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# Evaluation of genoprotection against malathion induced toxicity by *Orthosiphon thymiflorus* Sleesen

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ABSTRACT

*Background:* Pesticide toxicity is considered to be one of the significant reason for increased incidence of cancer. Plants are treasure troves of active phytochemical compounds which are used as herbal medicine as well as nutraceuticals.
*Objective:* To evaluate the genoprotective potential of *Orthosiphon thymiflorus* (Roth) Sleesen, (Lamiaceae) against the toxicity induced by malathion by a battery of four *in vivo* assays in Swiss albino mice.
*Materials and methods:* Micronucleus assay was performed for analyzing the micronuclei induction and ratio of polychromatic and normochromatic erythrocytes (PCE/NCE). Anticlastogenic and mito depressive effect of the methanol and hexane extracts of *O.thymiflorus* were evaluated by chromosome aberration assay. Alkali comet assay was performed to assess double strand DNA repair. DNA damage sensing ability of the bone marrow cells were assessed by γ-H2AX foci formation. Phytochemical screening of hexane and methanol extract was done by GC–MS analysis.
*Result: O. thymiflorus* extracts showed a dose dependant protective effect in all assays. It significantly decreased the frequency of micronuclei and improved PCE/NCE value in post treated groups of animals. Malathion induced clastogenic aberrations were effectively attenuated by methanol and hexane extracts. DNA comet assay showed that malathion induced damage can be protected by *O. thymiflorus* extracts. Multiple foci formation in γ-H2AX assay confirmed the activation of DNA repair proteins in post treated animals.
*Conclusion:* Genoprotective effect of *O. thymiflorus* against malathion induced toxicity was confirmed. This study would be helpful to initiate more research including clinical using *O. thymiflorus* extract against pesticide induced toxicity.


## 1. Introduction

Malathion, an organophosphate pesticide, is a probable carcinogen in human. It induces genotoxicity and oxidative stress [1] resulting in uncontrolled growth. It is reported to be mutagenic on bacterial strains, insects and human cell lines and carcinogenic in mice [2–6]. There are numerous reports on the anticancer, antitumor, antioxidant activities of crude extract and essential oil of aromatic herbs in the Lamiaceae. *Orthosiphon thymiflorus* (Roth) Sleesen, is an aromatic herb in the family, commonly seen in Western and Eastern Ghats of India. The plant is referred as 'Pratanika' in Sanskrit and is used in Ayurveda and folk preparations for various ailments like erysipelas, dermatopathy etc [7]. Bioactive compounds were reported from *O.thymiflorus* [8] and potential antioxidants including sinensetin, eupatorin, 3′-hydroxy-5, 6, 7, 4′-tetramethoxyflavone, rosmarnic acid were isolated from the genus [9]. In the present study the genoprotective efficiency of *O.thymiflorus* extracts are analyzed against the toxicity induced by malathion.

## 2. Materials and methods

### 2.1. Experimental design of in vivo studies in mice

Male Swiss albino mice of 6–8 weeks weighing 20–25 mg were purchased from Small Animal Breeding Station, College of

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Veterinary and Animal Sciences, Mannuthy, Thrissur. They were kept in standard conditions in registered animal house, Department of Life Science, University of Calicut. Five animals were housed in each polypropylene cage and fed with standard diet (Manufactured by Kamadhenu Agencies, Bangalore, India) and sterilized water *ad libitum.* Animals were allowed to acclimatize before the commencement of experiments. For experimentation animals were caged according to the approved experimental design (426/2/CPCSEA dated19/09/2014) of three mice per cage.

Assays consisted of nine groups of three animals each:- negative control (DMSO, 2%), positive control group Ethyl methanesulfonate (EMS-a DNA alkylating agent, ½ LD 50), malathion (LD 50) treated group, three groups of animals post treated with methanol and hexane extracts with different concentrations. Five concentrations (25, 50, 75, 100 and 150 mg/kg b. w) of the extracts were tested for its cytotoxicity *in vivo* (MN assay, CA assay & Comet assay) with five animals in a group (result is not included in this paper). Based on that result, in antimutagenic studies, three concentrations (25, 75 and 150 mg/kg b. w) were used which was expected to show the differences.

The animals were administrated with 0.5 mL of LD 50 concentration of malathion once as intraperitoneal (*i.p*) 2h before the exposure with different concentrations of methanol/hexane extract of *O.thymiflorus* to evaluate the genoprotective effect.

Four *in vivo* assays were done in mice namely; micronuleus (MN) assay chromosome aberration (CA) assay, comet assay and immunofluorescence (γ- H2AX) assay. Bone marrow cells from femur bones (MN assay, CA assay and Comet assay) and blood samples (only for γ-H2AX Assay) from peripheral veins were used for the experiment.

### 2.2. Micronucleus (MN) assay

For MN assay, the procedure described by Schmid [10] was adapted in this study. After 30h of exposure animals were sacrificed by cervical dislocation. Bone marrow cells were flushed into human AB serum and fixed in methanol for 3h. Smear of the cells were stained by Geimsa – May Grunwald staining method [11] and observed under Leica DM 500 microscope with an attached image analyzer. Thousand well identifiable polychromatic erythrocytes and corresponding monochromatic erythrocytes per animal were scored for micronuclei formation. The percentage of normochromatic erythrocytes (NCE), polychromatic erythrocytes (PCE) and micronucleated PCE (MN-PCE) were calculated and expressed as mean ± standard error.

### 2.3. Chromosome aberration (CA) assay

Animals were treated with 4 mg/kg b. w of colchicines (*i.p*), a cytostatic chemical, 2 h before sacrificing the animals. After 24 h of exposure to the extract, animals were sacrificed and bone marrow cells were flushed out into 3 ml of prewarmed 0.56% potassium chloride solution. The method described by Preston et al. [12] was followed in the study. The suspension was dropped forcefully on to a clean pre chilled slide and stained using 4% Giemsa for 10 min [13]. 100 well spread metaphase plates were examined for chromosomal aberrations and the values were expressed as mean ± standard error. Mitotic index among 100 cells were calculated per animal by using the standard formula [14].

### 2.4. Measurement of DNA damage by comet assay

Alkaline comet assay was performed according to the method of Singh et al. [15] with minor modifications as described in detail by Sasaki et al. [16] and Speit and Hartmann [17].

Bone marrow cells were collected after exposing to the extract for 24h and mononuclear cells (MNC) were isolated by density gradient centrifugation. 20 μl of bone marrow cells were embedded in 40 μl of 0.5% low EEO agarose at 37 °C, and mixed well and dropped on to the precoated slides and immediately covered with a long cover slip. These slides were kept on ice bags, later the cover slips were removed and the slides were kept in lysis buffer at 4 °C for 1 h and subsequently submerged in a horizontal electrophoresis apparatus containing fresh electrophoresis buffer. Slides were kept in the buffer for 20 min for equilibration and then electrophoresis was carried out at 25V and 300 mA for 30 min. Then the slides were neutralized by keeping in neutralization buffer. Later the slides were stained with Propidium Iodide. About 20 μLl of the stain was dropped on to the cell and kept for 10 min. Three slides per animal were prepared and 100 comets per slides were scored. Later the slides were observed under Olympus IX 72 fluorescent microscope with attached image analyzing system. Pictures of different classes of comets (class 0, 1, 2 and 3) from different treatment groups were saved and later it was scored for different features of comet like total intensity/DNA content, length moment and percentage of DNA in comet tail by CASP1.2.3 beta 2 (CASP lab- Comet assay software Project) comet assay analyzing software. In homogenous population of comets visual scoring was done and no cycling. Classification of comet was done as previously described [18]. Experiments were done in triplicate and mean values were calculated.

