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In-vitro Repair Ability of DNA Damage by *Anethum graveolens* L. Aqueous Extract Using Comet Assay

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Authors' contributions

This work was carried out in collaboration among all authors. Authors NSE and HAK designed the experiment and both authors were responsible for the experimental techniques, application of the experiment and statistical analysis. Author IHI was responsible for the chemical analysis of the work. All authors read and approved the final manuscript.

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ABSTRACT

Aim: the objective stated to assess the geno-repair ability of *Anethum graveolens* L. (Dill) aqueous extract against DNA damage caused by potassium bromate, a food additive.
Study Design: project has been done at Botany Department, Omer AL-Mukhtar University, Libya. Period from February to August 2023.
Methodology: Alkaline Comet assay was performed on whole blood samples of Ten, young healthy, non-smoking donors.
Results: current data showed that potassium bromate can significantly increase DNA damage after the application of selected doses 1, 1.5, and 2.5µg/ml of $KBrO_3$. In male cells, high significant

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differences between the control and $KBrO_3$ treated group were achieved, 0.5, 10.64, 29.64, and 82.66% are the averages of Comet DNA for control, low, middle, and higher doses of potassium bromate respectively. Also, 2.8, 8.18, 12.13, and 59.34% were attained in female lymphocytes. Whereas, the aqueous extract of dill significantly repairs the DNA impairment by declining the tail extent toward the control lymphocytes of both genders. The parameters illustrate that dill aqueous extract when applied as protective in males and females (11.88 and 10.5%) was significantly more effective than therapeutic (24.93 and 14.4 %) respectively. Total antioxidant content, DPPH free radical scavenging activity via IC_{50} value, total phenolic and total flavonoid content of dill extract were measured. The IC_{50} value was 67.2µg/ml compared to positive control, ascorbic acid (42.80µg/ml). Total antioxidant expressed in percentage was 62.0%, 70.2mg/g of dry weight of extract found to be the total phenolic content and the total flavonoid value was 72.0 mg/g. $KBrO_3$ was determined in bread samples collected from fifteen bakeries at Al-Bayda and Shahat cities, quantities more than the lowest amount that allowed by the FDA and WHO by Ten to several hundred folds.

Conclusion: The findings indicate the potential genotoxicity of $KBrO_3$ to human health because of the DNA damage achieved. Whereas, dill extract has a good anti-genotoxic effect against potassium bromate.

Keywords: Anethum graveolens L.; comet assay; anti-genotoxic ; potassium bromate ; DNA damage.

ABBREVIATIONS

DPPH: 2,2-diphenyl-1-picrylhydrazyl
HD: Higher Dose
LD: Low Dose
LMP: low Melting Point
MD: Middle Dose
NMP: Normal Melting Point
PBS: Phosphate Buffer Saline
SCGE: Single-Cell Gel Electrophoresis

1. INTRODUCTION

The usage of medicinal plants is probably as old as humankind itself. As per the estimation, more than 80% of the population in the world depend on traditional medicinal plants in their health care [1]. Traditional medicines are generally more acceptable from a cultural and spiritual perspective. the microsomal system/Salmonella was used to assess the extract of aromatic plants investigate whether they possess the direct or indirect anti-mutagens activities. Among aromatic herbs and spices, *Anethum graveolens* L. has a long history of use for its sensory and functional properties and having anti-mutagenic activity [2]. *Anethum graveolens* L. commonly known as dill, is a Libyan local plant, widely used for its medicinal properties, belonging to the family of Apiaceae [3]. In the current project, a water extract of *Anethum graveolens* L. was used to test the anti-genotoxic action of potassium bromate ($KBrO_3$), a food additive, has been classified by the International Agency for Research on Cancer (IARC) as a substance in the category of 2B carcinogens (potential human carcinogens) that poses a high risk for human kidney tumors [4,5]. Different biological assays were used to investigate the genotoxic effects of some chemicals, human lymphocyte is commonly used as a suitable genetic model for cytological studies. In general, lymphocytes are used in laboratories to assess the cytogenetic and genotoxic effects of harmful chemical substances by evaluating DNA [6,7]. In this study, alkaline Comet assay (Single-Cell Gel Electrophoresis; SCGE) was used for assessing DNA integrity (damage and repair) in human lymphocyte cells. Human cells was used as an applicable model to explore the damage in genetic material when exposed to genotoxic agents via Comet assay. This assay was used as bio-monitoring to assess both DNA impairment and recovery in genetic toxicology [8].

