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Amelioration of the cyclophosphamide induced genotoxic damage in mice by the ethanolic extract of *Equisetum arvense*

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ABSTRACT

In the present study, we evaluated the potential of the plant *E. arvense* against the cytotoxic and mutagenic effects induced by cyclophosphamide (chemotherapeutic agent) in the bone marrow cells of mice using the Chromosome assay (CA) and Mitotic index (MI) *in vivo* as the biomarkers. The study was performed following 3 protocols: pre-treatment, simultaneous treatment and post-treatment with the ethanolic extract of the plant. The results demonstrated that the plant extract was not cytotoxic and mutagenic and has a protective effect against the mutagenicity induced by cyclophosphamide in pre, simultaneous and post treatments and against its cytotoxicity as well. Because of its ability to prevent chromosomal damage, *E. arvense* is likely to open an interesting field concerning its possible use in clinical applications, most importantly in cancer as a chemopreventive agent or even as a coadjuvant to chemotherapy to reduce the side effects associated with it.

1. Introduction

Medicinal plants have always been on the vanguard whether regarding the treatment of a number of ailments or even cancer. Over decades plants have been prized for their medicinal properties and used pragmatically as drugs, initially as traditional preparations and then as pure active principles, with this knowledge and practice being passed from generation to generation [1]. It has been suggested that the use of antimutagens/anticarcinogens in everyday life can be the most effective way to avert human cancer and genetic diseases [2]. The bioactive compounds in medicinal plants act as a blueprint to block or reverse carcinogenesis at early stages [3]. Moreover, they are considered to be an inexpensive, effective and easily applicable approach to control cancer [4]. Herbal medicines remain an important component of the health care system. Medicinal plants are the food supplements which have not only nutritional value but therapeutic value as well. The medicinal value of plants is due to the presence of secondary metabolites which includes alkaloids, saponins, terpenoids, flavonoids, tannins, sterols and phenolic compounds. Hence the importance of any plant lies in its biologically active principles. Almost four decades ago, the antimutagens were reported. Many reports have shown the rising trends of antimutagenic studies with the plant extracts. [5–7].

Medicinal plants and their extracts have been used by man from prehistoric times to cure various diseases and this has resulted in the discovery of some very important drugs. It is now been well established that the traditional herbal therapies contain a diverse array of chemopreventive agents as well [8].

Equisetum arvense, commonly known as the field horsetail or common horsetail (Sehetband or Brahmgund locally in Kashmir), is a very common, bushy perennial herb native to the northern hemisphere. It is a member of a very primitive family of plants. It is distributed throughout Canada and the USA except the southeast (Florida, Georgia, Alabama, Louisiana, Mississippi, and Tennessee), throughout Europe and Asia south to Turkey, Iran, the Himalayas and across China (except the southeastern part), Korea and Japan [9]. *Equisetum* is the only living genus of the order Equisetales and the class Sphenopsida. The plant mostly occurs in marshes, swamps, ditches, river banks, open fields, open woods, and fill areas, such as road sides, and railroad embankments.

Horsetail is a strange looking plant with creeping, string like rootstock and roots at the nodes that produce numerous hollow stems. Phytochemically, the plant is found to have a wide array of secondary metabolites which contribute to the medicinal properties of the plant.

The plant *Equisetum arvense* is a folk medicine and its extract is used locally to treat tuberculosis, edema, kidney and bladder stones, urinary tract infections, incontinence, acidity and dyspepsia, ulcers and wounds, bleeding etc. Reports are available regarding its anti-inflammatory, antinociceptive [10], antioxidant and antiproliferative [11], antimicrobial [12], hepatoprotective [13], antidiabetic [14], coagulant and astringent properties [15]. Horsetail is mainly used as a diuretic

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[16]. The ancient Greeks used horsetail in the treatment of wounds.

Mutagenicity/antimutagenicity assays have been used to identify potential antimutagens and anticarcinogens providing opportunities for the development of new drugs that can be used in the prevention and treatment of neoplasies and other genetic diseases [17]. Among the bioassays to assess DNA damage in mammalian cells in vivo, the chromosomal aberration test is an effective short term assay because the occurance of the chromosomal aberrations is one of the most important biological consequence of exposure of an organism to the genotoxic agent. *In vivo* mammalian chromosomal aberration test has been successfully used for the detection of structural aberrations induced by the test substance in the bone marrow cells of animals, usually rodents [18–20]. The advantages of the CA assay are related to the cell by cell approach, an accurate identification of most of the chromosome mutation types, a possible co-detection of mitotic indices and the precise scoring by image analysis.