### 2.5. Immunochemistry/immunofluorescence (γ-H2AX assay)

Immunofluorescent staining and counting of γ-H2AX nuclear foci was performed as previously described [19]. The mononucleated cells (MNCs) collected from blood by gravity gradient centrifugation were checked for their viability and about 10 μl was pippetted out into an eppendorff tube and fixed with 4% paraformaldehyde in PBS for 20 min at room temperature. Then the cells were rinsed with PBS. The cells can be sterilized in 0.2% sodium azide in PBS at 4 °C and can be kept for several hours. Permiabilization of the cells were done by incubating the cells in 0.2% Triton x 100 in PBS for 5 min at room temperature. Then rinse the cells in PBS. The cells were incubated in the first antibody (γ-H2AX) at 4 °C overnight. Later the cells were washed with PBS and incubated the cells in second antibody (rabbit/goat anti histone H2AX) at room temperature for 1h. The cells were stained by incubating in DAPI (1 mg/ml) for 10 min. Cells were mounted on a clean slide and covered with cover slip and visualized under Olympus IX 72 fluorescent microscope with attached image analyzing system and imported into IP Labs image analysis software package (Scanalytics, Inc.). At least 50 cells were screened for analyzing γ-H2AX foci in each group and cells were classified as positive and negative based on the presence and absence of foci in the nucleus.

### 2.6. Gas chromatography and mass spectrometry (GC–MS)

Soxhlet extracted methanol and hexane extract of O.thymiflorus after evaporating the solvent completely was subjected to gas chromatography with a mass spectrometry to determinate the volatile components of the extracts. Varian model CP-3800 GC interfaced with a Varian Saturn 2200 Iron Trap Mass Spectrometer (ITMS) at 70eV and 250 °C was employed. It was equipped with a CP-1177 Split/splitless capacity injector and a combo auto sampler. Helium was the carrier gas (1 ml/min). 1 μl volume of sample at 1:20 split ratio and temperature 60 °C for 1 min initially and then heated up to a rate of 3 °C/min to 280 °C for 60 min run time.Peak area was calculated by integrated programme for quantification and NIST MS library search identified compounds in the volatile fraction of the extracts.

### 2.7. Statistical analyses

For statistical analysis mean values and standard deviation of each concentration with respect to parameters is calculated. Significance ($P < 0.05$) was analyzed by One-way ANOVA, Tukey's test and Duncan's post hoc test using SPSS 17. (SPSS Inc.,Chicago, Illinois, USA).

## 3. Results

The genoprotective potential of methanol and hexane extract of *O. thymiflorus* were tested on the bone marrow cells of Swiss albino mice by inducing micronuclei in erythrocytes. The induction of micronuclei in polychromatic and normochromatic erythrocytes were counted and tabulated in each treatment group. The result showed a significantly high count of MN in EMS (13.67 ± 0.9) and malathion (10.0 ± 0.57) treated group explicating their toxicity (Fig. 1). Single and double micronuclei in PCE and NCE cells, different types of abnormal morphology like budding of NCE, shrinking of NCE membrane etc were also noticed in positive control and malathion treated groups.

The data showed that all concentrations of both extracts decreased the frequency of MN induced by the insecticide. The effect was significant compared to malathion treated group at 5% probability. Hexane high dose (150 mg/kbw) in the study showed no significant difference from negative control clearly indicating the genoprotective potential of the extract against the mutation induced by malathion.

Similarly it was observed that both extract have significant effect on PCE/NCE (Fig. 2). The toxicity induced by EMS and malathion was not significantly different giving an explicit knowledge about its genotoxic effect in mammalian system. Both extracts (all concentrations) significantly increased the PCE/NCE in a dose dependant in a way revealing its genoprotective potential. The results revealed the effectiveness of all the tested concentration of both hexane and methanol extracts in maintaining the hematopoietic equilibrium of PCE/NCE and efficacy of the extract in genoprotection.

Result of CA assay is expressed as per cent aberrant cell, the types of aberrant cell and Mitotic index (MI). Animals showed a dose dependant effect on the tested parameters in the post treated groups in both extracts in the present study. There was a linear increase in the aberrant cells in both extract groups with high

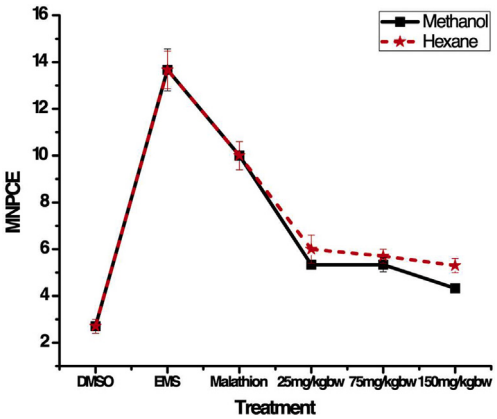


**Fig. 1.** Effect of methanol and hexane extract on MN induction in PCE. Negative control: Dimethylsulfoxide (DMSO) 0.1 mL/10 g body weight (b.w.), Positive control: Ethyl Methane Sulphonate (80% ½ LD50), Malathion (LD 50). M–malathion. All values are mean ± SE of three experimental data. Values were compared with malathion treated group and negative control. Significance: ($p > 0.05$).

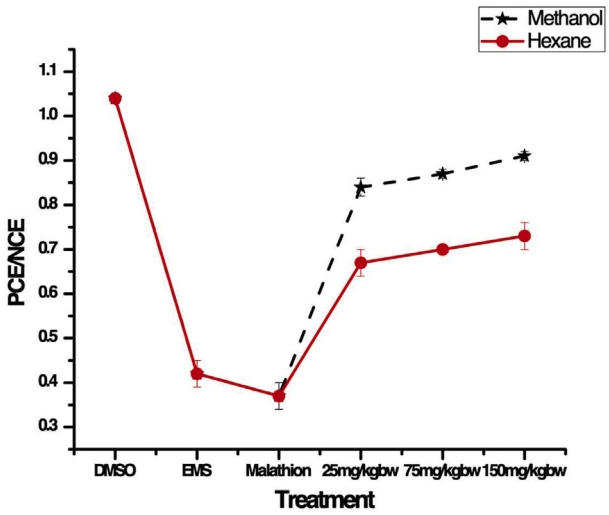


**Fig. 2.** Effect of methanol and hexane extract on PCE/NCE. Negative control: Dimethylsulfoxide (DMSO) 0.1 mL/10 g body weight (b.w.), Positive control: Ethyl Methane Sulphonate (80% ½ LD50), Malathion (LD 50). M–malathion. All values are mean ± SE of three experimental data. Values were compared with malathion treated group and negative control. Significance: ($p > 0.05$).

chromosome aberration in the highest dose of hexane extract (150mg/k.b.w). Methanol extract groups (all three concentrations) showed a better effect on alleviating the aberration induced by malathion (Table 1).