2. MATERIALS AND METHODS

2.1 Comet Assay

Lymphocytes from 10 healthy (5 females and 5 males), non-smoker patients with ages ranging from 25-30 years old were conducted to Comet assay [9]. The whole blood samples (100µl) were distributed into Eppendorf tubes that contain Phosphate Buffer Saline (PBS, PH=4.0), treated with chemicals, and incubated for 45 min at 37◦C. Tubes were centrifuged for 3 minutes, 3000rpm at 4◦C. Residual was suspended in 0.5 % low melting point (LMP) agarose and located on a prepared slide that layered with 1% of normal melting point agarose (NMP). Slides were covered with coverslip, formerly removed after 5-10 min and then slides were immersed in freshly prepared cold lysis solution at pH 10 (2.5 M NaCl, 10 mM Trizma base, 100 mM EDTA, 1 % Triton X-100 and 10 % DMSO) and left for 24 hours at refrigerator. Next day, slides were located in a flat gel electrophoresis chamber and kept for 30min at 4◦C in freshly prepared alkaline electrophoresis buffer (10MNaOH and 200mM EDTA, pH > 13). Then electrophoresis was conducted at 4◦C at ~0.75 V/cm (20–25 V, ~300 mA). Slides were neutralized (0.4M Trizma base, pH 7.5) and stained with ethidium bromide. 65 cells were documented 'blind' for each patient with Olympus Bx51, a fluorescence microscope connected to an imaging system of Leica. CaspLab- Comet Assay software was used to measuring the % tail of DNA in order to detect DNA impairment and recovery.

2.1.1 The Design of the Experiment

For each donor, blood was divided into seven main groups in Eppendorf tubes that already contained a Phosphate Puffer Saline (PBS). **Group I: The control,** blood was incubated only with distilled water for 45mins at 37 ◦C. **Group II, III, IV: The genotoxic substance,** three concentrations of potassium bromate 1, 1.2, and 2.5 mM (low, middle, and high) [10] were freshly prepared and incubated with the blood. **Group V: medicinal plant,** only water extract of dill was incubated with the blood at 800µg/ml [11]. **Group VI: Protective,** this group was divided into two stages; incubation with the plant extract primarily for 45mins followed by KBrO3 for another 45mins. **Group VII: Treatment,** the reverse of the last group was applied, firstly, blood was incubated with the highest dose of potassium bromate for 45mins and then plant extract was added and incubated for another 45mins.

2.2 Determination of total Antioxidant, DPPH Radical Scavenging Assay, Total Phenol and Total Flavonoid Content

Dill was attained from the resident shop in Al-Bieda City and the extraction was prepared [12]. The procedure of [13] was followed to assess antioxidant activity of the *Anethum graveolens* L. by DPPH scavenging assay. Singleton and Rossi, [14] were applied to determine total phenol and Park et al., [15] was followed to measure the total flavonoid content.

2.3 Gas Chromatography-Mass Spectrum Analysis

The extraction was kept in a dark bottle and transferred for Gas Chromatography- Mass Spectrum (GC-MS) examination at the Regional Center for Mycology and Biotechnology at Al-Azhar University

2.4 Determination of $KBrO_3$ Quantity in Some Bread Samples from AL-Bayda and Shahat Cities

Bread samples were randomly collected from Ten bakeries from Al-Bayda and five bakeries from Shahat city in the present study. Three bread samples were taken randomly per batch. Samples were prepared for analysis based on [16], based on the equation of the calibration curve, the amount of $KBrO_3$ per bread specimen was calculated [17].

2.5 Statistical Analysis

The experiment was a completely randomized design (CRD), data were combined from five independent replicates, and subjected to analyze the effects of different concentrations of potassium bromate on DNA damage. The Least Significant Difference (LSD) test was used at levels of 0.01 using the Gnestat statistical package program.

3. RESULTS

3.1 Alkaline Comet Assay of Human Lymphocytes

Data illustrated in Fig. 1 reveals the normal lymphocytes of healthy-untreated volunteers (a). Normally, the shape of control heads should be spherical with no tail. However, DNA fragments

migrated out of the head forming a comet tail, thus gradually increasing (b, c, and d) the percentage of DNA in the tail (damaged DNA) with increased potassium bromate dose. Notably, the spherical head was distinguished again (e) when the cell was treated with dill aqueous extract. The migration of the DNA fragments was less in both protective (e) and therapeutic dose (g) in comparison to (d) where cells were treated with a higher dose of $KBrO_3$. Under those experimental conditions, the significant reduction in Comet tail probably reflects the repair action of dill extract.

