetic information, mutations, chromosomal effects, sister chromatid exchange, unscheduled DNA synthesis different developmental, genetic and cancerous diseases [5]. For example, alkylating agents cause sublethal DNA damage and mutational events in hematopoietic progenitors that lead to malignant transformation from preleukemic to leukemic states [11]. Given widespread of exposure with these compounds for endogenous and exogenous sources, it will deserve to investigate preventive strategy against alkylating agents by chemopreventive agents like plant-derived bioactive compounds with antioxidant, antimutagen, anti-inflammation, anticancer properties.

It seems, cancer chemoprevention by natural compounds can be a promising strategy for blocking, reversing, or retarding carcinogenesis [12]. Chemoprevention by administration of some plant-derived bioactive compounds have been well studied in cellular and animal experimental models and published in literature [12]. Plant-derived bioactive compounds may be used to reduce the toxicity and carcinogenicity of chemical carcinogens and radiation. These natural compounds are biologically active, non-toxic, rational and safe [12]. It has been reported that the administration of plant-derived bioactive compounds obtained from edible fruits and vegetables is a promising way to prevent cancer development, especially in high-risk populations exposited to chemical carcinogens [12]. One of these plant-derived bioactive compounds is vanillic acid (an oxidized form of vanillin) is found in edible plants and fruits such as rye, almond skins and rice, and has biological activities, such as antimutagenicity anti-inflammation and antioxidant activities [13]. It has been reported that vanillic acid via its antioxidant potential protects UV- and H_2O_2-induced DNA damage [14, 15]. Nilnumkhum et al., reported that vanillic acid has antimutagenicity activity in a rat liver micronucleus test and Salmonella mutation assay [16]. In addition, Punvittayagul et al., reported that vanillic acid exhibits a significant protection against 1,2-dimethylhydrazine- and diethylnitrosamine-induced hepatocarcinogenesis, probably via the reduction of proliferation, the induction of the detoxifying enzyme and the induction of apoptosis [17]. Chemopreventive potential of vanillic acid in 7,12-dimethylbenz(a)anthracene -induced hamster buccal pouch carcinogenesis via improvement the phase I and phase II detoxification enzymes has reported by Vinoth et al. [18]. Vanillic acid improves the phase I and phase II detoxification enzymes in DMBA treated hamsters [18]. Due to above positive effects against DNA damage and chemical-induced carcinogenicity, in the current study we aimed to evaluate chemopreventive effect of vanillic acid on ENU-induced toxicity and carcinogenicity in a mice leukemia model. For this purpose, animals were pretreated with vanillic acid for 30 days and injuries induced by ENU on the 31st day of study. Weight change and mortality were monitored during 30 days, and cellular parameters like ROS formation, MDA production, GSH level, MMP collapse and lysosomal membrane integrity were studied in mouse isolated lymphocytes.

2. Material and methods

2.1. Chemicals

Bovine Serum Albumin (BSA), 2′,7′dichlorofluorescin diacetate (DCFH-DA), Acridine Orange, Fetal Bovine Serum (FBS), MTT 3–(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Antibiotic-Antimycotic Solution, Rhodamine123, Thiobarbituric acid (TBA), Trypan blue, 5,5 Edithiobis (2-nitrobenzoic acid (DTNB), N-ethyl-n-nitrosourea (ENU), Trichloroacetic acid (TCA) and Vanillic Acid were purchased from the Sigma Chemical Co (St. Louis, MO USA). RPMI1640 medium was purchased from GIBCO (Grand Island, NY, USA). Ficoll-paque PLUS was provided from Ge Healthcare BioScience Company.

2.2. Animals

Female Swiss albino, healthy, and adult mice (3–4 weeks old, 20–25 g, n=21) from the Laboratory Animal Center of Urmia University of Medical Sciences, Urmia, Iran were provided. The animals were housed and acclimatized for two weeks in the Laboratory Animal House of Faculty Pharmacy, Ardabil University of Medical Sciences (Ardabil, Iran) before the start of the experiments. The mice were maintained at a constant temperature (23 °C) and humidity (40–60%) with a 12 h light/dark cycle. All animals had free access to drinking water standard food during study. The experimental procedures were approved by the ethics committee of the Ardabil University of Medical Sciences, and were performed in accordance with the institutional guidelines (Approval No. IR.ARUMS.AEC.1400.030).

2.3. Treatment protocol

The chemoprotective role of vanillic acid on leukemia carcinogenesis was evaluated based on the model suggested by Aliyu et al. [19–21]. The concentrations of ENU and vanillic acid were selected based on previous study [19]. Mice were randomly assigned to three groups, each containing seven animals. Group I received normal saline given, groups II (ENU) received ENU a single dose of ENU (80 mg/kg body weight i.p) to initiate toxicity and CLL carcinogenesis on the 31th day of the study and group III (ENU + vanillic acid) pretreated with vanillic acid (20 mg/kg body weight/daily, i.p) up to 30 days before the carcinogen injection (a single dose of ENU with 80 mg/kg on the 31th day). Weight change and mortality were monitored during 120 days. All mice were anesthetized with injection of ketamine (60 mg/kg) and xylazine (3 mg/kg) and blood samples were directly collected from the heart of the treated mice for measurement of cellular parameters.

2.4. Lymphocyte isolation

Mouse lymphocytes were isolated from blood, which was obtained from the heart of the treated mice. Blood (1 mL) was gently mixed with normal saline (1 mL) added to on Ficoll-Paque in new tube and centrifuged at 800 × g for 20 min at 4°C. The buffy coat from the interface of plasma and Ficoll-Paque was collected and centrifuged at 400 × g for 10 min at 4°C. The obtained pellet was incubated in erythrocyte lysis buffer for 5 min at 37 °C and the supernatant was removed and the resulting lymphocytes was resuspended in medium of RPMI1640, containing L-glutamine, 25 μM of 2-mercaptoethanol, 10% FBS, 100 U/mL of penicillin and 100 μg/mL of streptomycin. The cell pellet was washed twice with RPMI 1640 complete medium at 650 g for 7 min and before each experiment, cell viability was estimated by trypan blue dye and the number of live cells should be at least 95% [22].