Positive control, insecticide and extract treatment resulted in a multitude of clastogenic and non clastogenic aberrations in bone marrow cells (Table 1, Fig. 3). Malathion and EMS caused high frequency of aberrant cells while methanol extract showed a dose dependant decrease in the frequency. On the other hand, there observed a dose dependant increase in CA in hexane extract but its toxicity was not statistically significant. Chromosome fragments, rings, centromeric fusion and attraction, translocation rings, ring chromosomes, deletion, pulverization, chromosome exchange etc were the frequently observed aberrations in all treatment groups.

Figure 4 shows the linear effect of methanol and hexane extract concentrations on MI. Hexane extract was observed to depress the mitotic activity in a significant way. MI values of all tested concentrations of both extracts differ significantly ($p < 0.05$). Results of CA assay manifested a potent genoprotective activity of the *O. thymiflorus* extracts on the mammalian cells.

Genoprotective of three different concentrations of methanol and hexane extract (25, 75 and 150 mg/kg.b.wt) of *O. thymiflorus* was analyzed by alkali comet assay. Single stranded DNA damage caused by malathion and post treatment group of animals in terms of comet head volume and tail length percentage gives a clear affirmation of the DNA damage which is in par with the earlier results about the genoprotective potential of the plant extracts (Table 2). All the tested concentrations of both methanol and hexane extracts of *O. thymiflorus* showed a dose dependant effect in the percentage of tail length indicating effective repair of DNA damage. Even though all tested concentrations of both hexane and methanol extracts showed significant effect in dwindling the toxic effect of malathion, methanol extract was comparatively more effective in DNA repair.

DNA damage sensing ability of the cells increases in response to *O. thymiflorus* extracts. Alkaline comet assay can detect cells with DNA single-strand and double-strand breaks induced by the chemical/drug molecules. γ-H2AX antibody staining is comparatively a sensitive technique to detect the DNA damage and repair by

**Table 1**
Effect of methanol and hexane extract of *O. thymiflorus* on chromosomal aberrations induced by post treatment with malathion in the bone marrow cells of mice.

| | DMSO | EMS | Malathion | M+Methanol | | | M+Hexane | | |
|---|---|---|---|---|---|---|---|---|---|
| Dose | 2% | LD50 | ½ LD50 | 25 | 75 | 150 | 25 | 75 | 150 |
| CA % | 6.93 ± 0.7 | 32.4 ± 4.7 | 38.76 ± 0.8 | 16.19 ± 0.68 | 14.77 ± 0.8 | 12.6 ± 1.1 | 17.47 ± 1.84 | 19.05 ± 0.85 | 21.85 ± 0.74 |
| Ring | – | 2.33 ± 0.6 | 2.33 ± 0.6 | 0.67 ± 0.6 | 1 ± 0 | 1 ± 1 | 0.67 ± 0.6 | 1 ± 0.6 | 1.33 ± 0.6 |
| Polyploidy | – | 2 ± 0 | 2.33 ± 0.6 | 0.33 ± 0.6 | 1.33 ± 0.6 | 0.33 ± 0.67 | 0.67 ± 0.6 | 0.67 ± 0.6 | 1 ± 0 |
| Aneuploidy | – | 1.33 ± 0.6 | 1 ± 0 | 0.67 ± 0.6 | 0.67 ± 0.6 | 1 ± 0 | 0.67 ± 0.6 | 0.67 ± 0.6 | 1 ± 0 |
| Haploidy | – | 1.33 ± 0.6 | 1.33 ± 0.6 | 0.67 ± 0.6 | 0.67 ± 0.6 | 0.67 ± 0.67 | 1 ± 0 | 1 ± 0 | 1 ± 0 |
| Centromere attraction | 0.33 ± 0.6 | 3.33 ± 0.6 | 4 ± 0 | 1.33 ± 0.6 | 1.67 ± 0.6 | 1.67 ± 0.67 | 1 ± 0 | 2 ± 0 | 2 ± 0 |
| Deletion | – | 1.67 ± 0.6 | 1.33 ± 0.6 | 0.7 ± 0.6 | 1 ± 0 | 1 ± 0 | 0.67 ± 1.2 | 1 ± 0 | 1 ± 0 |
| Fragments | – | 2 ± 0 | 2 ± 0 | 1 ± 0 | 0.67 ± 0.6 | 1 ± 0.6 | 0.33 ± 0.6 | 1 ± 0 | 1 ± 0 |
| Acentric chromosome | – | 1 ± 0 | 1.33 ± 0.6 | 0.33 ± 0.6 | 0.67 ± 0.6 | 0.33 ± 0.67 | 1 ± 0 | 1 ± 0 | 1.33 ± 0.6 |
| Chrormosome Break | – | 1 ± 0 | 1.33 ± 0.6 | 0.33 ± 0.6 | 1 ± 0 | 1 ± 0 | 1 ± 0 | 1.33 ± 0.6 | 1.33 ± 0.6 |
| Pulverisation | – | 1 ± 1 | 1 ± 0 | 0.67 ± 0.6 | 1.33 ± 0.6 | 0.33 ± 0.67 | 1 ± 1 | 0.67 ± 0.6 | 1 ± 0 |
| Translocational ring | – | 1.67 ± 0.6 | 1.67 ± 0.6 | 0.67 ± 0.6 | 0 ± 0 | 1 ± 0 | 0.33 ± 0.6 | 0.67 ± 0.6 | 1 ± 0 |
| Centromeric fusion | – | 1.67 ± 0.6 | 2.67 ± 0.6 | 0.67 ± 0.6 | 1.33 ± 0.6 | 1.33 ± 0.67 | 0.67 ± 0.6 | 1.33 ± 0.6 | 1.33 ± 0.6 |
| Gap | – | 1 ± 0 | 2 ± 0 | 0.67 ± 0.6 | 0.67 ± 0.6 | 1 ± 0 | 1 ± 1 | 1 ± 0 | 1 ± 0 |
| Chromosome exchange | – | 1 ± 0 | 1.33 ± 0.6 | 0.33 ± 0.6 | 0.67 ± 0.6 | 0 | 0.33 ± 0.6 | 0.67 ± 0.6 | 1 ± 0 |
| others | 0.33 ± 0.6 | 2.67 ± 2.1 | 2.33 ± 0.6 | 0 ± 0 | 1 ± 0 | 0 ± 0 | 0.33 ± 0.6 | 0 ± 0 | 0 ± 0 |