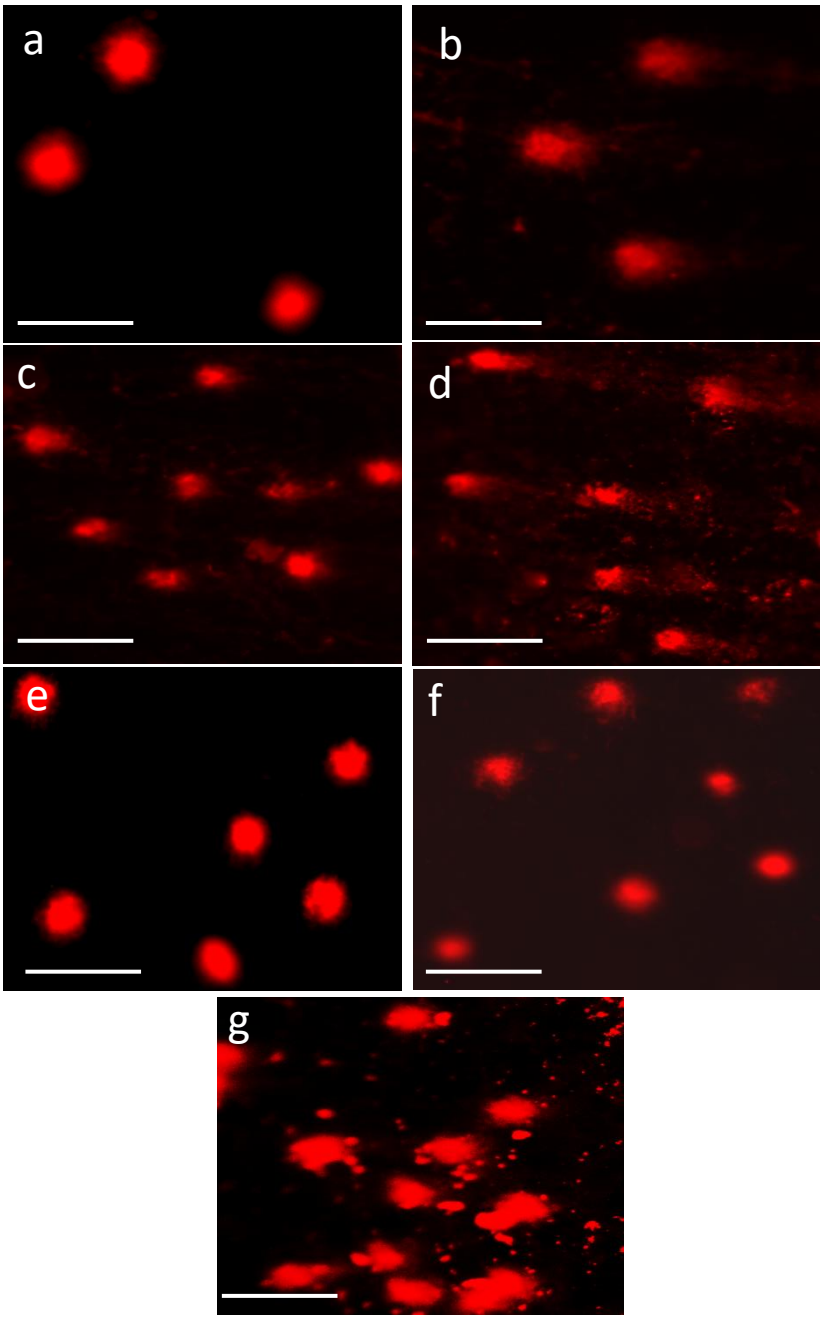

Fig. 1. Representative images of control and treated lymphocytes stained with ethidium bromide isolated from healthy volunteers

Small letters illustrate different treatments; a: control, b, c, and d: selected doses of $KBrO_3$, e: dill extract, f: dill then $KBrO_3$, and g: $KBrO_3$ then dill, (Scale bar: 20µm).

