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Melissa officinalis: A potent herb against EMS induced mutagenicity in mice

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Abstract. *Melissa officinalis* (L) is used traditionally for different medical purposes such as tonic, antispasmodic, carminative, diaphoretic, surgical dressing for wounds, sedative hypnotic, strengthening the memory and relief of stress induced headache. The methanolic extractof *Melissa officinalis* (Mo-ME) was investigated for antimutagenic activity. The extraction was done by Soxhlet extraction method and the extract was evaluated for antimutagenic assay against EMS induced mice by micronucleus and chromosomal aberration assay. Briefly, mice were treated with methanolic extract of *Melissa officinalis* (Mo-ME) (100, 200 300 & 400 mg/kgbw) for 15 days. Without the doses of EMS, no and mutagenic effects were observed in blood and bone marrow samples of the mice. Micronucleus and chromosomal aberration test revealed the protective effects of Mo-ME when administered at high doses.The reduction profiles in the MN induction of methanolic extract of *Melissa officinalis* at the concentration (100, 200, 300 and 400 mg/kgbw) with EMS were estimated as 14.5%, 28.0%, 47.7% and 81.5% respectively. The methanolic extract of *Melissa officinalis* exhibited no cytotoxic and mutagenic effects but only have antimutagenic effects, an effect that can be attributed the presence of majorital compounds, and the antimutagenic property of Mo-ME is an indication of its medicinal relevance.

Keywords. *Melissa officinalis*, GC-MS, EMS, mice, micronucleus test, chromosomal aberration, antimutagenicity.

INTRODUCTION

Medicinal plants with antioxidant and antimicrobial properties are gaining a lot of attention as these properties are commonly assumed to play an important role in preventing diseases caused by oxidative stress, such as cancer, coronary arteriosclerosis and the ageing processes (Haraguchi *et al.* 2009). Derived forms of medicinal plants (extracts, syrups, etc.) have been the basis of medical therapy for centuries. Traditionally used in the treatment of several human disorders, their pharmacological and therapeutic properties are attributed to various chemical constituents isolated from their crude extracts (Pereira *et al.* 2009, Kwak and Ju, 2015; Liu *et al.* 2015). Notwith-

standing, their correct use requires the manipulation of plants selected for their efficacy and safety, based either on folk tradition or scientific validation (Tovart, 2009). The use of herbal infusions to cure various disorders is very common in folk medicine especially to those who live in upper reaches of Kashmir Himalayas (Dutt *et al*. 2015). Although the diversity of plant species in Kashmir Himalayas is a potential source of biologically active compounds, the effects on human health and genetic material are often unknown. There are indications that the protective action on genetic material can lead, not only to its repair, but also the preservation of its integrity (Berhow *et al*. 2000; Fernandes and Vargas, 2003; Souza *et al*. 2004). Not all are harmless, some even presenting toxic and mutagenic substances in their phytochemical composition (Bresolin and Vargas, 1993; Sa-Ferreira and Vargas, 1999). Interest in such popular usage has recently gained strength, through recent knowledge that chemicals, such as proteases and antioxidants may prevent or reduce the development of cancer by blocking genetic damage (Hernandez-Ceruelos *et al*. 2002).

Melissa officinalis belongs to Lamiaceae family, a large group of medicinal plants. *M. officinalis* is native to southern Europe and northern Africa; although, over the last several centuries it has been successfully cultivated all over the world. Today it can be found growing wildly throughout North America, Europe, Asia, and in the Mediterranean. The leaves of *M. officinalis* have been used in folk medicine especially in Turkey and Iran, for the treatment of some disease (Sadraei *et al*., 2003). Also, the leaves of *M. officinalis* are often used as herbal teas. *M. officinalis* contains some phenolic and flavonoid compounds such as rosmarinic acid (Herodez *et al*., 2003). The phenolic contents in plants have some antioxidant properties (Chen *et al*., 2001). Essential oils and extracts of this plant have been reported to have antiviral (Schnitzler *et al*., 2008), antimicrobial and antioxidant properties (Dastmalchi *et al*., 2008). As little has been done on the antimutagenicity of *Melissa officinalis*, therefore, the purpose of this study was to determine the antimutagenic activities of methanolic extract of *Melissa officinalis*.