There have been improvements in the cancer treatment which have increased the survival rates dramatically among patients in the recent time [21]. But the treatments like radiotherapy and chemotherapy have proven genotoxic and mutagenic and have led to the development of secondary, treatment related conditions. This includes further cancers, as well as predisposes survivors to various non-malignant diseases that can often evince in the offspring [22]. The efficacy of present chemotherapeutics has been limited by its toxicity and for the cells developing resistance against the therapy [23]. Studies on mice have also shown that anticancer drug exposure can result in the genomic instability acting across multiple generations [24]. Chemotherapy cancer treatment cases involve invariable usage of antineoplastic agents which kill neoplastic tissue as they are toxic to rapidly proliferating cells. However in the process, they can damage normal proliferating cells as well. This lowers their therapeutic index. Thus long term use of these antineoplastic agents has become a subject of increasing concern [25].

More than 100 chemotherapy drugs are used in treating cancer, either alone or in combination with other drugs or treatments. One such drug is Cyclophosphamide. Cyclophosphamide (CPA) which belongs to the class of oxazaphosphorines is an alkylating agent. It had been listed as one of the most successful chemotherapy drugs on the World Health Organizations List of Essential Medicines. Cyclophosphamide possesses marked immunosuppressant properties against both humoral and cellular immunity because of which it has been extensively used to treat a variety of childhood and adult malignancies [26–28] including breast cancer, lymphomas and leukemias, retinoblastoma, small cell lung cancer, ovarian cancer, sarcomas and multiple myeloma [29], since its initial synthesis in 1958. But over the time its use has been declining because of its adverse genetic effects. The chemical is activated by the hepatic P-450 cytochrome and its metabolites phosphoramide mustard and acrolein are linked to its antineoplastic and other toxic side effects [30,31].

CPA has been recommended to be used as a positive control chemical in genetic toxicity tests [32,33]. CPA has also been extensively tested by various mutagenicity/antimutagenicity assays to induce dominant lethal mutations, micronuclei, DNA damage and generation of free radicals or Reactive Oxygen Species (ROS) *in vivo* [2].

Thus based on all the pharmacological properties of *Equisetum arvense*, especially the antioxidant effect which is desirable for the antimutagenic property, in this study we evaluated the anticytotoxic and antimutagenic effects and its corresponding benefits to humans with the possible mechanisms of actionof this plant against the damages induced by antineoplastic agent CPA.

Certain plants, however, can also directly induce mutations and/or chromosome aberrations under certain conditions. It is reasonable that while some medicinal plants may suppress the effects of mutagens, others may have toxic or mutagenic effects [34]. However, it is very important to investigate any negative effect of the plants to the animals, if any. So the mutagenicity of the plant *E. arvense* was also studied before evaluating its antimutagenic potential.

2. Materials and method

2.1. Plant material

Equisetum arvense was collected at the sterile stage from Hajibal area of Baramulla district, 1577 mt above sea level, J & K. The plant was collected randomly from the area. The plant was identified and authenticated by the Centre of Biodiversity and Plant Taxonomy, Department of Botany, University of Kashmir. A specimen under voucher number KASH-2348 was preserved in the respective department for future reference.

2.2. Preparation of the extract

The aerial part of the sterile stem was air dried in the dark at 20 ± 5 °C for 12 days. The shade dried plant material was fine powdered using a domestic blender. After being macerated to fine powder, 1 kg powdered *Equisetum arvense* was subjected to hot extraction using the soxhlet apparatus. The powdered material was packed in the soxhlet apparatus and exhaustively extracted with ethanol at a desired temperature for 16–20 h continuously. The extract was filtered using Whatman's filter paper no. 1. The residue was discarded and the filtrate collected and concentrated to dryness under reduced pressure using Heidolph rotary evaporator (R-215). The final yield of the extract was also calculated and found to be 98.6 g (on dry weight basis of the crude material).

2.3. GC–MS analysis

GC–MS analysis of the extract 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 m) with helium as a carrier gas, at a flow rate of 3 ml/min with 1 µl injection volume. Samples were analyzed 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 flameionization 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.