3.1.1 The genotoxicity of potassium bromate and repair ability of dill extract on DNA damage (tail DNA %) of lymphocytes

Current test was aimed to explore the genotoxic effects of $KBrO_3$ and the possible repair ability of dill aqueous extract on the Comet tail (DNA damage) of male and female lymphocytes. Results are shown in Figs. 2 & 3 the control (C) group was compared against the selected doses of $KBrO_3$; 1, 1.5, and 2.5µg/ml which are designated in the figure as low dose (LD), middle dose (MD) and higher dose (HD) respectively. In general, increased levels of DNA damage (Comet tail) were gained in all of the different doses of KBrO3, whereas in all cells that were exposed to various treatments of dill extract, the DNA damage was significantly less as shown in Fig. 2 & 3. In male lymphocyte assessment (Fig. 2), high significant differences ($P < 0.001$) between the control and KBrO3 treated group were achieved. 0.5, 10.64, 29.64, and 82.66% are the averages of Comet DNA for control, low, middle, and higher dose of potassium bromate respectively. Predictable, no significant differences were found in assessed DNA damage between control (0.57%) lymphocytes and those exposed to dill extract (1.24%). In addition to that, the damage of DNA was significantly less in both protective and therapeutic groups. 24.93% of DNA tail was achieved when the cell was incubated with a higher dose of KBrO3 and then dill extract. Once dill extract was applied followed by potassium bromate, the reduction of DNA impairment was significantly ($P < 0.001$ at DSL= 2.42) less (11.88%) than the reverse application which reflects the anti-genotoxic ability of *Anethum graveolens* L. aqueous extract. Comparable signs were obtained in the female (Fig. 3) compared to the male lymphocyte. High significant differences ($P < 0.001$) were observed between the control and $KBrO_3$-treated female group. 2.8, 8.18, 12.13, and 59.34 are the averages of Comet DNA for control, LD, MD, and HD of potassium bromate respectively. Similarly, our assay revealed that the effect of dill extract resulted in recovering the DNA content. Resulting in a significant decrease in the tail length ($P < 0.001$ at DSL= 3.9), with 10.5 as protective and 14.4 values as therapeutic compared with 60% in HD of $KBrO_3$.

3.1.2 A Comparison between Male and Female response

In general, we found that both genders expressed similar responses but surprisingly males exhibited higher values of DNA damage (Fig. 4) than female donors. Regarding DNA damage and repair, there were no differences between untreated lymphocytes of males

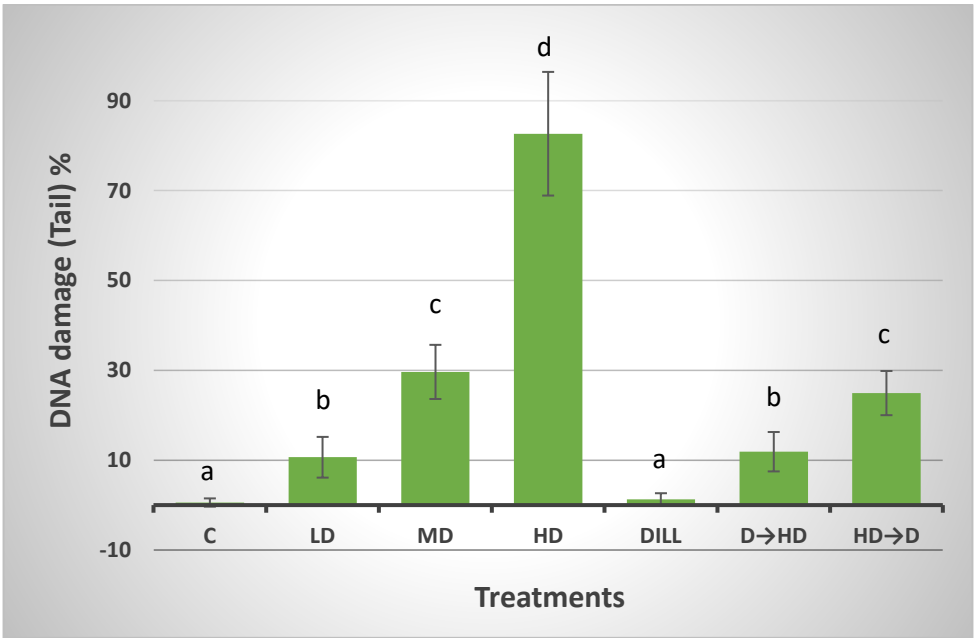

Fig. 2. Percentage of DNA damage (tail) in lymphocytes from male donors via comet assay

Capital letters represent the following: C: control, LD, MD & HD: low, middle & higher dose of $KBrO_3$ (µg/ml), D: Dill. All data were expressed as means (325 cells), and bars represent standard deviation (SD). Small letters represent significant differences (at $p < 0.01$; LSD= 2.42).

and females, as well as in the dill group. A significantly (P < 0.01) higher level of DNA damage was noticed when cells were treated with MD, HD of potassium bromate whereas results from the tail percentage showed a significant (p < 0.001) reduction in DNA damage in the therapeutic group.

