Antimutagenic Effects of Habak against Cyclophosphamide Induced Genotoxicity and DNA Damage in Mouse Bone-Marrow Cells

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Abstract: The pre- and post- treatments effects of Habak, one of *Mentha longifolia* subspecies, as one of the famous folk medicine was studied in mouse bone-marrow cells in contrary to the mutagenicity of cyclophosphamide (CP). Male Balb/C mice were pre- and post-treated orally with aqueous extract of Habak at variable concentrations (250, 500 and 1000 mg/kg b.wt) for 24h and 48h respectively. Mice were injected intraperitoneally with CP for 24h pre- and post-treatment with Habak. Assessment of cell viability of animals treated with Habak aqueous extract and/or CP was performed using trypan blue exclusion assay. Investigating the protective effects of pre- and post-treatments with Habak aqueous extract on genotoxicity of CP were evaluated with chromosome aberrations and DNA damage using the comet assay. Cell viability of mice bone-marrow cells was activated with the low and medium concentrations of Habak, however, the highest concentration induced cytotoxicity significantly. When animals treated with Habak prior to CP, the chromosome aberrations were reserved highly significant in comparison with animals injected with CP. Also, Habak reduced the chromosome abnormalities induced by CP when mice pre-treated with CP. The efficacy of Habak was recorded when it reduced DNA damage induced by pre- and post-treatment with CP as assessed with percentage of DNA damage, tail length and tail moment ratio. In conclusion, the whole aqueous extract of Habak has the ability to reduce the mutagenic effects induced by toxins, besides its ability to ameliorate the cell viability and prevent DNA damage. Thus, we recommend using Habak with the traditional methodology. Besides, studying its constituents and evaluate their bioactivity to prohibit mutagenesis.
[Abo-Zeid MAM, Aly FA, Farghaly AA. **Antimutagenic Effects of Habak against cyclophosphamide induced genotoxicity and DNA damage in mouse bone-marrow cells.** *Stem Cell* 2016;7(4):34-40]. ISSN: 1945-4570 (print); ISSN: 1945-4732 (online). http://www.sciencepub.net/stem. 7. doi:10.7537/marsscj070416.07.

Keywords: *Mentha longifolia*; Habak; DNA damage; chromosome aberrations; Comet; mouse; bone-marrow

1. Introduction

Mentha longifolia is one of the Lamiaceae family, which is used in traditional and folk medicine (Hedge, 1992; Gulluce et al., 2007). This genus was used for a very long time since the ancient Hebrews, Romans and Greeks were well-known for its high efficiency in medications. It is named also as Wild Mint (Biblical Mint), and includes at least 21 subspecies and about 150 types with different common names according to the plant breeding region (Duke et al., 2008). From these subspecies, our study was concerned with the unique subspecies existing in the Arab region, especially in Palestine and Al-Madinah Almunawwarah- Saudi Arabia (Duke et al., 2008; El-Badry et al., 2010) and famous with the common name "Habak" (Duke et al., 2008; Ghazanfar, 1994). In general, *Mentha longifolia* species are cultivated in moisture places and temperate areas such as those found in the Middle East, central and southern Europe, southwest Asia and Australia (Gulluce et al., 2007; El-Badry et al., 2010). The species of *Mentha longifolia* is considered as one of the oregano herbs which are characterized with their aerial parts. All parts of the grass subspecies including Leaves, flowers and stems are used in herbal tea or as an additive in commercial spice constituents of flavor foods (Gulluce et al., 2007). Some studies evaluated its traditional use to treat fibrosis and cervical cancer after decoction (Duke, 2001; Marderosian, 2001). Also, it has been used to treat nausea, bronchitis, emphysema, and anorexia (Iscan et al., 2002). Mentha genus is an aromatic perennial plant, categorized as one of the carminative, decongestant, antioxidant, anti-inflammatory, antibacterial, anti-fungal, antispasmodic remedy (Gulluce et al., 2007; Mimica-Dukic et al., 2003; Mkaddem et al., 2009; Van Wyk and Gericke, 2000) and HIV-1 inhibitory effect (Amzazi, et al., 2003). It is used in herbal medicine, to strength the immune system, treat cold, influenza, enlarged glands and injuries (Van Wyk, et al., 1997), besides, its efficacy to repel mosquitoes and rodents (Hutchings and Van Staden, 1994; Phillips and Foy, 1999).