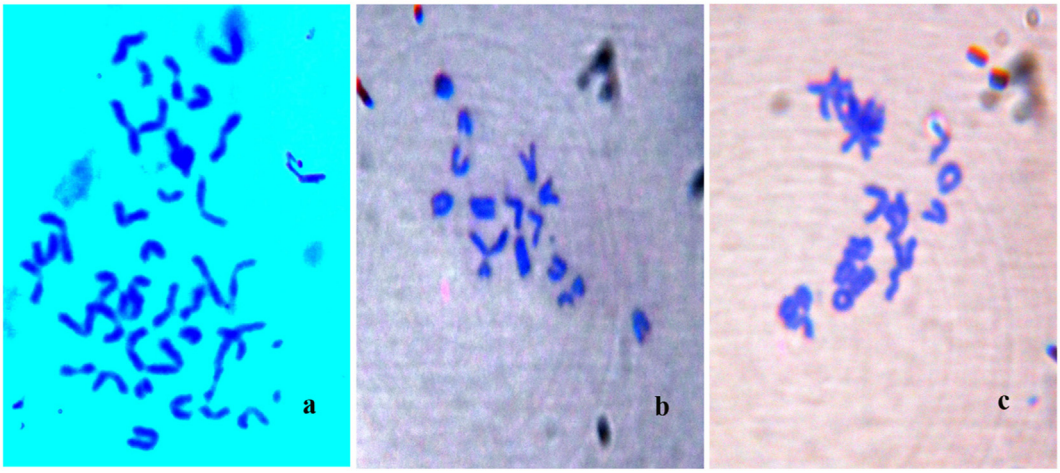


**Fig. 3.** Chromosome aberration induced by EMS and malathion in bone marrow cells of Swiss albino mice. a. chromsome fragments b. Cenromere fusion c. Translocation ring.

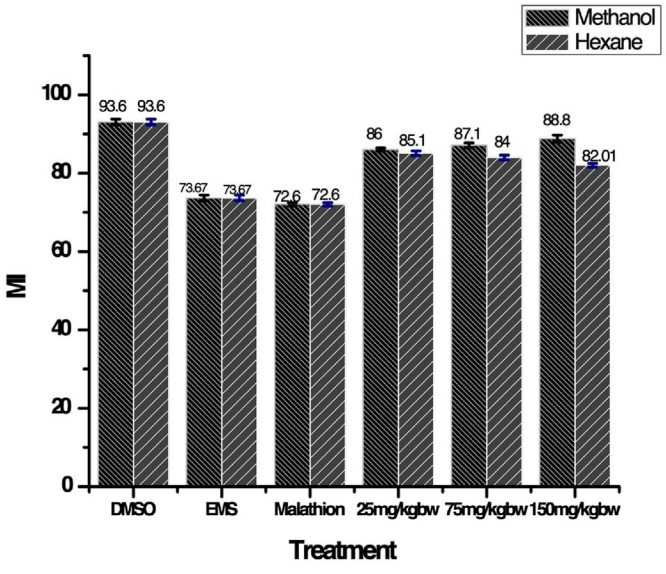


**Fig. 4.** Comparison of Mitotic depression induced by methanol & hexane extract of *O. thymiflorus* in bone marrow cells of Swiss albino mice.

chemicals and drugs. The result from antibody binding techniques showed the magnitude of toxicity caused by malathion and EMS by analyzing the accumulation of γ-H2AX ($P^{S139}$– H2AX) (Fig. 5). It was observed that there was no significant foci formation in the nuclei by both positive control and insecticide treatment (Fig. 5.a-red arrow) which clearly stating the absence of any repair proteins in the nuclei. Post treatment with extract groups showed the presence of multiple foci formation (Fig. 5 c,d-yellow arrow) in the nuclei unfolding the genoprotective efficiency of both methanol and hexane extract of *O. thymiflorus* unambiguously. The result unravel the genotoxicity of malathion by inducing double strand breaks in DNA and it will not be repaired and stay as a permanent aberration or lesion in the nuclei.

The chromatogram of methanol and hexane extract of *O.thymiflorus* by GC–MS analysis showed numerous prominent peaks. From the gas chromatogram of methanol extract (Fig. 6), about 100 peaks were detected among which 68 compounds matched (˂95) with the database in NIST library search. The tentatively identified compounds (Table 3) include different diterpenoids, sesquiterpenoids, alkaloids, phenolics, flavanoids, phytosterols, quinolone etc. Presilphiperfolan-1-ol (3.288%), Nalidixicacid (2.62%), 10s, 11s-Himachala-3(12),4 diene (9.13%), 2,2′,6,6,6′,6′,9,9′-Octamethyl-8,8′ bi(tricyclo[5.4.0.02,9]undecane (1.776%), 4-Phenylisoquinoline (1.678%), Eudesma-3,7(11)-diene (1.617%), Estra-1,3,5(10)-triene-

**Table 2**
Genoprotective effect of methanol and hexane extract of *O. thymiflorus* against ssDNA and dsDNA damages induced by malathion in bone marrow cells of *Swiss albino* mice detected with alkaline Comet assay after 24 h of exposure.

| Treatment | Methanol | | Hexane | |
|---|---|---|---|---|
| | Head DNA (%) | Tail DNA (%) | Head DNA (%) | Tail DNA (%) |
| DMSO | 99.66 ± 0.2$^{b}$ | 0.34 ± 0.2$^{a}$ | 99.66 ± 0.2$^{c}$ | 0.34 ± 0.2$^{a}$ |
| EMS | 57.30 ± 7.6$^{a}$ | 42.7 ± 7.6$^{b}$ | 57.3 ± 7.6$^{a}$ | 42.7 ± 7.6$^{c}$ |
| Malathion | 57.97 ± 5.6$^{a}$ | 42.02 ± 5.6$^{b}$ | 57.97 ± 5.6$^{a}$ | 42.02 ± 5.6$^{c}$ |
| 25mg/kgbw+Malathion | 89.22 ± 1.1$^{b}$ | 10.77 ± 1.1$^{a}$ | 84.17 ± 0.8$^{b,c}$ | 15.83 ± 0.8$^{b}$ |
| 75mg/kgbw+Malathion | 87.29 ± 0.5$^{b}$ | 12.7 ± 0.5$^{a}$ | 74.89 ± 3.4$^{a,b}$ | 25.11 ± 3.4$^{b}$ |
| 150mg/kgbw+Malathion | 86.09 ± 2.3$^{b}$ | 13.9 ± 2.3$^{a}$ | 76.42 ± 1.8$^{b}$ | 23.58 ± 1.8$^{b}$ |

Negative control: Dimethylsulfoxide (DMSO) 0.1 mL/10 g body weight (b.w.), Positive control: Ethyl Methane Sulphonate (80% ½ LD50), Malathion (LD 50). All values are mean ± SE of three mice. Statistical analyses: one-way ANOVA and the Tukey's test. 5 Statistical analyses: Duncan's post hoc test. Mean values followed by the same letter in the column do not present significant difference at 5% probability. Letter-a, b, c, d in common in the same column do not present significant difference ($p > 0.05$). Different letters in the same column present significant difference ($p < 0.05$).