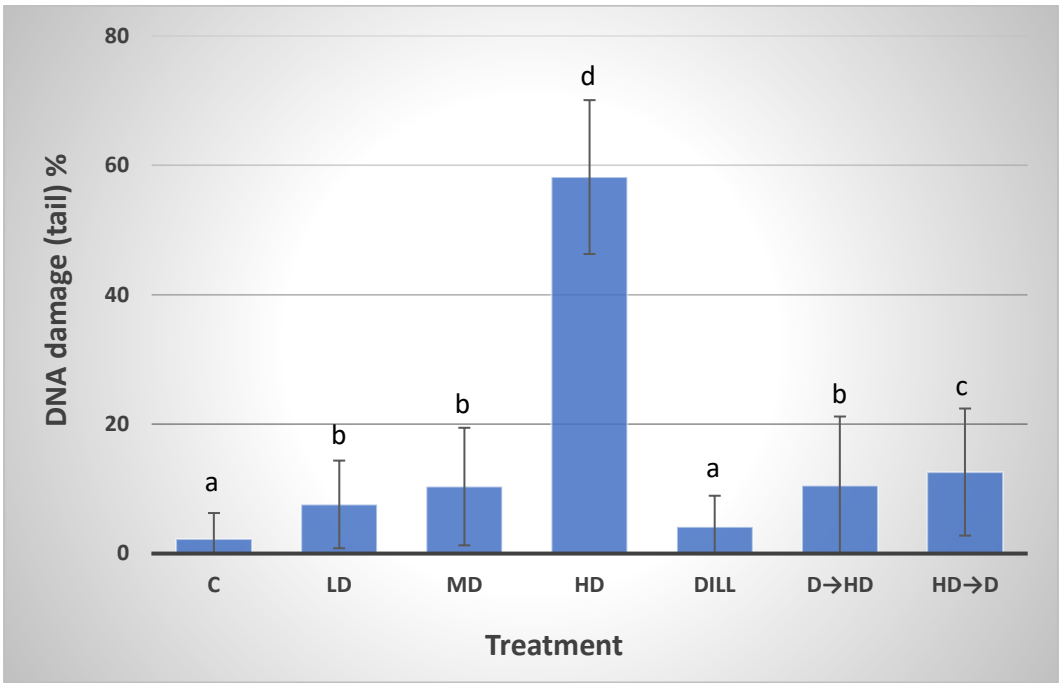

Fig. 3. Percentage of DNA damage (tail) in lymphocytes from female donors via comet assay
Capital letters represent the following: C: control, LD, MD & HD: low, middle & higher dose of $KBrO_3$ (µg/ml), D: Dill. All data were expressed as means (325 cells), and bars represent standard deviation (SD). Small letters represent significant differences (at p < 0.01; LSD= 2.42).

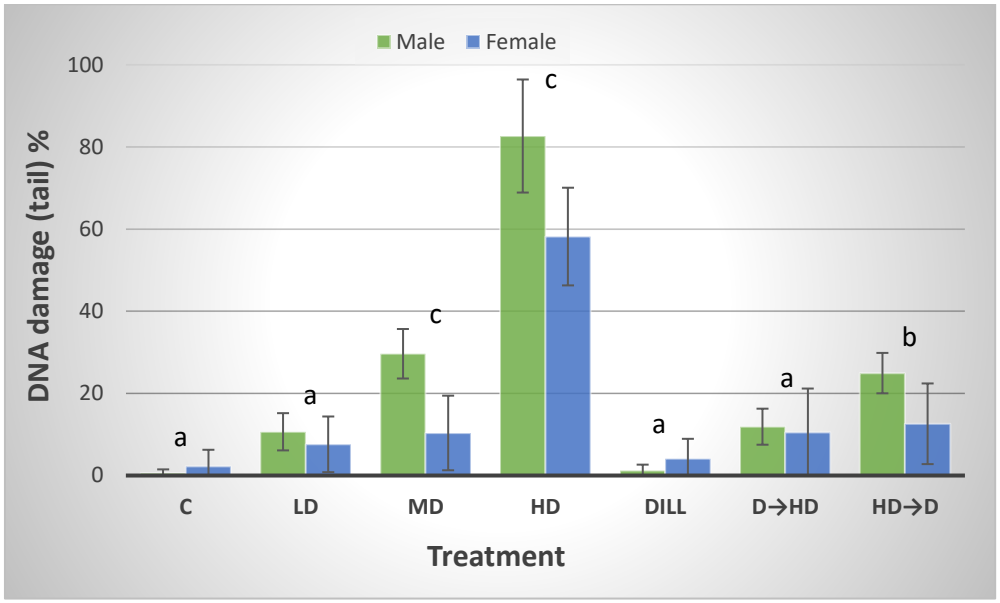

Fig. 4. A Comparison of Male and Female Percentages of DNA damage (tail) via comet assay
Capital letters represent the following: C: control, LD, MD & HD: low, middle & higher dose of $KBrO_3$ (µg/ml), D: Dill. All data were expressed as means (325 cells), and bars represent standard deviation (SD). Small letters represent significant differences (at p < 0.01; LSD= 3.9).