To assess the antimutagenic efficacy of "Habak" as one of the *Mentha longifolia* subspecies on mouse bone-marrow cells, we used one of the chemotherapeutic agents characterized by its potential to induce genetic alterations and chromosome instability. Cyclophosphamide (CP) is one of the alkylating agents used as a potent anti-inflammatory

and immunosuppressive cytostatic and cytotoxic drug to treat diverse medical problems such as neoplasia, tissue transplantation and inflammatory diseases (Gershwin et al., 1974; Oboh et al., 2011; Stankiewicz and Skrzydlewska, 2003). It is characterized by its inactive form, once reaches to the liver; it converts to the active metabolites and generates reactive oxygen species (Gershwin et al., 1974; Oboh et al., 2011; Stankiewicz and Skrzydlewska, 2003), which in turn induces genetic alterations, chromosomal breakages, rearrangements and aneuploidies and other mutagenic effects (Ben-Yehuda et al., 1996; Povirk et al., 1994; Gamal-Eldeen et al., 2013), and subsequently could induce secondary neoplasia (i.e. Bernatsky et al., 2008; Pryor et al., 1996).

In the present study, we aimed to investigate the cytogenetic bioactivity of whole plant extract of *Mentha longifolia* subspecies "Habak" prepared by the traditional method to reduce and ameliorate the cytogenetic alterations and DNA damages induced by CP as a neoplastic agent in mouse bone-marrow cells.

2. Material and Methods

2.1. Plant aqueous extract preparation

One of the subspecies of *Mentha longifolia*, which is known by the common name "Habak" was collected from Al-Madinah Almunawwarah, Saudi Arabia. The leaves of the plant material were harvested on dry and sunny day. They were kept in air to completely dry, then separated from stems, and well grinded to fine dust. The plant aqueous extract was prepared according to the traditional method described by Dyson (1988) with trivial modifications. We added 10g from the leaves dust to 500ml boiled deionized distilled water for about 1 h, then left overnight to cool before filtered and freeze dried. The collected Habak powder extract was preserved in packaged aliquots at -20°C, and was prepared with the required concentrations just before oral administration to animals by gavage

2.2. Animals and dosage

Laboratory-bred strain Swiss albino male mice of 12-15 weeks old with an average weight of 25g obtained from the NRC, Cairo, Egypt, were used. Animals were housed in groups (8 animals/ group) and maintained under standard conditions of temperature, humidity and light according to the animal care statements of the Ethical Committee, NRC, Cairo, Egypt. The animals were given standard food and water ad libitum. Animals were fed by different concentrations (250, 500, 1000 and 2000 mg/kg b.wt) of Habak by gavage for 24h pre- or post-intraperitoneal (i.p.) injection with CP at a concentration of 20 mg/kg b.wt for 24h or 48h respectively. Animals were divided into six groups.

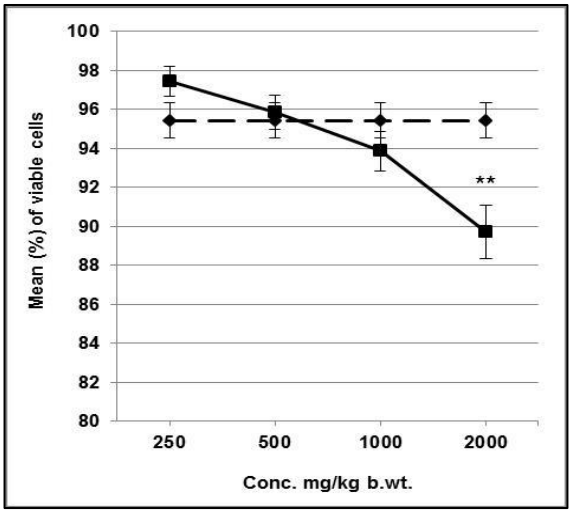

Figure 1. Assessment of cell viability of different concentrations of Habak (solid line) in comparison with control (dash line) in mouse bone-marrow cells using trypan blue exclusion assay. Data were presented as means ± SEM, n=8, and considered significant when $p<0.05$; [**$p<0.01$].

The 1st group was treated with vehicle and considered as non-treated group; the 2nd group was treated with Habak at 1000 mg/kg b.wt; the 3rd group was injected with CP for 24h; the 4th group was administered Habak for 24h followed by CP for 24h; 5th group was injected with CP for 48h; and the 6th group was injected with CP for 24h followed by Habak for 24h and samples were collected after 48h from starting injection of CP.