2.4. Selection of doses

Ethanol extract was dissolved in distilled water and the choice of the concentration was based on the maximum dose used in other experimental works on this plant i.e. 500 mg/kg of body weight (bw). The treatments were performed orally (gavage). The alkylating agent cyclophosphamide (CAS No. 6055-19-2, Himedia) was used as the positive control. It was diluted with water and administered intraperitonealy at a dose of 50 mg/kg of the body weight.

2.5. Animals and treatment

Swiss albino mice (*Mus musculus*), 5–6 weeks old and weighing 30 ± 5 g were randomly selected and supplied by the Indian Institute

of Integrative Medicine (IIM). The mice were housed in polypropylene cages under the controlled light and temperature conditions and were given food (commercial mouse pellets) and water *ad libitum* throughout the experiment. The whole experiment was approved from Institutional Animal Ethical Committee. All the protocols and the experiments were conducted in strict compliance according to the ethical principles and guidelines provided by Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA).

Before evaluating *Equisetum arvense* as an agent to protect and/or repair genetic material, it was first evaluated for any cytotoxic or mutagenic activity. For the antimutagenic activity of the plant the experiment was carried out using the protocols for pre-treatment, simultaneous treatment and post-treatment The experiment consisted of 6 groups: the treatment groups (pre, simultaneous and post and ethanolic extract alone treatment group),the positive control (cyclophosphamide), the negative control/vehicle (distilled water). Each group had 5 animals selected randomly irrespective of the sex.

2.5.1. Ethanolic extract alone

500 mg/kg bw of the ethanolic extract of the plant was given to the mice via gavage for 21 days. The animals were euthanized by cervical dislocation 24 h after the last treatment.

2.5.2. Pre-treatment

The mice received ethanolic extract of the plant for 21 days and cyclophosphamide (50 mg/kg bw) on the 22nd day. The negative control was given distilled water for 21 days. The positive control received only CPA (50 mg/ kg bw). After 24 h of the drug administration, the animals were euthanized.

2.5.3. Simultaneous treatment

The mice in this group received ethanolic extract of the plant and CPA simultaneously. The animals were euthanized after 24 h of the treatment.

2.5.4. Post-treatment

The mice received the mutagenic agent CPA on the first day and for the next 21 days the plant extract was administered to them. The euthanasia was held 24 h after the treatment ended.

2.6. Chromosome aberration assay in mouse bone marrow

Chromosome preparations were made from mitotically active bone marrow cells following the techniques of Preston et al. [18].

Two hours prior to the tissue sampling, animals were injected intraperitonealy with 0.4 ml of 0.05% colchicine, to arrest the metaphase stage. The mice were then euthanized using the cervical dislocation method. The euthanasia was held 24 h after the particular treatment ended. Both the femurs were excised, fleshed out and cleaned of any remaining tissue. Both the ends of the bone were cut open and a needle with syringe containing 3 ml, 0.56% KCl (hypotonic solution) from the distal end was inserted and aspirated forcefully in the centrifuge tube until the bone became visibly empty. The cell suspension was mixed gently with the help of Pasteur pipette and then incubated for 20 min at 37 °C. After the hypotonic treatment, the suspension was centrifuged at 800 rpm for 4 m. The supernatant was discarded and the cells were agitated with drop wise addition of 2 ml freshly prepared chilled Cornoy's fixative (Methanol: Acetic acid, 3:1). The content was kept for 1 h stabilization. The pellet was resuspended in the fixative and the above process was repeated 3–4 times until the whitish pellet was obtained. After the fifth centrifugation, the supernatant was discarded and the sediment mixed with 1 ml of fresh fixative and slides were prepared.

Fresh slides were soaked in ethanol and polished with tissue paper to make them grease free. They were then kept in the deep freezer 2–3 h prior to use. Cell suspension was dropped from a height of 30–40 cm on pre chilled slides so that the cells burst open onto the slides. The slides were heat fixed and 1–2 drops of fresh fixative was added on the slides to see more reliable pictures of the chromosomes. The slides were air dried and stained with 5% Giemsa stain for 15–20 min. Finally the slides were washed without agitation in the buffer solution and air dried again for the microscopic analysis. The microscopic observations were performed under microscope (OLYMPUS CH20*i*) at 100X oil immersion [35,18].