2.5. Detection of intracellular ROS formation in mouse isolated lymphocytes

Analysis of ROS formation in isolated mouse lymphocytes in different groups was determined by flow cytometry using DCFH-DA as a sensitive nonfluorescent precursor dye. Mouse isolated lymphocytes were suspended at RPMI 1640 complete medium and added to 12-well plates at 10^6 cells per well. Mouse isolated lymphocytes were incubated with 10 μM DCFH-DA for 15 min in the incubator. Following incubation, mouse isolated lymphocytes were washed with phosphate buffer (PBS) and the cells were then collected by centrifugation at 650 g for 5 min and resuspended in PBS. The fluorescence intensity of DCF for

10^4 particles was determined by flow cytometry (Cyflow Space-Partec, Germany), on the FL1 channel [22].

2.6. Detection of lipid peroxidation in mouse isolated lymphocytes

Lipid peroxidation was measured by estimating the amount of thiobarbituric acid reactive substances (TBARS), mostly formed from malondialdehyde (MDA) with the pink color, which is detected at 532 nm. Mouse isolated lymphocytes were suspended in RPMI 1640 complete medium and added to 12-well plates at 10^6 cells per well. Then using a glass homogenizer, the isolated lymphocytes were mechanically homogenized in 1 mL 0.1% (w/v) trichloroacetic acid (TCA) and centrifugated at 10,000 x g for 10 min. The supernatant was suspended in 4 mL of 20% TCA with 0.5% TBA and boiled for 15 minutes at 90 °C. After cooling on ice, the absorbance value of the mixture was determined at 532 nm [23].

2.7. Detection of GSH and GSSG levels in mouse isolated lymphocytes

Ellman's reagent (DTNB) is used to quantify the concentration of thiol groups in a sample. Mouse isolated lymphocytes were suspended in RPMI 1640 complete medium and added to 12-well plates at 10^6 cells per well. Then using a glass homogenizer, the isolated lymphocytes were mechanically homogenized and centrifuged at 10,000 g for 10 min at 4° C. For detection of GSH, 100 µL of supernatant was mixed with 3 mL of reaction solution containing GSH reductase (1 U for each 3 mL reaction solution), 3 mL 500 mM Tris-HCl (pH 8.0) buffer containing 10 mM DTNB. Also, for detection of GSSG, 100 µL supernatant was mixed with 3 mL in 150 µM NADPH, 3 mM $MgCl_2$, 10 mM DTNB, 1 mM EDTA and 500 mM Tris-HCl (pH 8.0). After 15 min of incubation at room temperature (25 °C), the absorbance of each mixture at 412 nm was measured [23].

2.8. Detection of alterations in activity of mitochondria in mouse isolated lymphocytes

Depending on the mitochondrial membrane potential, the fluorescent dye rhodamine 123 selectively stains mitochondria and detection of its fluorescence intensity by flow cytometry is used to evaluate alterations in activity of mitochondria. Mouse isolated lymphocytes were suspended at RPMI 1640 complete medium and added to 12-well plates at 10^6 cells per well. Mouse isolated lymphocytes were incubated with 1 µM rhodamine 123 for 15 min in the incubator. Following incubation, mouse isolated lymphocytes were washed with phosphate buffer (PBS) and the cells were then collected by centrifugation at 650 g for 5 min and resuspended in PBS. The fluorescence intensity of rhodamine 123 for 10^4 particles was determined by flow cytometry (Cyflow Space-Partec, Germany), on the FL1 channel [24].

2.9. Detection of lysosomal membrane disintegrity in mouse isolated lymphocytes

To measure lysosomal membrane destabilization, acridine orange was used. This fluorescent dye easily traverses the cell membrane and because of its weak basic property accumulates in lysosomes, which its redistribution is indicator of lysosomal damage. Mouse isolated lymphocytes were suspended at RPMI 1640 complete medium and added to 12-well plates at 10^6 cells per well. Mouse isolated lymphocytes were incubated with 5 µM acridine orange for 15 min in the incubator. Following incubation, mouse isolated lymphocytes were washed with phosphate buffer (PBS) and the cells were then collected by centrifugation at 650 g for 5 min and resuspended in PBS. The fluorescence intensity of acridine orange for 10^4 particles was determined by flow cytometry (Cyflow Space-Partec, Germany), on the FL1 channel [23].

2.10. Statistical analysis

At least five independent experiments were carried out. Data are presented as mean ± standard error of mean (SEM). One-way ANOVA and Tukey's multi-comparison tests were carried out by GraphPad Prism 9 software (San Diego, CA, United States) to determine the statistical differences while the level of significance had been set at $P<0.05$. Fow cytometry data were analyzed with FlowJo software.

3. Results

3.1. General observations and body weight changes of mice in response to ENU exposure and pretreatment of vanillic acid

The ENU toxicity symptoms began to appear as early as 1 week after its administration in the ENU group. The recorded signs of ENU toxicity included hypoactivity, drowsiness, immobility and decreased feeding. Also, ENU-exposed mice had a reduction in water intake, food intake, final body weight and body weight gain compared to control mice. Pretreatment of vanillic acid with ENU protected the duration and severity the of above signs and improved the water intake, food intake, final body weight and body weight gain compared to those in the ENU group (Fig. 1A).

3.2. Survival rate of mice in response to ENU exposure and pretreatment of vanillic acid

The survival rate in the control group and mice pretreated with vanillic acid and/or ENU for 120 days is presented in Fig. 1B. Accordingly, our results show that ENU exposure caused a significant decrease in survival of mice (100%) until the end of the first week of the study compared with that in the control group. ENU-induced mortality was significantly protected by pretreatment with vanillic acid (57%). During the study period, all mice (seven or 100%) in the ENU-exposed group, and only three in the ENU+ vanillic acid group died (three or 43%).

3.3. ROS formation in response to ENU exposure and pretreatment of vanillic acid

ROS formation results in isolated lymphocytes obtained from the control group and mice pretreated with vanillic acid and/or ENU after 120 days is presented in Fig. 1B. Isolated lymphocyte obtained from mice treated with ENU had a significant increase in the fluorescence intensity of DCF compared with that in the control group, which is a reflection of ROS formation in these cells. Importantly, vanillic acid protected the effect of ENU on ROS formation, which is determined by the decrease in fluorescence intensity of DCF compared to those in the ENU group. Fig. 2

3.4. Lipid peroxidation in response to ENU exposure and pretreatment of vanillic acid

The level of MDA in all groups is illustrated in Fig. 3A. The analysis of MDA content in mouse isolated lymphocytes after exposure to ENU revealed high levels ($P < 0.05$), when compared to those of control, which is reflected lipid peroxidation in these cells after exposure with a single dose of this full carcinogen agent. However, group pretreated with vanillic acid had levels similar to those of control. This data indicated that vanillic acid protects against ENU-induced MDA as sensitive indicator of lipid peroxidation in mouse isolated lymphocytes.