2.3. Cell viability with trypan blue

To assess the effect of Habak in mouse bone-marrow cell viability, animals were administered four concentrations of Habak (250, 500, 1000 and 2000 mg/kg b.wt.) orally by gavage.

Bone marrow cells were collected after 24h from starting treatments. To measure cell viability, 15µl of each sample containing a cell suspension was mixed with15µl of trypan blue (0.4%; Sigma). Cells were analyzed with BioRad automated cell counter (TC20), and the percentages of viable cells from control were recorded with all concentrations.

2.4. Chromosome aberrations

Three concentrations of Habak were used to assess its bioactivity (250, 500 and 1000 mg/kg b.wt.). All animals were injected i.p. with colchicine 2h prior to collecting samples from bone-marrow cells. The metaphase preparation was carried out according to Yosida and Amano (1965). In brief, mouse bone-marrow cells were collected from both femurs, cells were incubated in hypotonic solution (KCL 0.075 M)

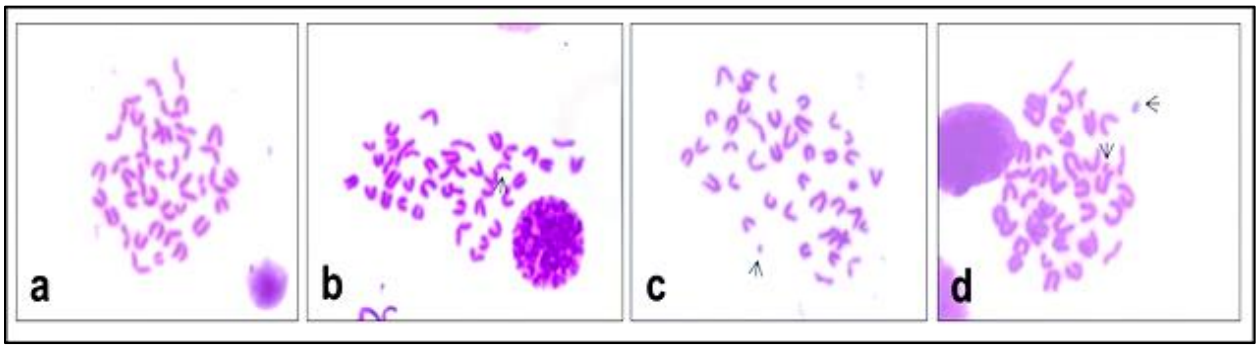

Figure 2. Metaphases spread from mouse bone marrow cells treated with aqueous extract of Habak and CP showing (a) normal metaphase, (b) gap, (c) fragment, (d) break and fragment.

for 20 min at 37°C, and then centrifuged. The cell pellets were resuspended in a fixative (methanol/glacial acetic acid). This step was repeated, then resuspended cells in fixative were spread onto frozen slides, air dried, stained with 10% Giemsa for 40 min, washed, and air dried again. Hundred well-spread metaphases were analyzed per animal for structural and numerical (tetraploidy) aberrations.

2.5. Comet assay

Assessment of DNA damage induced by CP and recording efficacy of Habak to reduce its genotoxicity was evaluated with alkaline comet assay according to Schlörmann and Glei (2009) with some modifications. Mouse bone-marrow cells were collected from femurs with phosphate buffer saline, centrifuged to collect the pellet cells with 50µl of 0.7% low melting agarose. The re-suspended cells were spread over 0.5% normal melting agarose pre-coated slides. Then the slides were immersed in lysis buffer for at least 1hour prior to dip them in the electrophoresis chamber containing electrophoresis buffer for 20 min, followed by another 20min with running current at 1.25V/cm and 300mA. Comets were recorded by scoring sixty cells per each animal under fluorescent microscope at 400X, analyzing images with Perceptive software V2, and recording the percentage of DNA damage, tail length (µm) and tail moment.

2.6. Statistical analysis

The statistical analysis of chromosome aberrations was done according to Chi-square-contingency test. Data of cell viability and comet assay were analyzed by one way ANOVA - Tukey's Multiple Comparison test. GraphPad Prism software was used for all statistical analysis. All data were presented as means ± SEM, and n=8 animals per group. Results were considered statistically significant when $p<0.05$.