The slides having brightly stained well spread metaphase chromosomes were independently coded and observed under the microscope for CA analysis. Four slides were selected per animal and a minimum of 25 metaphases were scored from each slide in each group including control. Since the number of mice per group was five, a total of 500 metaphasic complements were studied in each group. The types of aberrations were scored and recorded strictly in accordance with the method of Tice et al. [36]. All aberrations (fragments, chromatid breaks, chromosomal breaks, ring chromosomes, sticky chromosomes, and exchange) were considered equal regardless of the number of breakages involved. Regardless of whether it was a mono or poly aberration in one metaphase, it was counted as one aberrant metaphase. Other aberrations have not been counted for the statistical analysis of the data due to controversial genetic significance and also because some deletions and rearrangements (translocation, inversion) may not be lethal [37]. The gaps were recorded but not included with the total aberrations.

2.7. Mitotic index

The mitotic index is an important prognostic factor predicting both overall survival and response to chemotherapy in most types of cancer [38]). The Mitotic Index assay is useful as a tool for identification of novel anti-mitotic compounds, and also as a means for diagnosing and characterizing cancerous cells [39]. The bone marrow smears were made in the same way as in chromosomal aberration assay. The cell suspension was put drop wise onto the slides. For the study of MI, 1000 cells per animal were analyzed, totalling to 5000 cells per group. MI was calculated by using the formula [39]:

$$MI = (A/A + B)\ 100$$

Where,

A = No of dividing cells (Metaphase + Anaphase)

B = No of non dividing cells.

2.8. Statistical analysis

The calculated average data generated at different end points of the treated group of mice were compared with the respective data of vehicle/negative control and positive control groups and among themselves as well. Statistical analysis was performed with the SPSS (version 20) computer programme. To analyze the relationship between the variables, Mann-Whitney *U* test was performed ($p \leq 0.05$).

The percentage of damage reduction was calculated according to the following formula [40,41]:

$$\text{Reduction}\% = \frac{\text{Frequency of CA in A} - \text{Frequency of CA in B}}{\text{Frequency of CA in A} - \text{Frequency of CA in C}} \times 100$$

Where,

"A" is the group treated with CPA;

"B" is the group treated with plant extract and CPA; and

"C" is the negative control group.

Table 1
Phytoconstituents identified in ethanolic extract of *Equisetum arvense* by GC–MS.

S.no	Retention time	Compound	% Area	Molecular formula	Molecular weight
1	4.891	Cyclopentanol	1.91	$C_5H_{10}O$	86
2	6.118	Azulene	2.27	$C_{10}H_8$	128
3	6.317	4-Penten-2-one, 4-methyl	0.51	$C_6H_{10}O$	98
4	7.899	2-methoxy-4-vinylphenol	6.43	$C_9H_{10}O_2$	150
5	8.917	1-(2,6-dimethyl-4-propoxy-phenyl)-propan-1-one	0.57	$C_{14}H_{20}O_2$	220
6	10.018	4-(2,4,4-Trimethyl-cyclohexa-1,5-dienyl)-but-3-en-2-one	0.88	$C_{13}H_{18}O$	190
7	10.301	Phenol, 2,4-bis(1,1-dimethylethyl)	0.58	$C_{14}H_{22}O$	206
8	10.689	2,2,4-Trimethyl-5-oxo-2,5-dihydro-3-furancarboxylic acid	0.44	$C_8H_{10}O_4$	170
9	10.769	2(4H)-Benzofuranone, 5,6,7,7a-tetrahydro-4,4,7a-trimethyl	0.13	$C_{11}H_{16}O_2$	180
10	11.042	2,4-cyclooctadien-1-ol, 1-(1-methoxycyclopropyl)-6,6-dimethyl	0.37	$C_{14}H_{22}O_2$	222
11	11.252	Megastigmatrienone	0.89	$C_{13}H_{18}O$	190
12	13.049	1-Propanone, 1-(2-aminophenyl)-3-methoxy	0.63	$C_{10}H_{13}NO_2$	179
13	13.465	Phosphonic acid, dioctadecyl ester	0.38	$C_{36}H_{75}O_3P$	586
14	13.775	13-octadecenal	0.31	$C_{18}H_{34}O$	266
15	13.970	2,6,10-trimethyl,14-ethylene-14-pentadecne	16.36	$C_{20}H_{38}$	278
16	14.041	2-Hexadecene, 3,7,11,15-tetramethyl	1.57	$C_{20}H_{40}$	280
17	14.367	1,2-benzenedicarboxylic acid, bis(2-methylpropyl) ester	0.34	$C_{16}H_{22}O_4$	278
18	14.570	Hexadecane, 2,6,10,14-tetramethyl	0.15	$C_{20}H_{42}$	282
19	14.859	Hexadecanoic acid, methyl ester	1.99	$C_{17}H_{34}O_2$	270
20	15.092	1-hexadecen-3-ol, 3,5,11,15-tetramethyl	0.09	$C_{20}H_{40}O$	296
21	15.301	n-Hexadecanoic acid	15.82	$C_{16}H_{32}O_2$	256
22	15.524	Hexadecanoic acid, ethyl ester	2.39	$C_{18}H_{36}O_2$	284
23	15.615	Cis-9-Hexadecenal	0.64	$C_{16}H_{30}O$	238
24	15.822	Hexadecanal	0.17	$C_{16}H_{32}O$	240
25	16.433	Oxirane, tetradecyl	0.35	$C_{16}H_{32}O$	240
26	16.517	Hexadecane, 1-iodo	0.26	$C_{16}H_{33}I$	352
27	16.576	9-Octadecenoic acid, methyl ester	3.82	$C_{19}H_{36}O_2$	296
28	16.724	Phytol	15.2	$C_{20}H_{40}O$	296
29	17.054	Oleic acid	7.64	$C_{18}H_{34}O_2$	282
30	17.212	Ethyl (9z,12z)-9,12-octadecadienoate	7.16	$C_{20}H_{36}O_2$	308
31	17.653	2,6,10-trimethyl,14-ethylene-14-pentadecne	0.58	$C_{20}H_{38}$	278
32	18.373	Fumaric acid, 2-dimethylaminoethyl octadecyl ester	1.89	$C_{26}H_{49}NO_4$	439
33	18.566	9-octadecenoic acid (z)-, methyl ester	0.24	$C_{19}H_{36}O_2$	296
34	18.837	Eicosanoic acid, methyl ester	1.84	$C_{21}H_{42}O_2$	326
35	19.671	Heptadecanoic acid, ethyl ester	0.57	$C_{19}H_{38}O_2$	298
36	20.805	3-Cyclopentylpropionic acid, 2-dimethylaminoethyl ester	0.77	$C_{12}H_{23}NO_2$	213
37	21.474	cis-9-Hexadecenal	0.89	$C_{16}H_{30}O$	238
38	37.106	Stigmast-5-en-3-ol	2.97	$C_{29}H_{50}O$	414
		Total	100		