MATERIAL AND METHODS

Collection and air drying of plant material

Aerial parts of *M. officinalis* were collected from Bandzoo area of Pulwama from the garden of IIIM, Srinagar and from SKUAST-K in the month July, 2013. The plant was identified at the Centre of Biodiversity and Plant Taxonomy, Department of Botany, University of Kashmir, Srinagar, J&K and a voucher specimen (JKASH/CBT/227 Dated 08. 08. 2014) was deposited there. The parts were allowed to dry under shade (30 °C) for 8-10 days.

Preparation of extracts

After shade drying, the aerial parts were macerated to fine powder, 1 kg of leaves were extracted successively with hexane for defatenning and methanol for 16 h using Soxhlet apparatus. The extracts were filtered through a Buchner funnel using Whatman No. 1 filter paper, and all the extracts were concentrated to dryness under vacuum using a Heidolph rotary evaporator, yielding hexane, and methanol crude extracts of 65 and 48g respectively. All the extracts were stored at 4°C in air tight glass bottles before use.

GC-MS analysis

GC-MS analysis was carried out with GCMS-QP2010 Plus, Shimadzu, Japan fitted with programmable head space auto sampler and auto injector. The capillary column used was DB-1/RTX-MS (30 metre) with helium as a carrier gas, at a flow rate of 3 mL/min with 1 µL injection volume. Samples were analysed with the column held initially at 100°C for 2 min after injection, then increased to 170°C with 10°C/min heating ramp without hold and increased to 215°C with 5°C/min heating ramp for 8 min. Then the final temperature was increased to 240°C with 10°C/min heating ramp for 15 min. The injections were performed in split mode (30: 1) at 250°C. Detector and injector temperatures were 260°C and 250°C, respectively. Pressure was established as 76.2 kPa and the sample was run for 70 min. Temperature and nominal initial flow for flame ionization detector (FID) were set as 230 °C and 3.1 mL/min, correspondingly. MS parameters were as follows: scan range (*m/z*): 40-650 atomic mass units (AMU) under the electron impact (EI) ionization (70 eV). The constituent compounds were determined by comparing their retention times and mass weights with those of authentic samples obtained by GC and as well as the mass spectra from the Wiley libraries and National Institute of Standards and Technology (NIST) database.

Experimental Animals

Both sex of albino mice, Balb/c strain useful for research in cancer and immunology, age of 6 weeks, weighing 25-35 g were obtained from the Indian Institute of Inte-

grative Medicine (IIM), Canal Road Jammu-India, kept in plastic cages in an animal room under controlled conditions of temperature (22 ± 2°C), humidity (55 ± 10%), 12 h light/dark cycles and access to food and water. They were randomized at the beginning of the experiment.The study design was approved by the Institutional Animal Ethical Committee, and the experiments undertaken in accordance with the ethical principles of the CPCSEA norms.

Treatment protocol

The mice were divided into 8 groups, with 5 animals per group. Ethyl methane sulfonate (EMS, SigmaAldrich) was used to induce mutations. Just before use, the EMS was diluted in 0.9% NaCl. The exposure route was by gavage(1/4thof LD_{50} of EMS; 117.5 mg/kgbw). Evaluation of either DNA damage or protection by the methanolic extracts of *Melissa officinalis* was according to protocol developed by Azevedo *et al.* (2003), with the some adaptations. The mice in group 1 received only distilled water (10 mL/kg bw. per day by gavage) for 2 weeks and acted as negative control (Table 1). Mice in group 2 were exposed to EMS (1/4thof LD_{50}) for 24 h and this group acted as positive control. Group 3 and 4 were given different doses (100 & 400 mg/kgbw) of the extract to see the cytotoxic and mutagenic potential of *M. officinalis* and served as positive control of plant extracts. Group 5, 6, 7 and 8 were treated with dose of 100, 200, 300 and 400 mg/kgbw respectively for 15 days after treatment with EMS. The mice were killed by cervical dislocation on 16thday for evaluation of micronucleus and chromosomal aberrations.