3. Results

3.1. Cell viability

When mice administered Habak orally by gavage, Habak increased the mean percentages of cell viability non-significantly with 250 and 500mg/kg b.wt. in comparison with non-treated group. The higher concentration of Habak (1000 mg/kg b.wt.) reduced the mean percentage of cell viability without any significant. However, the highest concentration of Habak (2000 mg/kg b.wt.) reduced cell viability significantly (Figure 1).

3.2. Chromosome aberrations

The numbers and mean percentages of chromosome aberrations in mouse bone-marrow cells were recorded with all treatments (Table 1). When mice treated with Habak at 1000mg/kg b.wt., the mean percentages of chromosome abnormalities with and without gaps were increased non-significantly in comparison to non-treated group. The numbers of chromosome aberrations, including and excluding gaps, in animals treated with CP for 24h or 48h were increased extremely significantly regarding to control group. Habak enhanced a protective effect when administered to animals 24h prior to CP. This protective effect was observed in decreasing the numbers of chromosome aberrations, including and excluding gaps, in a concentration dependent manner highly significantly in comparison with animals injected with CP for 24h. When the animals injected with CP before administering Habak, the percentages of chromosome abnormalities were also decreased gradually in a significant manner with increasing the concentration of Habak in comparison with CP alone for 48h (Table 1). Recording different types of structural chromosome abnormalities including gap, break, fragment, deletion, and numerical aberrations represented by tetraploidy were assessed in our study (Table 1, Figure 2).

Table 1: Effect of Habak (Hab) on Cyclophosphamide-induced chromosome aberrations in mouse bone marrow cells *in vivo*.

	Conc. mg/kg b.wt.	Total Abnormal Metaphases				No. of metaphases with different types of Ch. Ab.					
		Including Gaps		Excluding Gaps		G.	Br. and/or Frag.	Del.	C.F.	M.A.	Polyp.
		No.	Mean (%) ± SE	No.	Mean (%) ± SE						
I. Control		35	4.4±0.45	21	2.6±0.48	14	13	8	0	0	0
II. Hab	1000	40	5.0±0.50	22	2.8±0.62	18	14	8	0	0	0
III. CP/24h	20	a 179	22.4±0.50	a 141	17.6±0.60	38	82	14	5	27	13
	250	ab 138	17.3±0.60	ab 112	14.0±0.48	26	77	13	3	16	3
IV. Hab/ CP	500	ab 119	14.9±0.54	ab 93	11.6±0.58	26	80	6	0	5	2
	1000	ab 97	12.1±0.50	ab 79	9.9±0.65	18	66	3	2	5	3
V. CP/48h	20	a 149	18.6±0.62	a 119	14.9±0.55	30	77	11	3	22	6
	250	a 135	16.9±0.40	a 108	13.5±0.58	27	78	6	6	13	5
VI. CP/ Hab	500	ac 106	13.3±0.50	ac 84	10.5±0.55	22	72	3	3	6	0
	1000	ac 83	10.4±0.55	ac 67	8.4±0.60	16	50	8	2	5	2

100 metaphases/animal; n=8; G.: Gap; Frag.: Fragment; Br.: Break; Del.: Deletion; C.F.: Centric Fusion; M.A.: Multiple Aberrations; Polyp: Polyploidy. a: Significance was compared to group (I) vehicle control; b: Significance of group (IV) was compared to CP for 24h; c: Significance of group (VI) was compared to CP for 48h); $p<0.05$; Chi-square contingency test.

3.3. DNA damage by the comet assay

To evaluate the efficacy of Habak to protect and/or reduce DNA damage induced in animal cells treated with CP as a mutagenic agent, we used alkaline comet assay version (Figure 3). When animals were treated with the highest concentration of Habak, the percentage of DNA damage, mean of tail length (µm) and a ratio of the tail moment were less than that observed in non-treated animals, which indicated that the aqueous extract of Habak induced ameliorative effects in mouse bone marrow cells (Table 2). The percentages of DNA damage induced by CP were observed to elevate extremely with more than 4 and 3.5 folds that induced in non-treated group after 24h and 48h respectively. Similarly the average of tail length and ratio of tail moment were recorded to rise highly significantly when animals injected with CP for 24h or 48h. The medium of tail length was higher than that induced in control groups with about 2.34 and 1.89 folds, and ratio of tail moment was larger than non-treated animals with nearly 6.70 and 6.06 folds after 24 h and 48 h respectively (Table 2).