3. Results

3.1. GC–MS analysis

In order to find out the phytocomponents responsible for antimutagenic activity, ethanolic extract of *E. arvense* was subjected to GC–MS analysis. The active principals present in the ethanolic extract of *E. arvense* along with their retention time (RT), molecular formula, molecular weight (MW) and peak area (%) are presented in Table 1. The chromatogram of Ea-EE showed 7 major peaks (Fig. 1): 2,6,10-trimethyl, 14-ethylene-14-pentadecne (16.36%), *n*-Hexadecanoic acid (15.82%), Phytol (15.2%), Oleic acid (7.64%), Ethyl (9z, 12z)-9,12-octadecadienoate (7.16%), 2-methoxy-4-vinylphenol (6.43%), 9-Octadecenoic acid, methyl ester, (E) (3.82%) comprising 72.43% of the total peak area.

Table 2 summarizes the results of the % chromosomal aberrations as well as the percentage of damage reduction by the plant extract following the protocols of pre-treatment, simultaneous treatment and post-treatment recorded alone and the Table 3 summarizes the results of the % mitotic index in the bone marrow cells. Figs. 2 and 3 show the comparative% age reduction in CA and% mitotic index for the different treatment groups.

3.2. Treatment using ethanolic extract alone

No significant difference from the negative control was shown by the extract given alone by gavage to all the animals in the group in terms of both chromosomal aberrations (9.4 ± 0.54) and mitotic index (7.1 ± 0.84)in the bone marrow cells.

3.3. Pre-treatment

All the animals treated with the ethanolic extract of the plant reduced the frequency of the chromosomal aberrations compared to the positive control group indicating its antimutagenic activity. There was also a reduction in the mitotic index percentage.

The percentage of reduction of chromosomal damage of the group treated with ethanolic extract of the plant was observed to be 85.91%.

The analysis of the effect of extract on the cytotoxicity induced by CPA showed a significant improvement by the extract (6.16 ± 2.30).

3.4. Simultaneous treatment

Animals treated with the extract and CPA simultaneously also reduced the chromosomal damage to some extent compared to the positive control. The percentage reduction in the chromosomal damage was observed to be 92.95%.