3.5. GSH and GSSG levels in response to ENU exposure and pretreatment of vanillic acid

The effects of pretreatment of vanillic acid, on the levels of GSH and GSSG are shown in Fig. 3B–C. It was found that the levels of GSH and

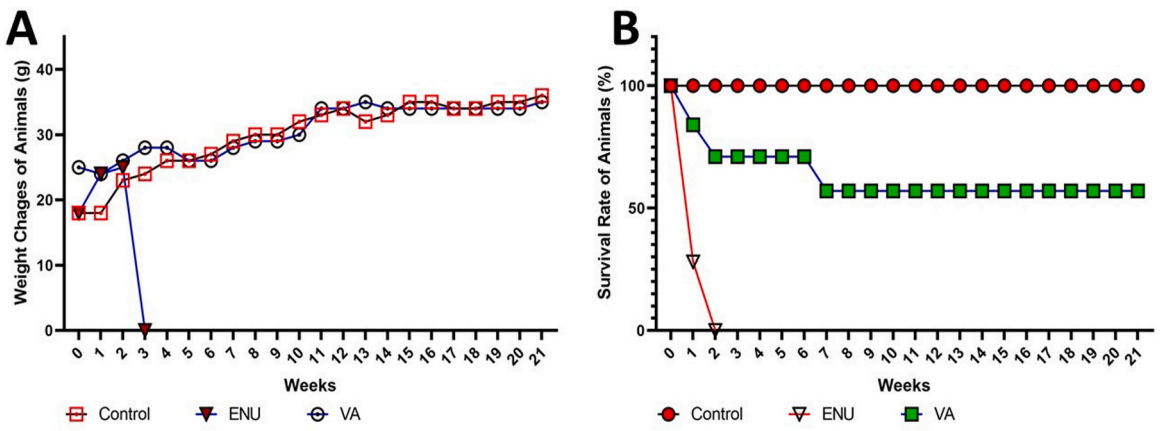

Fig. 1. Effect of ENU exposure (a single dose of 80 mg/kg) and pretreatment of vanillic acid (20 mg/kg/daily/ for 30 days) on the weight changes (A) and survival rate (B) in treated mice during the experimental period (120 days); Data were collected every week for each treatment group. The number of surviving mice is represented as % control group and weight changes are also presented as gram (g). ENU, N-Ethyl-N-Nitrosourea; VA, Vanillic acid.

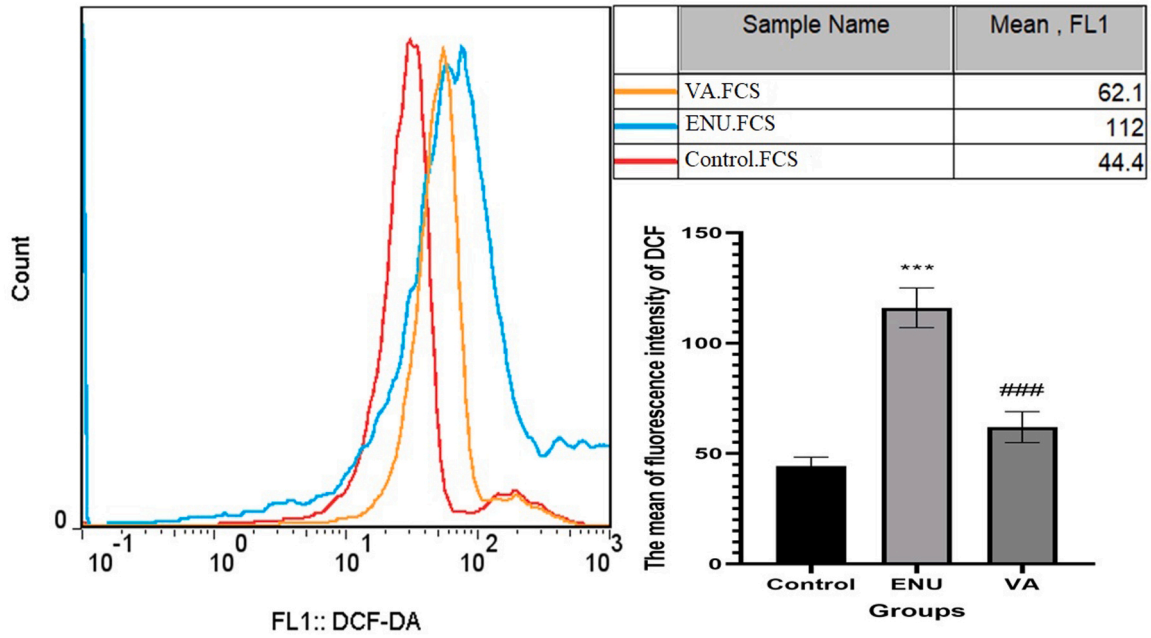

Fig. 2. Intracellular ROS formation levels were determined by flow cytometry and representative histograms of ROS fluorescence are illustrated. The fluorescence intensity of different groups was calculated, and the results were presented as areas under the curve of relative light units (RLU). ROS formation increased in the ENU-alone group. However, this increase in ROS formation was significantly inhibited in the ENU exposure after vanillic acid-pretreatment group. *** represents a significant difference in relation to the control group ($P < 0.001$); ### represents a significant difference in relation to the ENU group ($P < 0.001$). Values present means ± SEM, n = 5 and were analyzed by one-way ANOVA and Tukey's multi-comparison test. FL1, Fluorescence channel; ENU, N-Ethyl-N-Nitrosourea; DCFH-DA, 2′,7′dichlorofluorescin diacetate; VA, Vanillic acid; SEM, Standard error of the mean.

GSSG was close in control group in vanillic acid + ENU and ENU groups. Our analyzed data indicated there were no significant difference between groups and not consistent with ROS formation and lipid peroxidation tests. Probably the low sensitivity of the measurement method or the small number of cells be the reason for this inconsistency or long period of study (120 days) reduces the level of glutathione to baseline and close to the control group.