The animal groups treated orally with Habak aqueous extract with two concentrations (250, 1000 mg/kg b.wt.), and divided into two groups according to sequence of Habak before- or after CP- treatments. The first group treated with Habak for 24h followed by CP for another 24h as a protective period. Habak reduced the percentages of DNA damage, tail length and ratio of tail moment in a concentration dependent manner highly significantly in comparison with that induced with CP for 24h. The reduction observed with comet test reached to the limit that there were no significance between animals treated with Habak followed by CP and non-treated animals (Table 2). The second group, animals treated with CP for 24h followed by Habak for another 24h. In this group, Habak reduced dramatically the percentage of DNA damage, tail length and ratio of tail moment in comparison with animals treated with CP alone before collecting cells after 48h. But, post-treatment with Habak for 24h was not sufficient period to ameliorate the side effects induced by CP in mouse bone-marrow cells effectively as detected with the DNA damage, which was highly significant in comparison with non-treated animals (Table 2).

Table 2: Effect of Habak (Hab) on Cyclophosphamide-induced DNA damage in mouse bone marrow cells *in vivo* as evaluated by Comet assay. Data represented as mean±SE.

		Conc. mg/kg b.wt.	% DNA damage	Tail Length (µm)	Tail Moment
I.	**NC**		2.257±0.091	0.547±0.014	0.027±0.002
II.	**Hab**	1000	2.776±0.367 a	0.826±0.116 a	0.062±0.014 a
III.	**CP/24h**	20	11.219±0.242 b	1.637±0.021 ab	0.416±0.003 ab
IV.	**Hab/CP**	250	3.240±0.654 b	1.043±0.111 b	0.076±0.009 b
		1000	2.587±0.047 a	0.716±0.085 a	0.046±0.009 a
V.	**CP/48h**	20	9.701±0.184 ac	1.567±0.037 ac	0.376±0.008 ac
VI.	**CP/Hab**	250	5.834±0.292 ac	1.228±0.035 ac	0.157±0.007 ac
		1000	4.983±0.095	1.068±0.056	0.148±0.021

60 cells/animal; n=8; a: Significance was compared to group (I) vehicle control; b: Significance of group (IV) was compared to CP for 24h; c: Significance of group (V) was compared to CP for 48h); $p<0.05$; One way ANOVA-tukey's multiple comparison test.

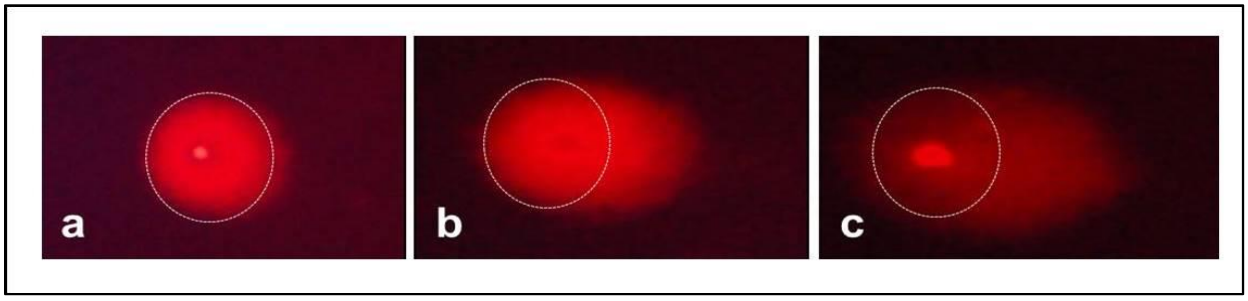

Figure 3. Comet pictures representing (a) intact DNA, (b) highly damaged DNA, and (c) extremely damaged DNA from mice bone-marrow treated with aqueous extract of Habak and the mutagenic agent Cyclophosphamide.