The micronucleus test

The method of MacGregor *et al.* (1987) was used for micronucleus test. Mice were sacrificed by cervical dislocation. Slides were prepared with blood collected from the jugular vein. The slides were air-dried, fixed in absolute methanol, stained in 10% Giemsa and then coded for blind analysis. One thousand polychromatic erythrocytes (PCE) were analysed per mouse. The proportion of PCE and normochromatic erythrocytes (NCE) in 1000 erythrocytes/group was calculated, to detect possible cytotoxic effects. The slides were scored blindly, using a light microscope with a 45x and 65x objectives. Photography was done using 100x immersion objective.

Chromosomal aberration

Mice were injected intraperitoneal with 0.5 ml of 0.06% colchicine and two hours later, were sacrificed by cervical dislocation. Both the femurs were fleshed out from the muscles and kept in HBSS (Hank's balanced salt solution). The femurs were then rinsed with 3 ml 0.056% KCl solution in a centrifuge tube. The tube was then incubated at 37°C for 20 minutes. After incubation, centrifugation at 800 rpm for 4 minutes was carried out. Supernatant was discarded and fresh Carnoy's fixative was added (3:1 methanol: acetic acid). The process of centrifugation was repeated three times. Then slides were prepared, stained with 4% Giemsa, air dried and studied under compound microscope.

Statistical analysis

Variable normality was assessed using the Kolmogorov-Smirnov test. Micronucleus testing and chromosomal aberration involved multiple pair-wise comparison between experimental groups and positive and negative controls, with the Mann Whitney U test at a significance level of <0.05. Lower the Mann Whitney statistic value and Z score value, higher the difference.

Table 1. Grouping, dose (distilled water, EMS and Ab-ME in concentrations of 100, 200, 300 and 400 mg/kg bw) and duration of experiment.

Group	Dose	Purpose of group	Duration
Group 1	Distilled water	Negative control	15 days
Group 2	1/4th LD_{50} EMS	Positive control EMS	24 h
Group 3	Mo-ME 100 mg/kg bw	Positive control *Melissa officinalis*	24 h
Group 4	Mo-ME 400 mg/kg bw	Positive control *Melissa officinalis*	24 h
Group 5	Mo-ME 100 mg/kg bw + EMS	Treated Group	15 days
Group 6	Mo-ME 200 mg/kg bw + EMS	Treated Group	15 days
Group 7	Mo-ME 300 mg/kg bw + EMS	Treated Group	15 days
Group 8	Mo-ME 400 mg/kg bw + EMS	Treated Group	15 days

Mo-ME = Methanolic extract of *Melissa officinalis.*

RESULTS

GC-MS analysis

In order to find out the phytocomponents of *Melissa officinalis,* the methanolic extract was subjected to GC-MS analysis. The active principals present in the methanolic fraction of *Melissa officinalis* along with their retention time (RT), molecular formula, molecular weight (MW) and peak area (%) are presented in Table 2. The chromatograms of methanolic extract of *Melissa officinalis* (Mo-ME) showed three major peaks (Fig. 1): 2, 3-dihydro-3, 5-dihydroxy-6-methyl-4H-pyran-4-one (51.62%), 5-(hydroxymethyl)-2-furan carboxaldehyde (29.46%), hexadecanoic acid, methyl ester (8.24%), constituting 89.32% of the total peak area. The minor fractions of Mo-ME include octadecanoic acid (3.26%), stigmast-5-en-3-ol (1.44%), tetradecanoic acid (1.43%), 2, 4- cresotaldehyde (1.17), comprising 7.30% of the total peak area. The phytoconstituents identified in the methanolic fraction of *Melissa officinalis* along with their retention time (RT), molecular formula, molecular weight (MW) and peak area (%) are presented in Table 3.6.