3.6. Mitochondrial alterations in response to ENU exposure and pretreatment of vanillic acid

In order to identify whether vanillic acid pre-treatment protects ENU-induced mitochondrial changes, the mitochondrial membrane potential (the mean of the fluorescence intensity of rhodamine 123) was measured in mouse isolated lymphocytes. The mean of the fluorescence intensity of rhodamine 123 values were 11.70 ± 1.62 in the control group, 33.4 ± 3.24 in the ENU-alone group, 16.70 ± 2.31 in the ENU exposure after vanillic acid pre-treatment group. This data indicated that the fluorescence intensity of rhodamine 123 as indicator of mitochondrial depolarization was increased significantly by ENU in mouse isolated lymphocytes and that this increase was significantly inhibited by vanillic acid pre-treatment ($p < 0.001$, Fig. 4).

3.7. Lysosomal alterations in response to ENU exposure and pretreatment of vanillic acid

In order to identify whether vanillic acid pre-treatment protects ENU-induced lysosomal alteration, the lysosomal membrane

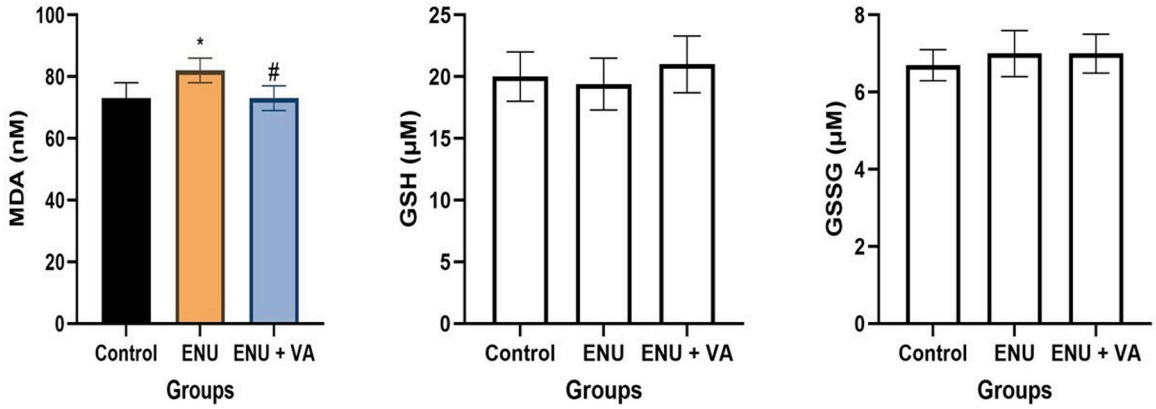

Fig. 3. Effects of ENU and vanillic acid on cellular MDA level (A), GSH (B) and GSSG (C) in isolated lymphocytes obtained from different groups. MDA production increased in the ENU-alone group. However, this increase in MDA production was significantly inhibited in the ENU exposure after vanillic acid-pretreatment group. In the GSH and GSSG results bars do not differ significantly. * represents a significant difference in relation to the control group ($P < 0.05$); # represents a significant difference in relation to the ENU group ($P < 0.05$). Values present means ± SEM, n = 5 and were analyzed by one-way ANOVA and Tukey's multi-comparison test. ENU, N-Ethyl-N-Nitrosourea; MDA, Malondialdehyde; VA, Vanillic acid; GSH, Reduced glutathione; GSSG; Oxidized glutathione; SEM, Standard error of the mean.

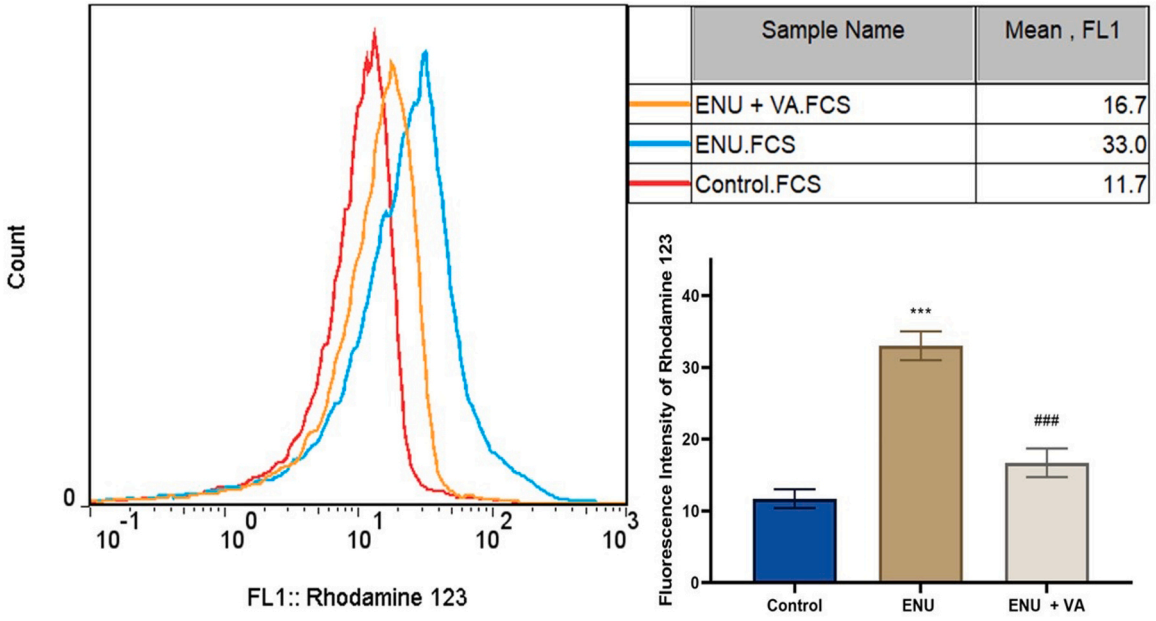

Fig. 4. Mitochondrial membrane potential was determined by flow cytometry and representative histograms of the fluorescence intensity of rhodamine 123 are illustrated. The fluorescence intensity of different groups was calculated, and the results were presented as areas under the curve of relative light units (RLU). Exposure to a single dose of ENU (80 mg) alone increased mitochondrial depolarization in mouse isolated lymphocytes. This increase in mitochondrial depolarization was significantly inhibited in mice pretreated with 20 mg/kg vanillic acid before ENU exposure. *** represents a significant difference in relation to the control group ($P < 0.001$); ### represents a significant difference in relation to the ENU group ($P < 0.001$). Values present means ± SEM, n = 5 and were analyzed by one-way ANOVA and Tukey's multi-comparison test. FL1, Fluorescence channel; ENU, N-Ethyl-N-Nitrosourea; VA, Vanillic acid; SEM, Standard error of the mean.

destabilization (the mean of the fluorescence intensity of acridine orange) was measured in mouse isolated lymphocytes. The mean of the fluorescence intensity of acridine orange values were 38.82 ± 3.12 in the control group, 81.60 ± 3.75 in the ENU-alone group, 54.23 ± 2.58 in the ENU exposure after vanillic acid pre-treatment group. This data indicated that the fluorescence intensity of acridine orange as indicator of lysosomal membrane destabilization was increased significantly by ENU in mouse isolated lymphocytes and that this increase was significantly inhibited by vanillic acid pre-treatment ($p < 0.001$, Fig. 5).