Also the analysis of the effect of the simultaneous treatment on the cytotoxicity caused by the alkylating agent also showed an increase in the mitotic index (6.98 ± 2.17) statistically found to be different from the positive control.

3.5. Post-treatment

The animals in this treatment group showed the highest reduction in chromosomal aberration frequency (97.65%) induced by the cyclopho-

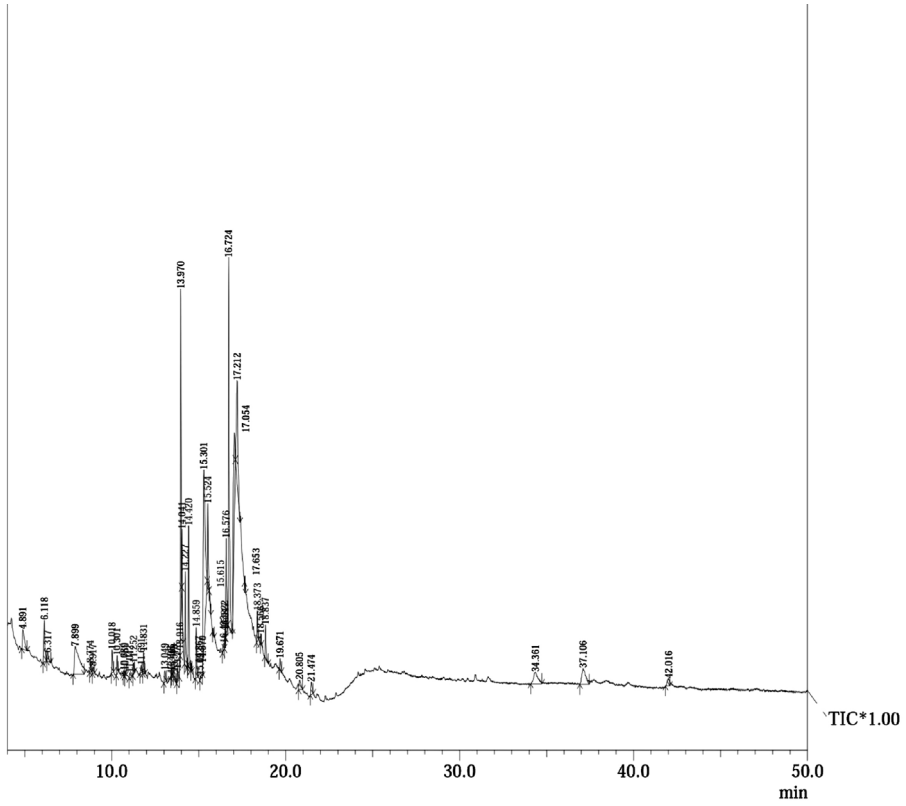

Fig. 1. GC–MS chromatogram of Ethanolic extract of *Equisetum arvense*.

sphamide compared to the other two treatment groups. The analysis of the effect on cytotoxity also showed more significant effects. The post treatment via gavage was the most effective in bringing up the mitotic index percentage (7.1 ± 1.22) lowered initially by the cyclophosphamide given.

4. Discussion

With the growing realization that the natural products are a healthier match to that of successful drugs, the interest in applying natural chemical diversity to drug discovery appears to be increasing [42]. Hence more extensive collection of plants could provide many novel chemicals in drug discovery assays for various diseases.

The healing properties of many substances found in nature such as aspirin, digitalis, penicillin etc. have been acknowledged for long. Medicinal plants have played a paramount role in the prevention and treatment of various ailments. With the advanced knowledge of molecular science and refinement in isolation and structure elucidation techniques, various anti-cancer plant products have been identified as

Table 3
Mitotic index of mice bone marrow cells observed during ethanolic extract sub chronic treatment.

Treatment Group	DOSE mg/kg	Total cells analyzed	% Mitotic Index ± SD
Distilled water (NC)	–	1000	7.36 ± 1.14
Cyclophosphamide (PC)	50	1000	4.64 ± 1.14
Eth-EA alone (gavage)	500	1000	7.1 ± 0.84[1]
Eth-EA pre-treatment (gavage)	500	1000	6.16 ± 2.30[a]
Eth-EA sim-treatment (gavage)	500	1000	6.98 ± 2.17[a]
Eth-EA post-treatment (gavage)	500	1000	7.1 ± 1.22[a]

NC: negative control, PC: positive control (Cyclophosphamide), Eth: ethanolic extract, EA: Equisetum arvense. Values with different letter superscript differ significantly ($p < 0.01$: highly significant) from the positive control whereas values with numeric superscript do not differ significantly ($p > 0.05$) from the negative control (**Mann Whiteny U Test**).