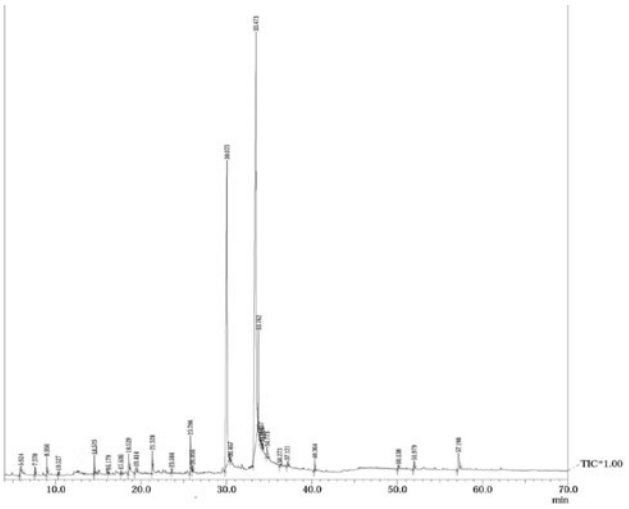

Figure 1. GC-MS chromatogram of methanolic extract of *Melissa officinalis.*

Micronucleus test

According to MN testing of mouse blood cells the low frequencies of micronucleated cells presumes the too little effects of methanolic extract of *Melissa officinalis* (Mo-ME) 100 and 400mg/kg (Table 3, Fig. 2), thereby indicating the virtual absence of mutagenic or cytotoxic effects. In other words, no statistically significant difference in the frequency of MN polychromatic erythrocytes (PCE) or the ratio of PCE to normochromatic erythrocytes (NCE), between the negative control and the groups that ingested extracts could be detected. When evaluating antimutagenicity inMo-ME, a significant decrease in the frequency of EMS-induced MNPCE was observed only in mice that had received 100, 200, 300 and 400 mg/kg ofMo-ME (p = 0.05 –Mann Whitney U test). In the present study, the methanolic extract of *M. officinalis* showed antimutagenic activities by reducing the % age of micronuclei with increase in the dose of the extract (Fig. 3).

Table 2. Phytocomponents identified in the methanolic extract of *Melissa officinalis* (Mo-ME) by GC-MS.

S. No.	Compound	RT	% Area	MF	MW
1	2,4-Cresotaldehyde	18.52	1.17	$C_8H_8O_2$	136
2	D-allose	19.41	0.56	$C_6H_{12}O_6$	180
3	Dodecanoic acid	21.32	0.58	$C_{12}H_{24}O_2$	200
4	Tetradecanoic acid	25.79	1.43	$C_{14}H_{28}O_2$	228
5	2,6,10-Trimethyl,14-ethylene-14-pentadecne	27.37	0.49	$C_{20}H_{38}$	278
6	Hexadecanoic acid, methyl ester	29.13	8.24	$C_{38}H_{68}O_8$	652
7	5- (hydroxymethyl)-2-furan carboxaldehyde	30.07	29.46	$C_6H_6O_3$	126
8	2, 3-dihydro-3, 5-dihydroxy-6-methyl-4H-pyran-4-one	33.47	51.62	$C_6H_8O_4$	144
9	Octadecanoic acid	33.76	3.26	$C_{18}H_{36}O_2$	284
10	Malonic acid, 3-hexyl tridecyl ester	34.05	0.38	$C_{22}H_{42}O_4$	370
11	9-Octadecenoic acid	34.22	0.12	$C_{18}H_{34}O_2$	282
12	9,12-Octadecadienoic acid	34.77	0.50	$C_{18}H_{32}O_2$	280
13	1,2-Benzenedicarboxylic acid	40.36	0.34	$C_{24}H_{38}O_4$	390
14	Tochopherol	51.97	0.41	$C_{29}H_{50}O_2$	430
15	Stigmast-5-en-3-ol	57.19	1.44	$C_{29}H_{50}O$	414

Table 3. Frequency profile of micronuclei induced alone by ethyl methanesulphonate and *Melissa officinalis* methanolic extract and their simultaneous exposure for different doses to evaluate antimutagenicity in *Mus musculus*.

	Treatment	Total No. of cells analysed per mice	No. of cells with micronuclei	% age of MN	% Reduction
Group 1	Negative Control (Distilled water)	1000	2.35 ± 0.12	-	-
Group 2	Positive control (EMS)	1000	7.23 ± 0.89	-	-
Group 3	Mo- ME 100 mg/kgbw	1000	2.28 ± 0.10	-	-
Group 4	Mo- ME 400 mg/kgbw	1000	2.27 ± 0.09	-	-
Group 5	Mo- ME 100 mg/kg + EMS	1000	6.52 ± 0.70	90.1	14.5
Group 6	Mo-ME 200 mg/kg + EMS	1000	5.86 ± 0.58	81.0	28.0*
Group 7	Mo-ME 300 mg/kg + EMS	1000	4.90 ± 0.50	67.7	47.7**
Group 8	Mo-ME 400 mg/kg + EMS	1000	3.25 ± 0.43	44.9	81.5***