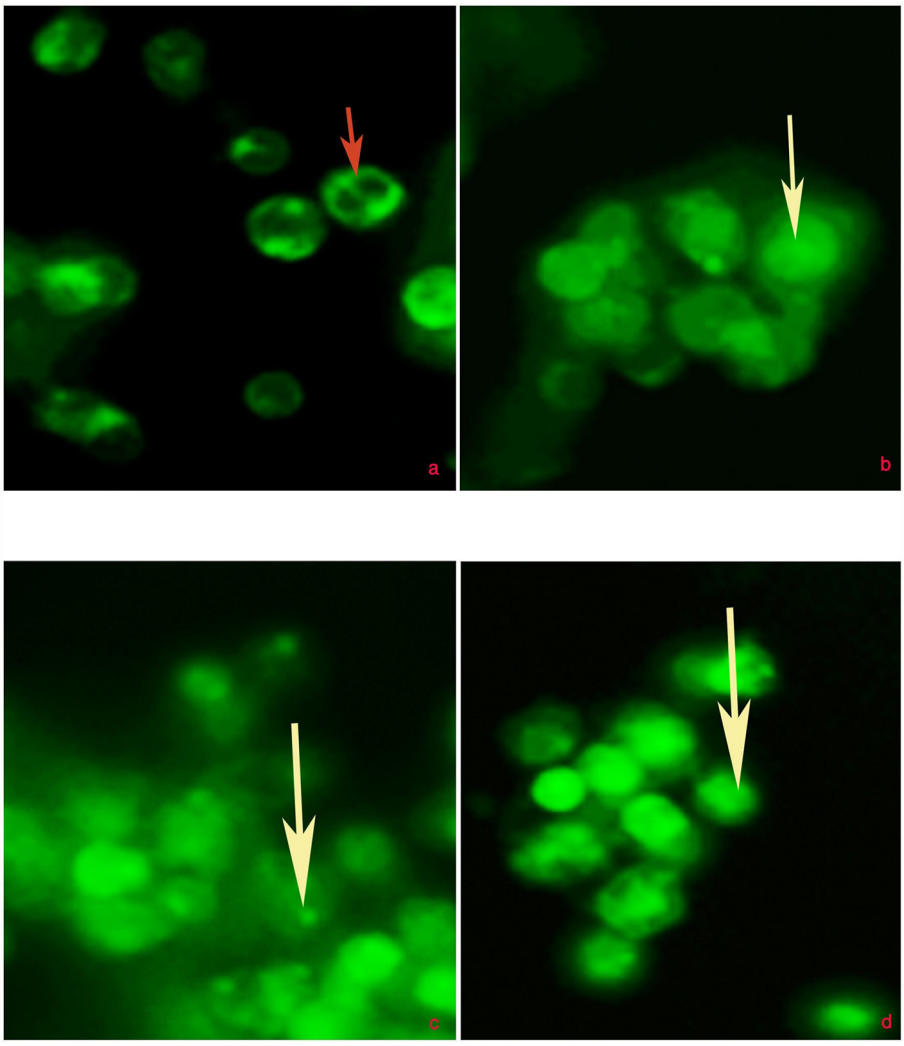


**Fig. 5.** a–d: Foci formation showing the presence of repair protein complex in the bone marrow cells of Swiss albino mice at the DNA double strand breaks induced by malathion (a) Malathion (b) *O.thymiflorus* methanol extract (150mg/kg. b.wt) (c) Effect of treatment with *O.thymiflorus* methanol extract (150mg/kg. b.wt) after treatment with malathion (d) Effect of treatment with *O.thymiflorus* hexane extract (150mg/kg. b.wt) after treatment with malathion; red arrow shows absence of foci formation, yellow arrow shows foci formation.

4,17-diol (1.243%), Vincadifformine (1.201%) etc. were the major compounds identified by the library search. Other biologically active compounds like Cis-phytol, Podocarpa-6,8,11,13-tetraen-12-ol etc. were also detected from methanol extract but they were present below 1%. The gas chromatogram of hexane extract of O.thymiflorus (Fig. 7) showed about 68 peaks among which 68 was identified with NIST library search. The extract was rich in a large number of bioactive compounds (Table 4) which includes alkaloids, phenolics, terpenoids, terpenes, flavanois etc.The major compound detected was Morphinan-14-ol (13.08%). Other compounds include Chromone

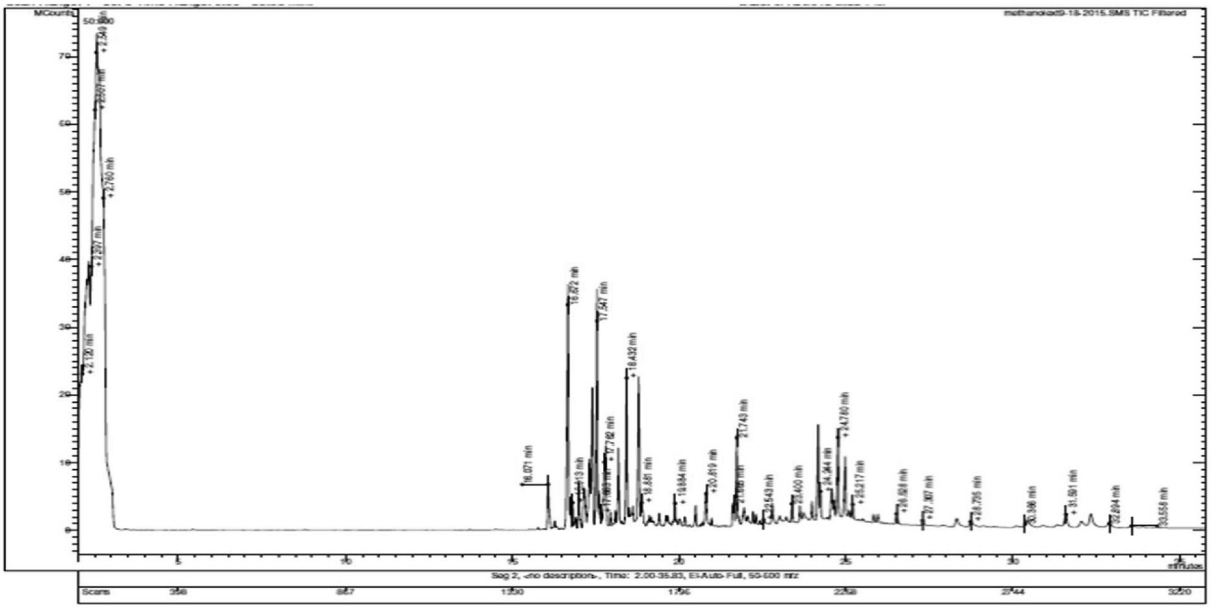

**Fig. 6.** Gas chromatogram of methanol extract of *O.thymiflorus* of aerial parts.

**Table 3**
Tentatively identified secondary metabolites in methanol extract of *O. thymiflorus* and its biological activity[a] by GC/MS analysis.