4. Discussions

Habak is one of the subspecies of *Mentha longifolia* Lamiaceae. It is considered one of the ancient plants used usually in the Arab region such as the folk medicine, food ingredients and flavor to various delightful drinks (Hedge, 1992; Gulluce et al., 2007; Duke et al., 2008). Therefore, it was necessary to investigate the cytogenetic activity of Habak in normal cells and assess its bioactivity to prevent or reduce the genotoxic effects could be induced by mutagenic agents such as CP. We used mice-bone marrow cells to be a model in the present study. We prepared Habak by the traditional way, which usually are used in the Arab region, then administered the aqueous extract to animals to assess its bioactivity. The low concentration of Habak (250 mg/kg b.wt.) activated cell proliferation effectively. The moderate concentrations (500, 1000 mg/kg b.wt.) preserved the bioactivity of the cells around the normal state. However the highest concentration of Habak (2000 mg/kg b.wt.) lessened greatly cell viability. Thus, it is recommended to use Habak with the proper concentrations that enhance bioactivity and do not induce cytotoxic effects on cells. Also, we found that the whole aqueous extract of Habak IC50 reached to 3991.5µg/ml in hepatocellular carcinoma (HepG2) cell line (data not shown). When Perez Raya et al. (1990) injected mice intraperitoneally with the essential oil of Spanish *Mentha longifolia*, they found that LD50 of the essential oil reached to 437.4mg/kg b.wt., but they did not assess the toxicological effects of the whole herb. Odeyemi et al. (2009) mentioned that the essential oil from *Mentha longifolia* may increase the liver activity in rats at the lowest dose and advised to use the moderate doses of the oil since it may not be completely "safe". Also, Murad et al. (2016) found that low and moderate concentrations of *Mentha longifolia* methanol extract reduced cytotoxic effects of acetic acid induced colitis in rat's colons; however the higher concentration did not prevent colitis or inhibit anti-inflammatory and antioxidant

effects in rat's colon cells treated with acetic acid. They mentioned that most components of the whole aqueous plant extract give it the efficacy to reduce cytotoxicity highly significantly rather than methanol plant extract.

The efficacy of Habak to induce bioactivity in normal cells was illustrated when animals treated with CP post- or pre- treatment with Habak. Habak stimulated the protective effect on animal cells before treating them with CP. Our study affirmed this protective effect when chromosome aberrations and percentages of DNA damage by comet test were declined highly significantly in a concentration dependent manner in animals pre-treated with Habak (250:1000 mg/kg b.wt.) followed by CP in comparison with those treated with CP alone.

The worth value was established when Habak declined the mutagenic effects of CP denoted with chromosome aberrations and DNA damage dramatically with concentration dependent manner when animals injected with CP then feed with the moderate concentrations of Habak (250:1000 mg/kg b.wt.). The efficacy of Habak to protect cells from the oxidative stress induced by CP (Oboh et al., 2011; Stankiewicz A. and Skrzydlewska, 2003) could be result from the antioxidant activity of the *Mentha longifolia* subspecies. Many studies illustrated the bioactivity of Mentha genus such as reducing oxidative stress and decreasing malonaldehyde (MDA) level (Ahmed et al., 2015; Al-Ali et al., 2013; Stanisavljević et al., 2014). Also, existence of monoterpenoins components in *Mentha longifolia* enhanced its efficacy to fight protozoa and microbes (El- Badry et al., 2010; Hussain et al., 2010; Stanisavljević et al., 2014). Mentha genus contain many compounds have antioxidant activity able to scavenge free radicals, reduce lipid peroxides and improve glutathione (GSH) levels induced by oxidative stress resulted from hydrogen peroxide (H2O2) in human keratinocytes (NCTC2544) (Berselli et al., 2010), reduce TNF-α, nitric oxide and scavenge free radicals could be produced in macrophages (Karimian et al., 2013). Furthermore, Fathi et al. (2015) found that the ethanol extract of *Mentha longifolia* at low concentrations reduced oxidative stress and lipid peroxidation by their antioxidant activity in rat's neuronal cells, which consequently squeezed blood-brain barrier and brain edema. Also, moderate doses of methanol extract and high doses of eucalyptol (one of *Mentha longifolia* essential oils) reduced the colitis induced in rat's colon cells treated with acetic acid due to their antioxidant and anti-inflammatory efficacy (Murad et al., 2016).

In conclusion, the aqueous extract of the *Mentha longifolia* "Habak subspecies" is characterized by its bioactivity and its potential to prevent and reduce the mutagenicity induced by toxins such as cyclophosphamide in mouse bone-marrow cells. We recommend using the whole herb extract since it is obtainable, easier to prepare and safer in comparing with methanol or ethanol extracts which require lower concentrations. Further investigations to detect the bioactivity of Habak aqueous extract including its effects on the immune system, circulation and other biological activities are suggested.

Acknowledgements:

The research work was financially supported by the NRC, Cairo, Egypt.

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