NC: Negative control (distilled water), PC: Positive control [Ethyl methane sulfonate (EMS) 117.5 mg/kgbw; dose is 1/4th LD_{50}], Mo-ME: *Melissa officinalis* Methanolic Extract. Values with different asterisks ($^{*}p < 0.05$: significant, $^{**}p < 0.01$: highly significant, $^{***}p < 0.001$: extremely significant) differ significantly from the positive control (Mann-Whitney U test).

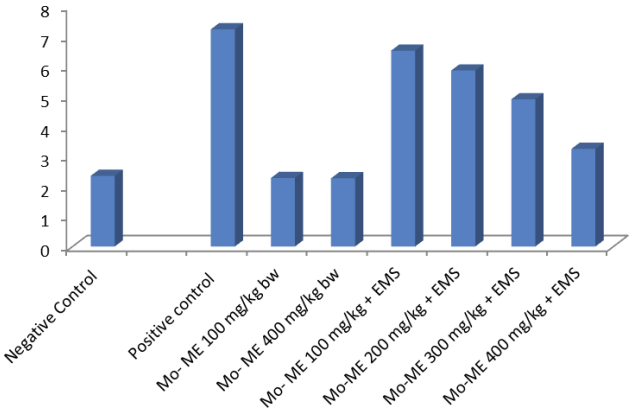

Figure 2. Graph showing number of micronucleated cells in different groups of mice treated with EMS alone, EMS + Mo-ME and Mo-ME alone in different concentrations.

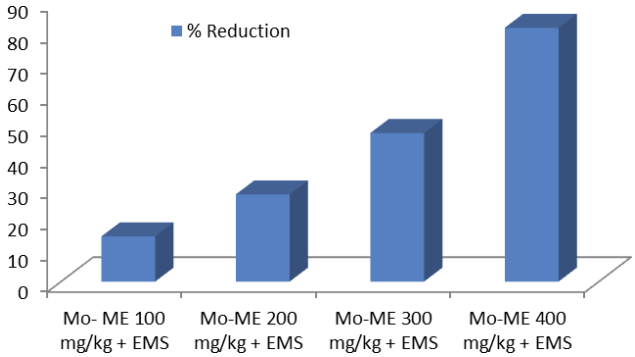

Figure 3. Bar diagram showing percentage reduction in EMS treated micronuclei with increase in concentration of Melissa officinalis methanolic extract (Mo-ME).

Chromosomal aberrations

The chromosomal aberrations induced by ethyl methanesulphonate (EMS 117.5 mg / kg body weight; positive control) were significant ($p<0.05$) to that of the control group. The frequency of breaks per cell in the EMS treated group at 24 h was 0.12 ± 0.010, which was significantly higher when compared to that of total number of breaks per cell in the control group (0.014 ± 0.002) ($p<0.05$). Our results also showed that in the Mo-ME and EMS combined treatment group, the frequency of chromosomal aberrations was significantly lower in comparison to those observed for the EMS only treated group at 24 h. The methanolic extract of *Melissa officinalis* reduced the chromosomal aberrations by 38.1%, 60.1%, 74.5% and 91.5% at 100, 200, 300 and 400 mg/kgbw (Table 4, Fig. 4). The chromosomal aberrations induced by EMS are mainly chromatid breaks, exchanges, gaps, fragments and rings (Fig. 5).

DISCUSSION

From ancient times, medicinal plants are being used as remedies for various diseases in human. In today's industrialized society, the use of medicinal plants has been traced to the extraction and development of several drugs as they were used traditionally in folk medicine (Shrikumar and Ravi, 2007). Medicinal plants have potent phytoconstituents which are important source of compounds and are responsible for the therapeutic properties (Jeeva *et al*., 2011; Florence *et al*., 2014; Sumathi*et al*., 2014, Ganaie *et al*, 2016; 2017). These phytoconstituents endow them with medicinal properties. Many plants possess antioxidant properties because of the presence of phenolic compounds (Brown and Rice-

Table 4. Frequency profile of chromosomal aberrations induced by ethyl methanesulphonate and *Melissa officinalis* methanolic extract separately and by their combination for different doses to evaluate the antimutagenicity in *Mus musculus*.