Table 2
Chromosomal aberrations observed during ethanolic extract sub chronic treatment in the bone marrow cells of Cyclophosphamide treated mice.

Treatment Group	Dose mg/kg	TMS	Chromosomal Aberrations%							Total abb. Mean(%) ± SD	% Reduction
			Frg	Cr	Csb	Ctb	Cg	St	Ex		
Distilled water (NC)	–	500	2.4	3	0.6	3	1.6	0.2	–	9.2 ± 0.84	–
Cyclophosphamide (PC)	50	500	6	16.2	20.6	21.2	7.2	4.2	0.6	68.8 ± 1.48	–
Eth-EA alone (gavage)	500	500	2.4	2.2	0.8	2.6	2.2	1.2	0.2	9.4 ± 0.54[1]	–
Eth-EA pre-treatment (gavage)	500	500	5.4	5.4	0.4	2.8	2.8	2.4	1.2	17.6 ± 0.55[a]	85.91
Eth-EA sim-treatment (gavage)	500	500	3	4.6	1	3	2.4	1	0.8	13.4 ± 0.89[a]	92.95
Eth-EA post-treatment (gavage)	500	500	4.2	1.8	0.4	2.4	1.4	1.2	0.6	10.6 ± 1.14[a]	97.65

Eth: ethanolic extract, EA: E.arvense, TMS: total metaphasic plates studied, Frg: fragment, Cr: chromosomal ring, Csb: chromosome break, Ctb: chromatid break, Cg: gap, St: sticky chromosomes, Ex: exchange. Values with different letter superscript differ significantly ($p < 0.01$: highly significant) from the positive control whereas values with numeric superscript do not differ significantly ($p < 0.01$: highly significant) from the negative control (Mann Whiteny U Test). Gaps have been mentioned but not included in the total aberrations.

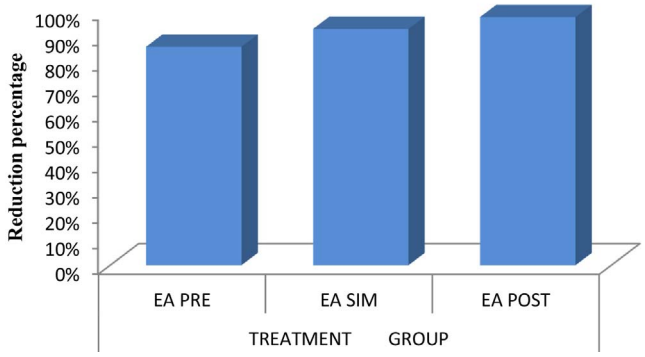

Fig. 2. Percentage reduction in chromosomal aberrations by different treatments with ethanol extract of *Equisetum arvense*.

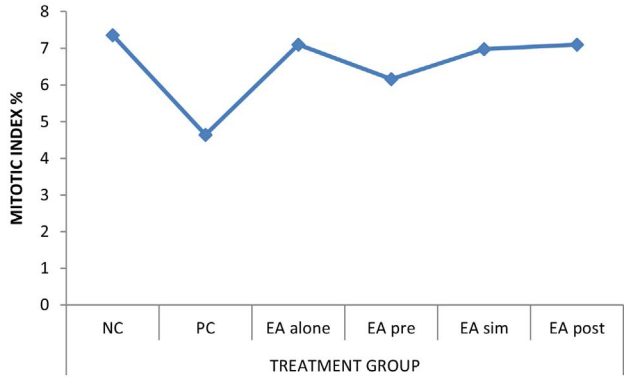

Fig. 3. % Mitotic Index of the bone marrow cells in different treatment groups.

well. Many clinical trials focusing on the anti-cancer effects of herbal formulas have been conducted. Many of them demonstrated that herbs are helpful against cancer, especially useful in survival and quality of life. After research for several decades, antimutagenic effect of many naturally occurring compounds extracted from plants has been well established in bacterial and mammalian cells [43]. Plants like *Acacia salicina* [44], *Mangifera indica L.* [45]), *Phellinus rimosus* [46], *Terminalia arjuna* [47], *Uncaria tomentosa* [48]), *Heterotheca inuloides* [49], *Ajuga bracteosa* [50] and many more have shown effective antimutagenic properties.