4. Discussion

Scientific evidence states there is a significant relationship between hygiene levels, life styles, spread of communicable diseases (e.g., HIV, HPV, or hepatitis B), environmental pollution, occupational exposure and the economic resources dedicated to preventive medicine and cancer incidence [25]. It has been reported that above cancer risk factors collectively contribute to the development of 70–95% of all cancers in human, which might be largely preventable [26]. One of the main cancer risk factors is exposure to chemical carcinogens through consumer products, work, environment and therapeutic agents [27,28]. Of the 1000 compounds studied by The International Agency for Research on Cancer (IARC) more than 50% of them are truly, probably or possibly carcinogenic to humans [25]. It has been reported that more than 300 million tonnes of chemicals per year are dealt in the European Union, which 12–15% of them are classified as mutagenic, carcinogenic or toxic to reproduction [25]. This suggests that humans are faced with a significant number of chemical carcinogens form different sources, and in

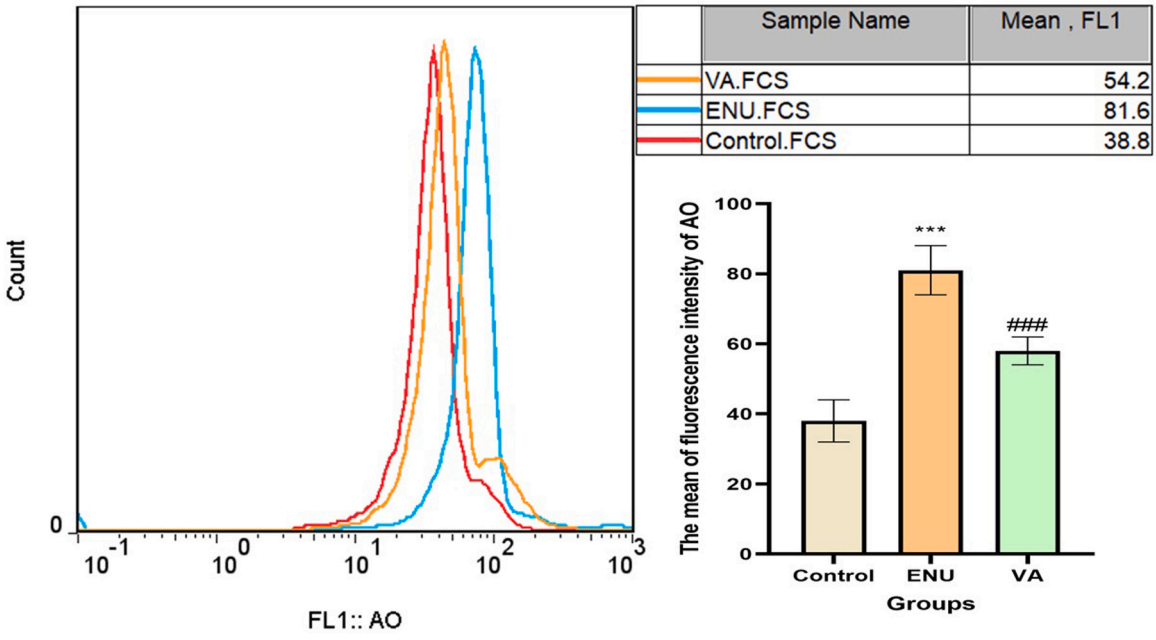

Fig. 5. Lysosomal membrane destabilization was determined by flow cytometry and representative histograms of the fluorescence intensity of acridine orange are illustrated. The fluorescence intensity of different groups was calculated, and the results were presented as areas under the curve of relative light units (RLU). Exposure to ENU alone increased lysosomal membrane disintegrity in mouse isolated lymphocytes. This increase in lysosomal membrane disintegrity was significantly inhibited in mice pretreated with vanillic acid before ENU exposure. *** represents a significant difference in relation to the control group ($P < 0.001$); ### represents a significant difference in relation to the ENU group ($P < 0.001$). Values present means ± SEM, n = 5 and were analyzed by one-way ANOVA and Tukey's multi-comparison test. AO, Acridine orange: FL1, Fluorescence channel; ENU, N-Ethyl-N-Nitrosourea; VA, Vanillic acid; SEM, Standard error of the mean.

many cases, exposure to these compounds is unavoidable [29]. Therefore, reducing the exposure to chemical carcinogens and the use of chemopreventive strategy can be useful in decreasing the incidence of various types of cancers [30]. One of well-known chemical carcinogens is ENU which was selected for induction of CLL in mouse model in the current study. A recent study reported that IP injection of 80 mg/kg ENU caused CLL in 3-week-old male Institute of Cancer Research (ICR)-mice 12 weeks after administration [19]. We used the same protocol with above study for induction of leukemia carcinogenesis by ENU in the current work. Surprisingly we observed that IP administration of 80 mg/kg ENU led to death all treated mice in ENU group. It seems that difference of our study with Aliyu et al. work may be related to the type of animal (Swiss albino versus ICR-mice) and gender (female versus male). As previously reported the difference in species, gender and genetic may affect toxic responses induced by chemical carcinogen [31, 32]. We showed that the gender of the female and the Swiss albino mouse species are more sensitive than the model used in the study of Aliyu et al., as well as this may be correct about human exposure to ENU. As previous mentioned ENU-induced DNA damage can occur from endogenous or exogenous sources, and it has been demonstrated that ENU produces in the human gastrointestinal by nitrosation processes [4]. Therefore, inhibition of carcinogenic and toxic effects of ENU and other alkylating agents is essential via cancer chemoprevention strategies. In the current study we observed that daily exposure to vanillic acid interestingly showed chemopreventive effects against the lethal toxicity of ENU. Similar effects of vanillic acid with chemopreventive potential have previously reported by other studies and are consistent with our results in the current study [14,17,18].