3.2 Analysis of plant extract

3.2.1 Determination of total antioxidant, DPPH radical assay, total phenolic content and Total flavonoid content

Total antioxidants capacity was expressed as percentage and found to be 62.0%. To evaluate the ability of antioxidants of the extract to act as free radical scavengers or hydrogen donors, the DPPH (2,2-diphenyl-1-picrylhydrazyl) assay was performed. The half maximal inhibitory concentration (IC_{50}) was 67.2µg/ml in comparison to ascorbic acid, 42.8µg/ml. The total phenolic content (TPC) was determined using Folin-Ciocalteu reagent, results are expressed as milligrams of gallic acid equivalents per gram dry plant weight (72.2mg GA/g). The Total flavonoid content (TFC) was 72.0mg QE/g of quercetin equivalents per gram dry weight plant material.

3.2.2 Analysis of plant extract by GC–MS technique

The aqueous extract of *Anethum graveolens* L. was dried, dissolved in ethanol, and then analyzed by gas chromatography–mass spectrometry (GC–MS). Fig. 5 reflect the highest compounds of dill aqueous extract that identified; 13-Docosenamide, (Z) - (Z)-Docos-9-enenitrile - 9-hexadecenoic acid, 9-octadecyl ester (Z, Z) - 1(3H)-Isobenzo furanone, 6,7-dimethoxy-3-[2-(2-methoxyphenyl)-2-oxoethyl] and 2 Benzene methane amine, 2-chloro-a-(2-quinoxalinylmethyene) with peak area (%) 22.64, 8.30, 4.00, 2.16, 1.15 respectively.

3.3 Determination of $KBrO_3$ Quantity in Some Bread Samples from AL-Bayda and Shahat Cities

The variation in the quantity of $KBrO_3$ between bread samples was noticed as shown in Table 1. Bakeries were coded as A, B, C, D, E, F, G, H, I, J, K, L, M, N, O, and P, the concentration of bromate in bread samples was 2.097, 0.466, 1.010, 3.077, 2.369, 2.663, 1.514, 1.701, 0.823, 1.865, 0.676, 0.217, 0.959, 2.578 and 3.524mg/kg respectively. The current result illustrates that the highest concentration was in sample O whereas sample L contained the lowest amount of bromate. When potassium bromate was calculated in judgment with the permissible limit of the US Food and Drug Agency (FDA; 0.02) and The World Health Organisation (WHO;0.025), the values exceeded the permissible limit by Ten to several hundred folds.