| Sl. No | Molecular formula | compound | RT | Area % | Class of compund | Bioactivity |
|---|---|---|---|---|---|---|
| 1 | $C_{15}H_{26}O$ | Presilphiperfolan-1-ol | 16.672 | 3.288 | Triquinane sesquiterpene | antimicrobial |
| 2 | $C_{12}H_{12}N_2O_3$ | Nalidixic acid | 17.547 | 2.62 | Quinolone | Antimicrobial |
| 3 | $C_{15}H_{24}$ | 10s,11s-Himachala-3(12),4-diene | 17.310 | 1.913 | sesquiterpenoid | anticancer |
| 4 | $C_{30}H_{50}$ | 2,2′,6,6,6′,6′,9,9′-Octamethyl-8,8′-bi(tricyclo[5.4.0.02,9] undecane) | 18.803 | 1.776 | | No activity reported |
| 5 | $C_{15}H_{11}N$ | 4-Phenylisoquinoline | 18.432 | 1.678 | alkaliod | Anticancer, Antitumor Antiproliferative |
| 6 | $C_{15}H_{24}$ | Eudesma-3,7(11)-diene | 17.806 | 1.617 | sesquiterpenoid | Anticancer, antitumor |
| 7 | $C_{19}H_{26}O_3$ | Estra-1,3,5(10)-triene-4,17-diol | 24.184 | 1.243 | Phytosterol | Antitumor, anticancer |
| 8 | $C_{21}H_{26}N_2O_2$ | Vincadifformine | 21.743 | 1.201 | alkaloid | anticancer |
| 9 | $C_3H_3OP$ | 1,2-Oxaphosphole | 24.244 | 0.715 | ketones | Cytotoxic, genotoxic |
| 10 | $C_{22}H_{30}O_2$ | Podocarpa-6,8,11,13-tetraen-12-ol | 24.663 | 0.601 | diterpenoid quinone | Anticancer, antitumor |
| 11 | $C_{20}H_{40}O$ | Cis phytol | 23.998 | 0.42 | diterpene alcohol | Anticancer, antitumor |
| 12 | $C_{15}H_{24}$ | a-Guaiene | 16.744 | 0.539 | Sesquiterpene lactones | Anticancer, antitumor, |
| 13 | C23H34O5 | Gitoxigenin | 18.194 | 0.929 | alkaloid | Anticancer |
| 14 | C20H40O | Phytol | 20.315 | 0.40 | diterpene alcohol | Antibacterial, Anticancer, antitumor |
| 15 | C37H76O | 1-Heptatriacontanol | 19.414 | 0.234 | phenolic | Anticancer, Antimicrobial Antiinflammatory, Diuretic |
| 16 | C20H23NO4 | Lauroscholtzine | 23.634 | 0.372 | | Anticancer |
| 17 | C30H48O | Urs-12-en-3-one | 32.339 | 0.57 | triterpenoid | Antitumor |

[a] Reference of only selected biological activity is sited in discussion to avoid excessive reference session.

(3.256%), Pregnane-7,8,9,11,20-pentol-18-oic acid (2.598%), α-Tocopherol (1.853%), 1,3,5-Tris(trimethylsiloxy)benzene(1.649%), 1,3-Dihydro-2-benzothiophene (1.64%), Cholesta-22,24-dien-5-ol 2.04%), α-Amyrin (1.44%) and Vincadifformine (1.33%). Other bioactive compounds like γ-Sitosterol, 8-Phenylisoquinoline, 4, 5, 7-Trimethoxy-3-(4 methoxyphenyl)-2H-1-benzopyran-2-one etc. were also detected but present below 2% in total area.Few unidentified prominent peaks incites the need of further purification and identification of the compounds in both extracts.

## 4. Discussion

Pesticide and insecticide toxicity is a severe problem nowadays because of the irrational usage of these chemicals in agronomy. It is reported that the high incidence of cancer and related diseases are caused by the consumption of fruits and vegetables containing pesticide and insecticide residues [20,21]. Malathion is a potent inhibitor of cholinesterase which interferes with the nerve impulse transmission manifested by headache, dizziness, nausea, vomiting, bradycardia etc. It causes cytotoxicity and genotoxicity by inducing oxidative stress on lymphocytes [22]. In literature there are innumerable reports on the protective effect of plant derived medicines and food which can effectively subdue the toxic effect of chemical mutagens in mammalian system. *O. thymiflorus*, member of a highly medicinal and aromatic herb family Lamiaceace is found to be a good candidate to analyze its genoprotective potential because of the reports on the phytochemicals of pharmacognostic interest from its related species *Orthosiphon stamineus* [23–25]. It was reported to be less cytotoxic because of the presence of bioactive flavonoids and polyphenolis. Even though cytotoxicity of

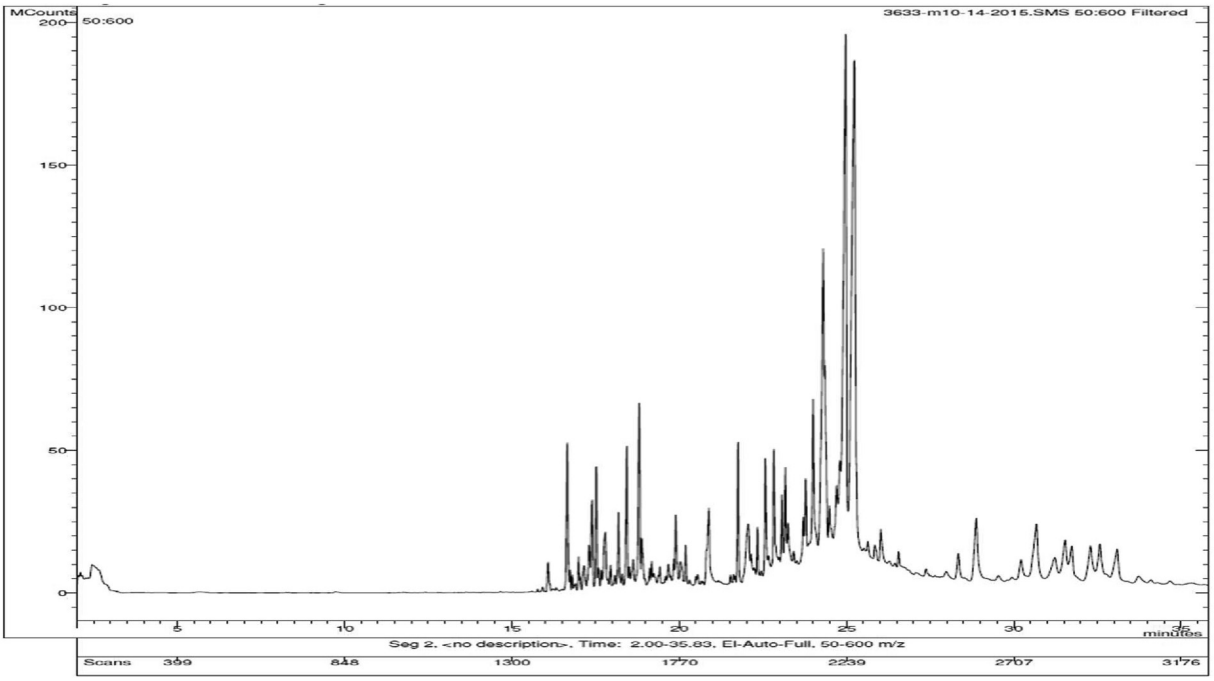


**Fig. 7.** Gas chromatogram of hexane extract of *O.thymiflorus* of aerial parts.

**Table 4**
Tentatively identified secondary metabolites in hexane extract of *O. thymiflorus* and its biological activity by GC/MS analysis.