ROS production is balanced by the intracellular antioxidant system by various enzymatic and nonenzymatic moieties and it maintains cellular homeostasis under normal conditions. However, under pathological or toxic conditions, the ROS formation results in depletion of the antioxidants, MDA production, oxidative stress and ultimately distorted homeostasis [33]. It is well proved that excesses ROS higher than potential of intracellular antioxidant system can lead to DNA damage, deregulated gene expression, signaling, functioning and stemness in the cells [34]. Therefore, ROS formation can play an integral role in carcinogenesis and the development of various cancers after exposure with chemical carcinogens [35]. It is well established that alkylating agents such as ENU contribute to carcinogenesis and toxicity via induction of oxidative stress due to increase in reactive oxygen and nitrogen species, glutathione depletion and lipid peroxidation [6,36]. Our results consistent with these studies showed that ENU as a full carcinogen agent induces ROS formation, MDA production and oxidative stress in lymphocytes as target cells in CLL. Enhanced ROS formation has been also reported for some leukemic cells, such CLL in previous studies [37,38]. With regard to the nature of ROS in carcinogenesis, strategies to downregulate it in cells can be a promising approach for cancer prevention [38,39]. In the current study we demonstrated that vanillic acid as antioxidant reduced ROS formation and MDA production in isolated lymphocytes. It is well studied that the antioxidant activity of vanillic acid via free radical scavenging activity, reducing power, and the inhibition of lipid peroxidation can help to inhibit chemical-induced toxicity and carcinogenicity [18,40–43]. Our findings in the present study, consistent with previous studies suggest that vanillic acid is an antioxidant with chemopreventive activity that can inhibit the cancer development induced by chemical carcinogens.

The pleiotropic implications of mitochondria in the cancer development and carcinogenesis have been recently demonstrated [44]. It has been reported that mitochondria participate in the process of carcinogenesis by oxidative stress, regulating cell metabolism, dialogue with other organelles, dynamics of fusion and fission and mitophagy [44]. In the initiation stage, mitochondrial ROS raise the transformation of healthy cells to preneoplastic cells mainly via oncogenic mitochondrial DNA (mtDNA) and nuclear DNA mutations [45]. Enhanced mitochondrial ROS reported has been reported for some leukemic cells, such CLL [37]. These mutations in mtDNA and nuclear DNA lead to oncometabolites accumulation, cell respiration alteration and activation of oncogenic pathways [45]. In addition, in the cancer progression stage, mitochondrial alterations are associated with metabolic reprogramming

which stimulated by mitochondrial dynamics, oxidative stress and oncogenes [45]. One of the main mechanisms of causing cancer by alkalizing agents is mitochondrial disruption [8], which was also proven in the our study. It seems that mitochondrial protection can reduce or inhibit the above outcomes during the cancer development process. In the current we observed the beneficial effects of vanillic acid on ENU-induced mitochondrial dysfunctions in mouse isolated lymphocytes. Our findings, consistent with previous studies [46–48] suggest that vanillic acid via its mitoprotective effects can protect mitochondrial dysfunction induced by different chemicals, which may finally lead to toxicity and cancer development induced.

In addition to the importance of intracellular ROS and mitochondria in carcinogenesis stages, lysosomes also play a prominent role in the cancer development. It is well established that the distribution and functional status of lysosomes in cell regulate the cancer development and progression [49]. In addition, lysosome plays an important role in immune system activities through the expression of important immune checkpoint receptors, immune escape and mitogen signal transduction processes, which can help the function of this system against cancer cells [49]. Therefore, use of lysosomal protection agents may inhibit or slow the transformation of normal cells to preneoplastic cells. Our finding in the present study demonstrated that ENU as chemical carcinogen and alkylating agent caused lysosomal dysfunction in mouse isolated lymphocytes, which is protected with pretreatment of vanillic acid. These data portrayed toxic effect of ENU on lysosomes and lysosomal protective activity of vanillic acid.

In conclusion, our study highlights for the first time the chemoprotective effect of vanillic acid on ENU-induced carcinogenicity and toxicity in mice as an animal model of CLL. Hopefully, vanillic acid revealed a potential impact against deadly toxicity induced by ENU in the present study. The obtained results demonstrated that vanillic acid can assist as a superior choice for chemopreventive treatment of alkylating agents-induced carcinogenicity and toxicity via the reduction of ROS generation, oxidative stress and mitochondrial and lysosomal protective effects in exposed individuals. From the safety point of view, vanillic acid (1000 mg/kg/day for two weeks) is safe and has no adverse effect on the process of leukopoiesis, erythropoiesis or on internal organs [50] and this bioactive compound can be safely used in the dose suggested in this study (30 mg/kg). However, further detailed investigations are needed to fully recognize the exact chemopreventive mechanism of vanillic acid against chemical-induced carcinogenicity. Also, the limitation of the current study such as the number of used animals, specie type, the administration route and single dose of vanillic acid, should be considered in the next studies.

Ethical approval

All experiments were performed based on standard protocols approved by the ethic committee of Ardabil University of Medical Sciences (No: IR.ARUMS.AEC.1400.030).

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CRediT authorship contribution statement

Saleh Khezri: Methodology, Investigation, Data curation. **Shadi Haddadi:** Methodology, Data curation. **Ahmad Salimi:** Writing – review & editing, Writing – original draft, Software, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Mahshad Pourgholi:** Data curation. **Bahare Asgari:** Data curation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

No data was used for the research described in the article.