Although an antimutagen is not necessarily an anticarcinogen, yet it is an indication of a possible anticarcinogen [51,52]. It was reported earlier that several antitumor compounds act through the antimutagenic mechanism [53–55]. Hence, searching for the antimutagenic compounds represents a rapidly expanding field of cancer research [56,57,7,58]. Plants having anti-mutagenic potential can also be used as an adjuvant with the chemotherapeutic drugs so as to reduce the side effects associated with the chemotherapy.

Antimutagens may be effective against a single mutagen or a class of mutagens; may act by multiple, sometimes strictly interconnected or partially overlapping mechanisms; may be even mutagenic at certain concentrations or in certain test systems, which implies a discriminative approach in antimutagenesis studies, as well as careful interpretation of the results [59]).

Antimutagenic activity can occur by different mechanisms either inside or outside the cell by three possible mechanisms. First, by intercepting the mutagenic agent before it reaches its target. Second, by preventing the formation of an active intermediate. Third, by interfering with the mutagenic process after the primary damage to DNA has been initiated.

An issue of considerable controversy in cytogenetic evaluations is the level of significance of gaps in chromatid or chromosomes. Some investigators feel that identification of gaps is extremely subjective and that they do not constitute true aberrations [60,61]. These investigators have suggested that gaps may be quantified and reported, but should not be used in the evaluation of the study. The gaps are defined as achromatic lesions or faintly stained regions perhaps connected between both distal parts. Therefore, these gaps are not considered to be real discontinuity and hence were mentioned separately and not included in the total structural aberrations in the present study.

In the present study mice treated with CPA showed significant ($p < 0.05$) increase in aberrant metaphases, CAs (including and excluding gaps), while decreased cellular proliferation rate (MI) compared to the control group. The toxic effect of CPA may be due to its metabolic activation by the hepatic microsomal cytochrome P450 mixed functional oxidase system via two different pathways [62,63]. In the first pathway, CPA is catalyzed by cytochrome P–4502 B and P-4502C enzymes to form the DNA cross- linking agent, phosphoramide mustard, and the toxic metabolite, acrolein (AC). The second pathway involves a CYP3A4-mediated N- dechloroethylation of CPA to form the inactive metabolite, 3-dechloroethylcyclophosphamide (DECP), and the toxic by-product, chloroacetaldehyde [64,29,65]. CPA metabolites can generate free radicals that can lead to oxidative stress.

The ethanolic extract when given alone did not influence the parameters measured. It was not cytotoxic or mutagenic at the time of treatment to the mice.

The use of a simultaneous treatment appeared to identify mechanisms with direct action on the mutagen by inactivating it, which may be classified as desmutagenicity effect [66]. Previously, it had been reported that some drugs, dietary components and endogenous biochemicals can function as antimutagens by altering the rates of mutagen absorption and uptake [67]. However, the present experimental design does not rule out the possibility of indirect effects of the *E. arvense* extract during CPA exposure. The post treatment could show its antimutagenic potential by playing a role in optimization of DNA repair. The conclusion obtained in the pre-treatment could reflect the effects on the prevention of DNA damage by affecting metabolic pathways, being antioxidant or acting on DNA replication. These action mechanisms, occurring in both pre- and post- treatments, could be called bio-antimutagenicity [68] or fidelogenesis. A great variety of antimutagenic agents act through multiple mechanisms to provide protection against diverse mutagens. Noteworthy, the ability of compounds to affect mutagens simultaneously in several different ways significantly increases antimutagenic effectiveness. Hence, searching for such multifunctionally acting antimutagens is of great importance.

5. Conclusion

A clear negative effect on the induction of chromosomal aberrations and increased mitotic index by the ethanol extract of *E. arvense* was found. The results of the present study clearly showed that the ethanol extract of *E. arvense* had an antimutagenic and anticlastogenic potential against the mutagenic activity of cyclophosphamide in mice.

Our results suggest that there may be several ways through which *E. arvense* extract can work against CPA. Selection of *E. arvense* for the present research topic 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.

From all it is reasonable to conclude that the suggested consumption of these medicinal plants as nutraceuticals can not only be safe but can also improve the quality of life by protecting against several environmental mutagens.However, further studies are needed in other test systems so that in the future *E. arvense* can be used in reducing the occurrence of cancers or even as a coadjuvant to chemotherapy to reduce its side effects.

Conflict of interest

The authors declare that there are no conflict of interest.

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