| Sl. no | Molecular formula | compound | RT | Area % | Class of compund | Bioactivity |
|---|---|---|---|---|---|---|
| 1 | $C_{29}H_{50}O_2$ | αTocopherol (Vitamie E) | 28.876 | 1.853 | Vitamine | Antioxidant, anticancer |
| 2 | $C_{18}H_{23}NO_3$ | Morphinan-14-ol | 25.224 | 13.08 | Alkaliod | antitussive effect, antitumor, anticancer |
| 3 | $C_9H_8O_2$ | Chromone | 23.998 | 3.256 | Pyridine derivative | antitumor |
| 4 | C28H48O | Cholestan-3-ol, 2-methylene-, (3á,5à) | 30.678 | 2.04 | Steroid compound | Antimicrobial Antiinflammatory Anticancer Diuretic Antiasthma Antiarthritic |
| 5 | $C_{21}H_{17}N_3S$ | 5-(p-Aminophenyl)-4-(4-biphenylyl)-2- -thiazolamine | 22.573 | 1.29 | - | Not reported |
| 6 | $C_{15}H_{30}O_3Si_3$ | 1,3,5-Tris(trimethylsiloxy)benzene | 22.824 | 1.649 | Aromatic polyethers | Not reported |
| 7 | $C_{30}H_{52}O_2$ | Tricyclo[20.8.0.0(7,16)]triacontane, | 22.067 | 1.39 | - | Not reported |
| 8 | $C_{28}H_{46}O$ | Ergosta-7,24(28)-dien-3- | 25.635 | 1.33 | sterol | Not reported |
| 9 | $C_{15}H_{26}O$ | Presilphiperfolan-1-ol | 16.066 | 1.29 | Triquinane sesquiterpene | Antimicrobial |
| 10 | NA | 7-METHOXY-3,4,5,6-TETRAM | 18.808 | 1.79 | - | Not reported |
| 11 | $C_8H_8S$ | 1,3-Dihydro-2-benzothiophene | 23.708 | 1.64 | Benzothiazoles | Antitumor |
| 12 | $C_{32}H_{64}O_2$ | palmityl palmitate | 20.881 | 1.25 | Palmitic acid | 5-Alpha-Reductase-Inhibitor; Antiandrogenic; Antifibrinolytic antioxidant, Flavor; hemolytic, hypercholesterolemic, lubricant, nematicide, pesticide, propecic, soap |
| 13 | $C_{15}H_{11}N$ | 8-Phenylisoquinoline | 18.437 | 1.17 | alkaloid | Anticancer, antitumor |
| 14 | $C_{15}H_{24}$ | δSelinene | 17.517 | 0.920 | sesquiterpenoid | Not reported |
| 15 | $C_{30}H_{50}O$ | αAmyrin | 33.091 | 1.44 | sesquiterpenoid | Anticancer, antitumor, antimicrobialAnalgesic; Anti inflammatory; Anti nociceptive; Gastroprotective; Hepatoprotective; Insectifuge |
| 16 | NA | Pregnane-7,8,9,11,20-pen | 24.312 | 2.598 | - | Not reported |
| 17 | $C_{21}H_{26}N_2O_2$ | Vincadifformine | 21.757 | 1.33 | alkaloid | Anticancer, antitumor, cytotoxic |
| 18 | $C_{15}H_{24}$ | Epizonarene | 17.524 | 0.920 | Sesquiterpenoid | Not reported |
| 19 | $C_{29}H_{50}O$. | Gamma-Sitosterol | 31.535 | 1.28 | phytosterol | Anticancer, Antioxidant, antibacterial and prophylactic activities cytotoxic |
| 20 | $C_{30}H_{48}O$ | Urs-12-en-3-one | 31.735 | 1.127 | triterpenoid | Antitumor |
| 21 | $C_{21}H_{26}N_2O_2$ | Vincadifformine | 21.755 | 1.22 | alkaloid | Cytotoxic, antitumor |
| 22 | $C_{41}H_{84}O$ | 1-Hentetracontanol | 31.229 | 1.204 | phenolic | Antimicrobial |
| 23 | $C_{30}H_{50}O$ | αAmyrin | 33.091 | 1.2 | pentacyclic triterpenoid | Antimicrobial, Antitumor, Antioxidant, cytotoxic |
| 24 | $C_{15}H_{11}N$ | 8-Phenylisoquinoline | 18.430 | 1.172 | alkaliod | DNA-intercalating antitumor agents |
| 25 | $C_{22}H_{18}N_2O$ | Methyl-2,5,5-triphenyl-2-imidazolin-4 | 23.251 | 1.148 | Aromatic isocynates | Not reported |
| 26 | NA | 4,5,7-Trimethoxy-3-(4 methoxyphenyl)-2H-1-benzopyran-2-one | 23.166 | 1.134 | Flavanoid | Anticancer, cytotoxic, anti-inflamatory,antibacterial |

*Reference of only selected biological activity is sited in discussion to avoid excessive reference session.

*O. thymiflorus* extract was reported *in vitro* and plant model system [26,27], reports on the essential oil of *O. thymiflorus* [8,28] indicate the presence of same group of antimutagenic phytochemicals as in other medicinally important herbs in the family. In the light of the results from a battery of *in vivo* assays in Swiss albino mice, we report the genoprotective effect of methanol and hexane extracts of *O. thymiflorus*.

Genomic instability and loss of DNA damage response are considered to be the hallmark of malignancy [29]. MN formation in bone marrow cells occur as a result of such genomic instability indicating the basal level somatic cell mutation. MN assay in mice is sensitive and accurate since the aberrant cells are not cleared by spleen as in human [30]. High incidence of MN in malathion and EMS treated animals showed the high genomic toxicity of these chemicals. Methanol and hexane extract post treatment could effectively attenuate the instability caused by the insecticide. Decreased PCE/NCE is a direct and accurate measure of cytotoxicity as seen in insecticide and positive control groups. Both extract could effectively mitigate the toxicity in post treatment. Effect of the DNA repair loci on the chromosome or DNA repair proteins are responsible for the regulation of MN level in the cell. Phytochemical like alkaloids, flavonoids etc bring about the anticancer activity by several mechanisms which include inhibiting the activation of genes/proteins or enzymes and signalling pathways, activating DNA repair mechanisms (p21, p27, p51, p53 …) or/and stimulating protecting enzymes with antioxidant activity [31]. The potential of the polar extracts in minimizing the induced MN level leading to a conclusion that these crude extracts may contain potential phytochemicals that can influence any of the aforesaid cellular repair machinery of the cell. Findings from comet assay and γ-H2AX assay is in congruence with this assumption. Later gave an intelligible evidence of the presence of DNA repair proteins in post treatment groups. The high anticlastogenic effect of polar extracts imparts more coherence to the deduction.