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References

[1] G. Jenkins, S. Doak, G. Johnson, E. Quick, E. Waters, J. Parry, Do dose response thresholds exist for genotoxic alkylating agents? Mutagenesis 20 (6) (2005) 389–398.
[2] V. Sharma, L.B. Collins, J.M. Clement, Z. Zhang, J. Nakamura, J.A. Swenberg, Molecular dosimetry of endogenous and exogenous O6-methyl-dG and N7-Methyl-G adducts following low dose [D 3]-methylnitrosourea exposures in cultured human cells, Chem. Res. Toxicol. 27 (4) (2014) 480–482.
[3] J. Swenberg, Y. Lai, R. Yu, V. Sharma, B.C. Moeller, H. Hartwell, J. Gibson, J. Nakamura, The role of endogenous versus exogenous DNA damage in risk assessment. Thresholds of Genotoxic Carcinogens, Elsevier, 2016, pp. 83–102.
[4] H. Bartsch, H. Ohshima, D.E. Shuker, B. Pignatelli, S. Calmels, Exposure of humans to endogenous N-nitroso compounds: implications in cancer etiology, Mutat. Res./Rev. Genet. Toxicol. 238 (3) (1990) 255–267.
[5] J. Egea, F. López-Muñoz, O. Fernández-Capetillo, R.J. Reiter, A. Romero, Alkylating agent-induced toxicity and melatonin-based therapies, Front. Pharmacol. 13 (2022) 873197.
[6] J. Zhang, Z.W. Ye, K.D. Tew, D.M. Townsend, Cisplatin chemotherapy and renal function, Adv. Cancer Res. 152 (2021) 305–327.
[7] F. Mégnin-Chanet, M.A. Bollet, J. Hall, Targeting poly(ADP-ribose) polymerase activity for cancer therapy, Cell. Mol. Life Sci.: CMLS 67 (21) (2010) 3649–3662.
[8] K. Sahu, U. Langeh, C. Singh, A. Singh, Crosstalk between anticancer drugs and mitochondrial functions, Curr. Res. Pharmacol. Drug Discov. 2 (2021) 100047.
[9] M. Tian, D. Tian, X. Qiao, J. Li, L. Zhang, Modulation of Myb-induced NF-kB -STAT3 signaling and resulting cisplatin resistance in ovarian cancer by dietary factors, J. Cell. Physiol. 234 (11) (2019) 21126–21134.
[10] R.M. Escalona, G. Kannourakis, J.K. Findlay, N. Ahmed, Expression of TIMPs and MMPs in ovarian tumors, ascites, ascites-derived cells, and cancer cell lines: characteristic modulatory response before and after chemotherapy treatment, Front. Oncol. 11 (2021) 796588.
[11] S.L. Gerson, P.F. Caimi, B.M. William, R.J. Creger, Pharmacology and molecular mechanisms of antineoplastic agents for hematologic malignancies. Hematology, Elsevier, 2018, pp. 849–912.
[12] T. Tanaka, R. Aoki, M. Terasaki, Potential chemopreventive effects of dietary combination of phytochemicals against cancer development, Pharmaceuticals 16 (11) (2023) 1591.
[13] C.S.M. Bezerra-Filho, J.N. Barboza, M.T.S. Souza, P. Sabry, N.S.M. Ismail, D.P. de Sousa, Therapeutic potential of vanillin and its main metabolites to regulate the inflammatory response and oxidative stress, Mini Rev. Med. Chem. 19 (20) (2019) 1681–1693.
[14] S. Taqvi, E. Ahmed Bhat, N. Sajjad, J.S.M. Sabir, A. Qureshi, I.A. Rather, S. Rehman, Protective effect of vanillic acid in hydrogen peroxide-induced oxidative stress in D.Mel-2 cell line, Saudi J. Biol. Sci. 28 (3) (2021) 1795–1800.
[15] K. Sevgi, B. Tepe, C. Sarikurkcu, Antioxidant and DNA damage protection potentials of selected phenolic acids, Food Chem. Toxicol.: Int. J. Publ. Br. Ind. Biol. Res. Assoc. 77 (2015) 12–21.
[16] A. Nilnumkhum, C. Punvittayagul, A. Chariyakornkul, R. Wongpoomchai, Effects of hydrophilic compounds in purple rice husk on AFB 1-induced mutagenesis, Mol. Cell. Toxicol. 13 (2017) 171–178.
[17] C. Punvittayagul, A. Chariyakornkul, K. Jarukamjorn, R. Wongpoomchai, Protective role of vanillic acid against diethylnitrosamine- and 1,2-dimethylhydrazine-induced hepatocarcinogenesis in rats, Mol. (Basel, Switz.) 26 (9) (2021).
[18] A. Vinoth, R. Kowsalya, Chemopreventive potential of vanillic acid against 7,12-dimethylbenz(a)anthracene-induced hamster buccal pouch carcinogenesis, J. Cancer Res. Ther. 14 (6) (2018) 1285–1290.
[19] A. Aliyu, M.R. Shaari, N.S. Ahmad Sayuti, M.F.H. Reduan, S. Sithambaram, M. M. Noordin, K. Shaari, H. Hamzah, N-Ethyl-n-Nitrosourea induced Leukaemia in a mouse model through upregulation of vascular endothelial growth factor and evading apoptosis, Cancers 12 (3) (2020) 678.
[20] R. Ullah, M. Ikram, T.J. Park, R. Ahmad, K. Saeed, S.I. Alam, I.U. Rehman, A. Khan, I. Khan, M.G. Jo, M.O. Kim, Vanillic acid, a bioactive phenolic compound,