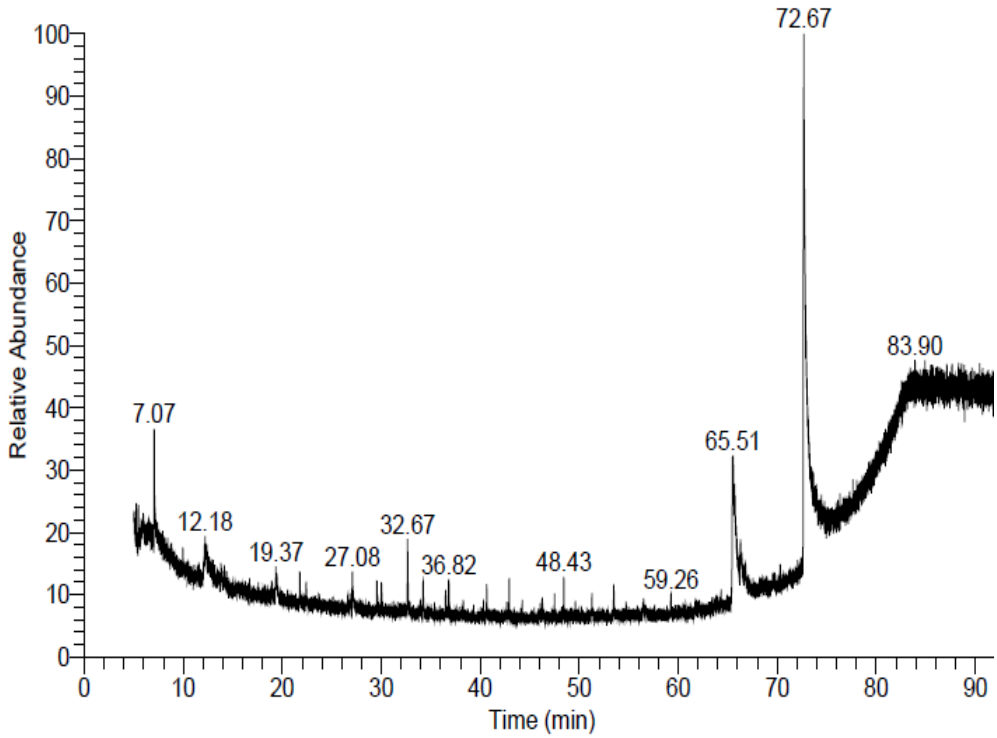

Fig. 5. Qualitative analysis of *Anethum graveolens* L. aqueous extract fresh leaves by Gas chromatography/Mass spectrometry (GC/MS)

Table 1. Coded beard samples, absorbance, and residual concentration of $KBrO_3$

Sample	Absorbance (Average ±SE)	ppm	Conc. mg/kg	Number of folds concerning limits	
				FDA	WHO
A	0.143±0.0096	419.417	2.097	104.8544	83.8835
B	0.047±0.0043	93.204	0.466	23.30097	18.64078
C	0.079±0.0048	201.942	1.010	50.48544	40.38835
D	0.200±0.0116	615.372	3.077	153.843	123.0744
E	0.386±0.0701	473.786	2.369	118.4466	94.75728
F	0.176±0.0067	532.686	2.663	133.1715	106.5372
G	0.108±0.0062	302.751	1.514	75.6877	60.55016
H	0.119±0.0033	340.129	1.701	85.03236	68.02589
I	0.068±0.0067	164.563	0.823	41.14078	32.91262
J	0.129±0.0046	372.977	1.865	93.24434	74.59547
K	0.059±0.0023	135.113	0.676	33.77832	27.02265
L	0.032±0.0022	43.366	0.217	10.84142	8.673139
M	0.076±0.0027	191.748	0.959	47.93689	38.34951
N	0.171±0.0069	515.696	2.578	128.9239	103.1392
O	0.227±0.00563	704.854	3.524	176.2136	140.9709
	Acceptable level (mg/kg)			**0.02**	**0.025**

FDA: Food & Drug Agency, WHO: World Health Organisation

4. DISCUSSION

4.1 The consequences of DNA damage (tail)

In the current study, the increase of DNA tail (Fig. 1, 2 & 3) confirms the impairment of potassium bromate on the genetic material in a dose-dependent manner. The influence of the dill extract was quite similar to the control with no differences, which reflects that there is no genotoxic influence of the dill selected quantity of 800µg/ml. The appearance of smeary DNA (Comet) in our results indicates that potassium bromate has been proven as a genotoxic chemical at all of the selected concentrations. Our indications agreed with those who reported that sunset yellow (SY), a synthetic food additive, had a genotoxic effect in rats. The genotoxic effects of SY may result either via interactions of intact molecules with cytosolic receptors [18, 19, 20] or like some other food additives via the formation of free radicals [21]. Also, it was reported that the chromatin fragmentation and DNA were noticed after application of butylated hydroxyl anisole on lung cancer cells [22]. In addition to that, Comet assay were used to evaluate mutagenic effect of synthetic pigments in drinks and DNA fragmentation displays DNA impairment. Their results indicated an increase in the tail length of Comet from blood lymphocytes [23]. Similar results were stated by [24] who applied Comet assay to investigate the effect of different food additives on DNA of human germ cells. The DNA damage induced by potassium bromate might be a result of inhibition or a delay in the activities of proteins that engaged in duplication of DNA, transcription, and repair [25]. The pre-treatment and also post-treatment of lymphocyte cells with dill extract clearly reduced the level of $KBrO_3$-induced DNA damage possibly through its anti-genotoxic action by induction of the DNA-damage response pathway. Dill extract might be like some other medicinal plants such that can induce DNA damage response (DDR) protein [26, 27]. Current results in the evaluation of the anti-genotoxic effect of dill aqueous extract suggest that the effects of $KBrO_3$ cannot be inhibited. However, when cells were incubated with dill extract (protective and therapeutic groups), the recovery action was noticeable (Fig. 2 & 3) which is reflected in the significant reduction of the tail percent. In the same context, [28] have shown that *A. graveolens* extract has exhibited cytotoxic/anticancer capabilities against leukemia L1210 cells in mice. Our findings also agree with previously published studies [29] which state that dill was able to inhibit micronuclei formation induced by benzo (a) pyrene in mouse bone marrow. Also, regulating expression levels of COX-2 and TNF-α in esophageal tissue indicates the protection ability of *A. graveolens* L. against esophageal damage [29]. Therefore, we believe that dill has good antioxidant activity and can be used as an efficient nutrition or as a supply for the inhibition and therapy of different dysfunctions and systemic disorders. A