Phytochemical analysis of methanol and hexane extract of *O.thymiflorus* by GC–MS accentuate the result from *in vivo* studies. Bothe extracts revealed a plethora of bioactive compounds with reported activity both *in vivo* and *in vitro*. Terpenoid and it derivatives were the most abundant group of compounds in hexane extract and alkaloids, phenolics etc were the most represented group from methanol extract. Both extracts were rich in phytosterols and flavonoids. These pharmaceutically important phytocompounds manifested a wide range of reported bioactivity including antimicrobial, antioxidant, anticancer/antitumor and cytotoxicity. Major compounds in methanol and hexane extracts like presilphiperfolan-1-ol [32], nalidixic acid [33], phytol [34], gamma-sitosterol [35], α-amyrin [36] are reported to be antibacterial/anti-fungal along with other properties. It has been reported that heptatriacontanol gives the antimicrobial activity to methanol extract of *Trilepisium madagascariense* [37] and cholestan-3-ol, 2-methylene-, is the compound present in the antibacterial fraction of *Z.officianale* [38] and *Citrus aurantifolia1* [39] These compounds were identified from the hexane extract of *O. thymiflorus*. The antibacterial, anti fungal activity of the compounds detected from the extract justify the use of the plant in traditional medicine against skin diseases.

We identified a spectrum of compounds with anticancer, antitumor, cytotoxic activities form hexane as well as methanol extract. Alkaloids like 4-Phenylisoquinoline and vincadifformine, were identified from both extracts. Gitoxigenin was detected from methanol extract where morphinan-14-ol was the major compound in hexane extract. Vincadifformine is a tubulin inhibitory indole vinca alkaloid which is an intermediate of clinically important antitumor anticancer bisindole drugs like vincristine and vinblastin [40]. Gitoxigenin is a cardinolide which is reported to be anti proliferative in tumour cell line [41]. Morphinan is a group of clinically important alkaloids and its derivatives based drugs are with industrial applications as narcotic drugs and antitumor agents [42]. Both extract revealed an array of sesquiterpenoids and sesquiterpene lactones which is a diverse group with pharmacological importance exhibiting cytotoxic and antineoplastic potentials by inducing apoptosis by ROS inhibition or arresting cell cycle or inhibiting cytokinins [43]. Presilphiperfolan-1-ol, 10s, 11s-Himachala-3(12), 4-diene, Cis phytol, a-Guaiene, αAmyrin, δ-Selinene etc were detected from the extracts. These compounds were reported from pharmacologically important Lamiaceae members with anticancer, antibacterial, antitumor efficacy [44]. Triterpenoids like Urs-12-en-3-one is identified from hexane extract. Ursolic acids and its derivatives are effective antioxidants which help in the modification of cellular redox status, excepting pro-oxidative action on tutor cells and reduce NF-kB kinase. It is reported to be showing pre-apoptotic effect and survival effectors in breast cancer [45].

αTocopherol, an isoform of Vitamin E and a fat soluble antioxidant was identified from hexane extract. There are numerous reports on the protective effect of vitamins especially Vit E against the cytotoxic effects of drugs and is used as a supplement or nutraceutical for cancer patients [46]. It is reported to enhance the anticancer efficacy of drugs [47]. Essential oils are usually rich in terpenoids and hexane extract well represents the group of compounds in essential oil. Phenolic diterpenoids in essential oil are antioxidants and prooxidants in higher concentration causing toxicity but essential oils are not genotoxic in any reported studies rather prevent the mutagenic effects by direct scavenging of radicles and/or activating enzymatic deactivation. Single strand DNA break caused by reactive oxygen species is prevented by phytol by its scavenging capacity [48]. In the present study, the decrease in the DNA damage (induced by the pesticide) in the methanol and hexane extracts of *O. thymiflorus* post treated group as demonstrated by comet assay and gamma H2AX assay explains and vindicates the anticancer activity of the extracts. Phytosterol rich vegetable oils are reported to be antioxidant because of stable radicle formation by an allylic free radicle in them and often used as neutraceuticals [49]. Hexane extract showed the presence few phytosterol among which gamma sitosterol is reported to be cytotoxic [50] and anti-proliferative in breast and lung cancer in human [51].

Essential oil of almost all members in Lamiacece is reported to exhibit antitumor antioxidant anticancer properties [52,53]. Previous studies, as cited earlier, reported the antioxidant potential of the methanolic extract of *O. thymiflorus* in MTT assay and the presence of active components in essential oil. Sesquiterpene, 2-isopropyl-5-methyl-9-methylenebicyclo-1-decene (4.4.0), linalool, pinenes, elemene, cymene, camphor etc were reported to be present in the essential oil of the plant. Even though very less report on the chemical profiling of this plant is available, reports from related species in the genera, *O. stamineus* showed a plentitude of active compounds [54,55]. It was reported that several compounds with medicinal value like rosmarinic acid, and caffeic acid derivatives had been isolated and being evaluated for its pharmacological use [56]. Polar extract of this species showed excellent antioxidant and radical scavenging activity [57]. Essential oil of *O. thymiflorus* also reported to have similar chemical profile which may attribute to its potent protective effect against a probably carcinogenic agent malathion.

Nowadays cancer treatment strategy is as challenging as drug discovery since the therapeutics used in chemotherapy affect rapidly multiplying other normal cells causing undesirable side effects and in some cases cancer genes mutate to drug resistant [58]. Usage of phytochemicals renders a green cancer therapy as these chemicals and its derived analogues stipulate less toxic

cancer treatment. Several chemopreventive phytochemicals like curcumin, ginger, capsaicin etc are very effective in down regulating the drug resistant genes and cell-signalling pathways that lead to neoplastic transformation [59]. Basil, Mint, Oregano, Thyme etc in Lamiaceae are used as nutraceuticals because of the presence of phenylpropanoids and polyphenols with high antioxidant properties [60]. In this scenario, the potential genoprotective phytochemicals present in polar and comparatively non polar extract of *O.thymiflorus* incites the use of this herb as a nutraceutical.

The efficacy of medicinal and folk preparation of the plant in polar and aqueous extract is suggested to be evaluated in human so that the high incidence of malignancies induced by chemical insecticides, pesticides can be minimized with low cost and minimum side effect.

## 5. Conclusion

This is the first time report to confirm the genoprotective effect of *O. thymiflorus* in *in vivo* system against the toxic effect induced by insecticides. It is suggested that the protective effect of methanol and hexane extract of *O. thymiflorus* by alleviating the MN level, reducing the cytotoxicity by improving the PCE/NCE, ameliorating the clastogenic effect induced by malathion toxicity, boosting DNA repair machinery in bone marrow cells of Swiss albino mice may be attributed to the synergetic action of biologically actively compounds in the plant with novel pharmaceutical potentials. Phytochemical analysis revealed a myriad of diverse group of compounds with pharmacognostic importance and industrial application. Literature survey on the class of compounds throw light to the possibility of using the plant as nutraceutical. The unidentified peaks incited more sophisticated phytochemical analysis of the extracts. The authors envisage further activity guided fractionation may lead to some promising green anticancer drug candidate from the plant.

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## Conflict of interest

None.

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