counteracts LPS-induced neurotoxicity by regulating c-Jun N-terminal kinase in mouse brain, Int. J. Mol. Sci. 22 (1) (2020).
[21] F.U. Amin, S.A. Shah, M.O. Kim, Vanillic acid attenuates Aβ1-42-induced oxidative stress and cognitive impairment in mice, Sci. Rep. 7 (1) (2017) 40753.
[22] A. Salimi, A. Motallebi, M. Ayatollahi, E. Seydi, A.R. Mohseni, M. Nazemi, J. Pourahmad, Selective toxicity of persian gulf sea cucumber holothuria parva on human chronic lymphocytic leukemia b lymphocytes by direct mitochondrial targeting, Environ. Toxicol. 32 (4) (2017) 1158–1169.
[23] A. Salimi, E. Baghal, H. Ghobadi, N. Hashemidanesh, F. Khodaparast, E. Seydi, Mitochondrial, lysosomal and DNA damages induced by acrylamide attenuate by ellagic acid in human lymphocyte, PloS One 16 (2) (2021) e0247776.
[24] M. Aghvami, F. Ebrahimi, M.H. Zarei, A. Salimi, R.P. Jaktaji, J. Pourahmad, Matrine induction of ROS mediated apoptosis in human ALL B-lymphocytes via mitochondrial targeting, Asian Pac. J. Cancer Prev.: APJCP 19 (2) (2018) 555.
[25] F. Madia, A. Worth, M. Whelan, R. Corvi, Carcinogenicity assessment: addressing the challenges of cancer and chemicals in the environment, Environ. Int. 128 (2019) 417–429.
[26] S. Wu, S. Powers, W. Zhu, Y.A. Hannun, Substantial contribution of extrinsic risk factors to cancer development, Nature 529 (7584) (2016) 43–47.
[27] M. Iwasaki, H. Itoh, N. Sawada, S. Tsugane, Exposure to environmental chemicals and cancer risk: epidemiological evidence from Japanese studies, Genes Environ.: Off. J. Jpn. Environ. Mutagen. Soc. 45 (1) (2023) 10.
[28] T. Kobets, B.P. Smith, G.M. Williams, Food-borne chemical carcinogens and the evidence for human cancer risk, Foods 11 (18) (2022) 2828.
[29] F.O. Adeola, Global impact of chemicals and toxic substances on human health and the environment, Handb. Glob. Health (2020) 1–30.
[30] M. Swetha, C. Keerthana, T.P. Rayginia, R.J. Anto, Cancer chemoprevention: a strategic approach using phytochemicals, Front. Pharmacol. 12 (2022) 809308.
[31] S. Kadekar, S. Peddada, I. Silins, J.E. French, J. Högberg, U. Stenius, Gender differences in chemical carcinogenesis in national toxicology program 2-year bioassays, Toxicol. Pathol. 40 (8) (2012) 1160–1168.
[32] J.L. Barnes, M. Zubair, K. John, M.C. Poirier, F.L. Martin, Carcinogens and DNA damage, Biochem. Soc. Trans. 46 (5) (2018) 1213–1224.
[33] A.Q. Khan, K. Rashid, A.A. AlAmodi, M.V. Agha, S. Akhtar, I. Hakeem, S.S. Raza, S. Uddin, Reactive oxygen species (ROS) in cancer pathogenesis and therapy: an update on the role of ROS in anticancer action of benzophenanthridine alkaloids, Biomed. Pharmacother. 143 (2021) 112142.
[34] A. Samimi, M.J. Khodayar, H. Alidadi, E. Khodadi, The dual role of ROS in hematological malignancies: stem cell protection and cancer cell metastasis, Stem Cell Rev. Rep. 16 (2020) 262–275.
[35] J.E. Klaunig, Z. Wang, X. Pu, S. Zhou, Oxidative stress and oxidative damage in chemical carcinogenesis, Toxicol. Appl. Pharmacol. 254 (2) (2011) 86–99.
[36] P. Nath, D. Maiti, A review of the mutagenic potential of N-ethyl-N-nitrosourea (ENU) to induce hematological malignancies, J. Biochem. Mol. Toxicol. 36 (7) (2022) e23067.
[37] R. Jitschin, A.D. Hofmann, H. Bruns, A. Giessl, J. Bricks, J. Berger, D. Saul, M. J. Eckart, A. Mackensen, D. Mougiakakos, Mitochondrial metabolism contributes to oxidative stress and reveals therapeutic targets in chronic lymphocytic leukemia, Blood 123 (17) (2014) 2663–2672.
[38] M. Romo-González, C. Ijurko, Á. Hernández-Hernández, Reactive oxygen species and metabolism in leukemia: a dangerous liaison, Front. Immunol. 13 (2022) 889875.
[39] H. Nakamura, K. Takada, Reactive oxygen species in cancer: current findings and future directions, Cancer Sci. 112 (10) (2021) 3945–3952.
[40] N. Sharma, N. Tiwari, M. Vyas, N. Khurana, A. Muthuraman, P. Utreja, An overview of therapeutic effects of vanillic acid, Plant Arch. 20 (2) (2020) 3053–3059.
[41] S. Surya, P. Sampathkumar, S.M. Sivasankaran, M. Pethanasamy, C. Elanchezhiyan, B. Deepa, S. Manoharan, Vanillic acid exhibits potent antiproliferative and free radical scavenging effects under in vitro conditions, Int. J. Nutr. Pharmacol. Neurol. Dis. 13 (3) (2023) 188–198.
[42] D.H. Yetiştirme, M.G. Erdem, N. Cinkılıç, Ö. Vatan, D. Yılmaz, D. Bağdaş, R. Bilaloğlu, Genotoxic and anti-genotoxic effects of vanillic acid against mitomycin C-induced genomic damage in human lymphocytes in vitro, (2012).
[43] A. Mentese, S. Demir, H. Kucuk, E. Yulug, N.T. Alemdar, E.A. Demir, Y. Aliyazicioglu, Vanillic acid abrogates cisplatin-induced ovotoxicity through activating Nrf2 pathway, Tissue Cell 84 (2023) 102161.
[44] H. Moindjie, S. Rodrigues-Ferreira, C. Nahmias, Mitochondrial metabolism in carcinogenesis and cancer therapy, Cancers 13 (13) (2021).
[45] S. Vyas, E. Zaganjor, M.C. Haigis, Mitochondria and cancer, Cell 166 (3) (2016) 555–566.
[46] M. Shabani, Z. Jamali, D. Bayrami, A. Salimi, Vanillic acid alleviates methamphetamine-induced mitochondrial toxicity in cardiac mitochondria via antioxidant activity and inhibition of MPT Pore opening: an in-vitro study, BMC Pharmacol. Toxicol. 24 (1) (2023) 33.
[47] M. Ay, Vanillic acid induces mitochondrial biogenesis in SH-SY5Y cells, Mol. Biol. Rep. 49 (6) (2022) 4443–4449.
[48] M. Mei, H. Sun, J. Xu, Y. Li, G. Chen, Q. Yu, C. Deng, W. Zhu, J. Song, Vanillic acid attenuates H2O2-induced injury in H9c2 cells by regulating mitophagy via the PINK1/Parkin/Mfn2 signaling pathway, Front. Pharmacol. 13 (2022) 976156.
[49] T. Tang, Z.-y Yang, D. Wang, X.-y Yang, J. Wang, L. Li, Q. Wen, L. Gao, X.-w Bian, S.-c Yu, The role of lysosomes in cancer development and progression, Cell Biosci. 10 (1) (2020) 1–18.
[50] A.C. Mirza, S.S. Panchal, Safety assessment of vanillic acid: subacute oral toxicity studies in wistar rats, Turk. J. Pharm. Sci. 17 (4) (2020) 432–439.