significant decrease in comet tail was observed after the supplementation of dill extract, indicating that dill plants have geno-protective properties and might be used as a worthy source of natural antioxidants through nutrition enhancements. Subsequently, the anti-genotoxicity/anticancer activities of dill probably as a result of its antioxidant activities [15]. We propose that those properties are possible because of the dill extract's components. The essential components of dill extract firstly work as antimutagenic factors acting outside the cell (desmutagenicity). Secondly inside the cell antimutagenic factors (antimutagenicity) according to the medical plant extractions classification [30]. The components of our extract might be able to block the DNA sensitive regions thus preventing the binding of potassium bromate. Enhancing the DNA repair system to recover the damage caused by potassium bromate is also proposed. Another possibility is inducing enzymes in some organs that can eliminate the toxicity and assist the cell in DNA replication reducing DNA damage and mutations [31, 32]. Multiple ways of anti-mutagenic compounds performance were previously proposed; prevent the activities of enzymes that elaborated in mutagen formation, rid cells from electrophiles, liberating of Reactive Oxygen Species (ROS), enhancing the enzymes responsible for converting mutagens to harmless compounds and protecting nucleophile sites of DNA [33]. Current data are possibly support the fact that the protective effect of dill extract is due to the attendance of multiple phyto-active compounds. Protection of DNA sites that may affected by mutagenic agents and scavenging free radicals are possibly as a result of the interaction between those multiple phyto-active compounds and mutagens [34]. Data presented in Fig. 4 reflect the significant differences (at $p < 0.01$) between males and females in DNA damage and recovery. The possible explanation of gender disparities was stated in different studies and was related to DNA Damage Response proteins (DDR) indicating that females are highly protected than males against several kinds of genotoxic agents [35]. One of those explanations is the telomere length, it was found that among eukaryotes, women have longer telomeres than men. Responding to genotoxic stress such as oxidative damage, ensuring proper protection for females [36]. Mitochondria present another possible reason for a gender-specific variance in regulation of oxidative stress between men and women [37]. The difference in sex hormone regulation could be an additional reason for our results. Indeed, the biology of both men and women relies on the ratio of their gender hormones and metabolism [38].

4.2 Analysis of potassium bromate in bread samples

Current results (Table 1) indicated that the concentration of potassium bromate in breads was particularly higher than the allowable limit of 0.02 and 0.025 mg/kg agreed upon by the US Food and Drug Agency (FDA) and World Health Organization (WHO) respectively [39,40,41,42]. Compared to some other studies in Libya, potassium bromate ranged between 0.1899-1.8415 µg/g in Benghazi, [43] in Tajora were ranged from 6.0 to 26.67 µg/g [44]. Based on the fact that bread is an essential food consumed regularly every day by residents of Al-Bayda and Shahat cities and the critical amount of potassium bromate that stated in bread samples, we able to conclude that, in long term, high bread consumption pointed to residents are exposure to great amount of potassium bromate. In addition, potassium bromate is a harmful food additive, it has been associated with neuro- and nephrotoxicity to consumers and another risk was stated for employees in bakeries via exposure to thermal product, potassium bromide, a toxic compound [45], Ototoxicity [46].

5. CONCLUSIONS

Taken all together, after assessing the genotoxicity of potassium bromate on the DNA of human lymphocytes and the potential anti-genotoxic action of *Anethum graveolens* L., Comet assay is efficient to investigate the impairment that resulted after exposure to genotoxic agents in the environment. The food additive (potassium bromate) induced DNA damage indicating that it is genotoxic to human blood cells. However, the findings of the present work support the anti-genotoxic effect of the aqueous extract of *Anethum graveolens* L. on human lymphocyte cells. The pretreatment with dill extract is more effective in decreasing the genotoxic effects of $KBrO_3$ than post treatment.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Authors hereby declare that NO generative AI technologies and NO text-to-image generators have been used during writing or editing of manuscripts.

CONSENT AND ETHICAL APPROVAL

As per Omar Al-Mukhtar University committee standard, the current research was given this reference number: NCB: 007. H. 23. 3. The agreements of all study patients were collected prior the commencement of the work.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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