Treatments		Chromosomal Aberrations							
Concentration (mg/kgbw)	No. of cells	Rings	Fragments	Exchange	Breaks	Gaps	Total Aberrations	%age of Aberrations	% Reduction
Distilled water	500	1	3	-	7	-	11	2.2	-
EMS 117.5 mg/kgbw	500	4	18	14	62	31	129	25.8	-
Mo-ME Alone100 mg/kgbw	500	1	3	-	7	-	11	2.2	-
Mo-ME Alone400 mg/kgbw	500	1	3	-	6	-	10	2.0	-
Mo-ME 100 mg/kgbw + EMS	500	3	13	10	44	14	84	16.8	38.1*
A-ME 200 mg/kgbw + EMS	500	2	9	7	32	8	58	11.6	60.1*
Mo-ME 300 mg/kgbw + EMS	500	1	7	5	22	6	41	8.2	74.5**
Mo-ME 400 mg/kgbw + EMS	500	-	4	3	10	4	21	4.2	91.5***

NC: Negative control (distilled water), PC: Positive control [Ethyl methane sulfonate (EMS) 117.5 mg/kgbw; dose is $1/4^{th}$ LD_{50}], Mo-ME: *Melissa officinalis* Methanolic Extract. Values with different asterisks ($^{*}p < 0.05$: significant, $^{**}p < 0.01$: highly significant, $^{***}p < 0.001$: extremely significant) differ significantly from the positive control (Mann-Whitney U test).

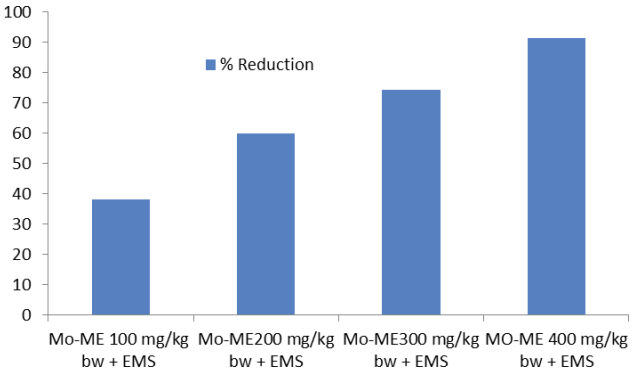

Figure 4. Bar diagram showing percentage reduction in chromosomal aberrations (CA) induced by EMS following post-treatment with methanolic extract of *Melissa officinalis* (Mo-ME).

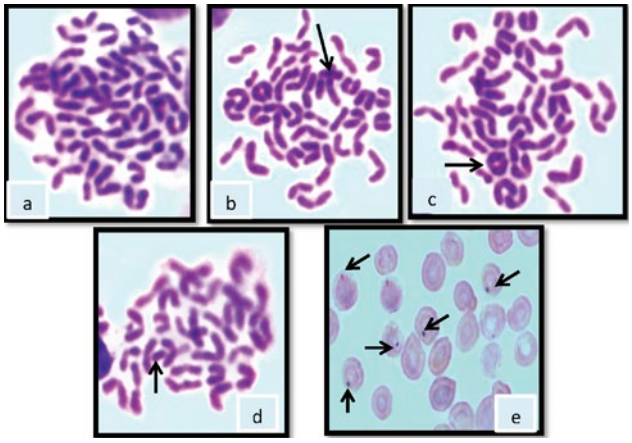

Figure 5. Photomicrograph showing metaphase chromosome preparation from bone marrow. a) Normal set of chromosomes, b) Exchanges (arrow), c) Ring (arrow), d) Fragment (arrow), e) Micronuclei (arrow).

Evans, 1998; Krings and Berger, 2001). These phenolic compounds possess biological properties such as anti-apoptosis, anti-aging, anti-carcinogen, anti-inflammation, anti-atherosclerosis, cardiovascular protection and improvement of endothelial function, as well as inhibition of angiogenesis and cell proliferation activities (Han *et al*., 2007). Tannins bind to proline rich protein and interfere with protein synthesis. Flavonoids are hydroxylated phenolic substances known to be synthesized by plants in response to microbial infection and they have been found to be antimicrobial substances against wide array of microorganisms *in vitro*. The activity is probably due to their ability to complex with extracellular and soluble proteins and to complex with bacterial cell wall (Marjorie, 1999). They are also effective antioxidant and show strong anticancer activities (Salah *et al*., 1995; Del-Rio *et al*., 1997).

Previous reports show that the essential oil of *M. officinalis* is composed of some important compounds like (E)-caryophyllene and caryophyllene oxide in addition to major constituents such as citronellal, neral and geranial (Sorensen, 2000; van de Berg *et al*., 1997). Literature reveals that the essential oil of *M. officinalis* subsp. *officinalis* contains significant amounts of citral and/or citronellal, whereas *M. officinalis* subsp. *altissima* contains only traces (van de Berg *et al*., 1997). Van de Berg *et al*. (1997) identified b-caryophyllene, germacrene-D, sabinene, and b-pinene as the main components in leaf oils of *M. officinalis* subsp. *altissima*.

Marnewick *et al*. (2000) found that the aqueous extracts of fermented and unfermented rooibos tea

(*Aspalathus linearis*) and honey-bush tea (*Cyclopia intermedia*) possess antimutagenic activity against 2-acetylaminofluorine and aflatoxin B_1. Vitamin C and E also significantly reduced the CA frequency in mouse bone marrow cells against rifampicin, an anti-tuberculosis drug, (Aly and Donia, 2002).

According to Kaur *et al*. (2010), the phytoconstituents from *Terminalia arjuna* suppressed the mutagenic effect of the aromatic amine, i.e., 2-aminofluorene (2-AF). The observed activity caused the inhibition of the metabolic activation of pro-mutagens. Hong *et al*. (2011) found that the extracts of *Acanthopanax divaricatus* were able to rapidly eliminate the mutagenic compounds from the cells before they induce the DNA damage. In a similar study, Nardemir *et al*. (2015) observed that the methanol extracts of the lichens have antimutagenic effects against sodium azide.In another study, Prakash *et al*. (2014) found that the different extracts of *Dioscorea pentaphylla* significantly inhibited the effects of methyl methanesulphonate (MMS) induced mutagenicity. They also found that the methanolic extract was highly mutagenic in comparison to Petroleum ether and chloroform. Entezari *et al*. (2014) compared the antimutagenic and anticancer activities of *Echinophora platyloba* DC on acute promyelocytic leukemia cancer cells and found that the methanolic extract of this plant prevented the reverted mutations and the hindrance was 93.4% in antimutagenic test. Akinboro *et al*. (2014) utilised the leaves of *Myristica fragrans* (Houtt.) for antimutagenic activity against benzo[a]pyrene and cyclophosphamide induced mutagenicity in *Salmonella typhimurium* and *Mus musculus* and found that the aqueous extract significantly suppressed more than 50 % of the mutations in all the tested concentrations. Sarac, (2015) utilised an edible wild plant, *Tragopogon longirostis* for the evaluation of antioxidant, mutagenic and antimutagenic properties and found that the ethanolic extract of its leaves exhibited antimutagenic properties at 2.5, 0.25, and 0.025 mg/plate concentrations. Habibi *et al*. (2014) found that the ethanolic extract of *Origanum vulgare* reduced the frequency of MN PCR from 10.52 ± 1.07 for CP to 2.17 ± 0.6 for the synergic test of CP and the ethanolic extract.

CONCLUSION

Based on the above results it can be concluded that the methanolic extractof *Melissa officinalis* possess some important phytoconstituents which possess antimutagenic activity.

The results of the present study clearly showed that the methanolic extract of *Melissa officinalis* had an antimutagenic and anticlastogenic potential against the mutagenic activity of ethyl methane sulphonate in mice. Our results suggest that there may be several ways through which *M. officinalis* extract can work against EMS. Selection of *M. officinalis* for the present study on the basis of its folklore usage seems to be justified as it is scientifically proved to have much potentiality. The extracts from such plants could be seen as a good source of useful drugs.However, further studies are needed in other test systems so that in the future *M. officinalis* can be used in reducing the occurrence of cancers or even as a coadjuvant to chemotherapy to reduce its side effects.

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