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Antigenotoxic effects of a polyherbal drug septilin against the genotoxicity of cyclophosphamide in mice

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ABSTRACT

Septilin (Spt) is a polyherbal drug formulation from Himalaya Drug Company, consisting of extracts from different medicinal plants and minerals. In the traditional system of medicine, septilin is being used as immunomodulatory, antioxidant and anti-inflammatory agent. In the present study, the protective effects of septilin against the genotoxicity of cyclophosphamide (CP) a widely used alkylating anticancer drug was evaluated by using *in vivo* micronucleus (MN) and sperm shape abnormality assays in Swiss albino mice. CP administered intraperitoneally at a dose of 50 mg/kg b.w. was used as positive mutagen. Different doses of septilin viz., 125, 250 and 500 mg/kg b.w. was orally administered for 5 consecutive days. CP was administered intraperitoneally on 5th day. MN and sperm preparations were made after 24 h and 35 days respectively. CP induced significant MN in both bone marrow and peripheral blood cells and also a high frequency of abnormal sperms. In septilin supplemented animals, no significant induction of MN and abnormal sperms was recorded. In septilin supplemented groups, a dose dependent significant decrease in CP induced clastogenicity was observed. Thus the current *in vivo* study revealed the antigenotoxic effects of septilin against CP induced damage, in both somatic and germ cells of Swiss albino mice.

1. Introduction

Ayurveda, the science of life, derived from spiritual visions were practiced by Indian Rishis since time immemorial. It still continues as an important system of medicine and drug therapy, in India. Throughout the history of civilization, plants have played major role in medication for the treatment of various kinds of human diseases. Plants and plant based medicine are the backbone of modern pharmaceutical preparations and they are the major contributors to the pharmaceutical industry both in India and other countries [102,14]. According to world health organisation more than 80% of the world population depends on the traditional medicines for their primary healthcare units. Plant secondary metabolites are the primary active ingredients of ayurvedic drugs. Plants harbour large number of active ingredients in valuable in controlling the diverse group of diseases. Several prescription drugs in the developed countries contain plant components and more than 100 important prescription drugs are derived from plants [49]. The medicinal values of plants are due to some specific chemical substances which produce definite physiological action on the human body [14]. A single plant may contain number of bioactive compounds which may act singly or synergistically to impart beneficial health effects. At global level, there is an increased demand for the pharmaceutical products of plant origin or other natural sources, because of the fact that the allopathic drugs have unwanted side effects which may be hindrance for their therapeutic use. Further, plant based therapy are marked due to its low cost, easy availability [58].

In recent years much attention is being given for the discovery of cell/genoprotective agents from the natural sources against the damaging effects of chemicals and radiations. Numerous studies have been carried out in the last four decades to identify the compounds that might protect the humans against the DNA damage and its consequences. In this line, more emphasis has been given to the medicinal plants and their isolated bioactive components. Septilin, is one of the ayurvedic herbo mineral [46] preparation of the Himalayan Drug Company containing extracts of six different plants and powders of *Blasmodendron mukul* and shankha bhasma. There are reports on the antibacterial, antiinflammatory,

Abbreviations: A, amorphous; B, banana shaped; BSA, bovine serum albumin; CMC, carboxymethyl cellulose; CP, cyclophosphamide; DH, double headed; DT, double tailed; F, folded; H, hookless; MN, micronucleus; MNNCE, micronucleus in normochromatic erythrocytes; MNPCE, micronucleus in polychromatic erythrocytes; NCE, normochromatic erythrocytes; PCE, polychromatic erythrocytes; Spt, septilin.

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immunomodulatory and immunopotentiating effects of septilin and it is extensively used for the treatment of several acute and chronic infections [62,45].

Among the currently available test systems to evaluate the genotoxicity/clastogenicity of the various test agents, micronucleus assay is the most widely applied methods due to its simplicity, reliability, sensitivity and proven suitability for genotoxicity evaluation [27,15].

Micronuclei (MN) are chromatin containing bodies, appearing as small satellite nucleus in the cell arising from acentric chromosomal fragments or from entire chromosome that is lagging at anaphase [56,89,81]. Presence of micronuclei in cells is an indicator of damage to the DNA. The *in vivo* micronucleus tests using haematopoietic bone marrow and peripheral blood cells are widely used methods for the assessment of genotoxicity of chemicals in exposed organisms. The rodent bone marrow micronucleus assay is the measure of both clastogenicity/aneugenicity of target chemical [59]. It has helped a lot to understand the dose response relationship for aneugens and clastogens [23]. Micronucleus is a biomarker, which is used widely in biomonitoring studies to determine the genetic risk due to the exposure to environmental chemicals [10]. Presence of micronuclei in the peripheral blood is found to be as an important biomarker, which shows the risk of cancer development [11,25]. Hernandez et al. [36] investigated the applicability of MN data to derive cancer potency information. They explored relationship between dose response data from genotoxicity tests and carcinogenicity studies and they observed a positive correlation between these two.

The number of mature (normochromatic) erythrocytes in the peripheral blood that contain micronuclei among a given number of mature erythrocytes can also be used as the end point of the assay when animals are continuously treated with the test agents [65]. Micronucleus assay is also being used as biological dosimeter of *in vivo* ionising radiation exposure [107]. There are many reports of clastogenecity and anticlastogenecity studies where bone marrow micronucleus assay was used as one of the parameters [57,95,67,73,74,63]. Several workers used peripheral blood micronucleus assay method to evaluate the clastogenic and anticlastogenic potency of different agents [21,50,64].

In animals, exposure of the males to toxic chemicals and radiation can result in wide variety and combination of reproductive dysfunction such as changes in the sexual behaviour, spermatogenic killing, diminished sperm quantity and quality, chromosomal defects in germ cells, reduced fertility *etc*. [108]. Epidemiological studies have shown cytotoxic and genotoxic effects of chemotherapeutic drugs and impaired fertility in males [7]. The mouse sperm morphology test is commonly used for measurement of spermatogenic damage induced by test agents. Studies have shown that induced changes in sperm morphology reflect the genetic damage in male germ cells. Sperm assays are commonly used to detect the causes of infertility. Here, mainly sperm counts, motility of sperms, and sperm morphology are being used as test parameters [93]. There are several reports on chemically induced abnormal sperms. [75] reported the sperm abnormalities in mouse germ cells after short term exposure to pesticides acetamiprid, propineb and their mixture. From various studies it has been concluded that chemicals yielding positive response in mouse sperm morphology test should be regarded as suspected germ cell mutagens in mammals and agent's positive responses in these sperm tests should be considered with high priority against human applications [110,111]. Sperm abnormality assay is extensively being used for the evaluation of genotoxicity of chemicals and also for the study of antigenotoxic protective effects of natural compounds [40,9,26,73,74,4,12,24,91]. In addition, sperm abnormality assay is also being used to study the endocrine mediated effects to assess the potency of endocrine disrupting chemicals on hormone homeostasis and also studies indicated the risk of lowered fertility due to decreased spermatogenesis in such exposed animals [114].

Cyclophosphamide (CP) is widely used anticancer and chemotherapeutic drug [90]. However, despite its wide spectrum of clinical benefits it can also induce cytotoxic effects on the normal cells in humans and experimental animals [44]. CP is an alkylating agent capable of inducing gene mutations, chromosomal aberrations, micronucleus (MN), sister chromatid exchanges, as well as other genotoxic effects [51,1]. Since CP is a well-known mutagen/genotoxin, in the present study it was used as a positive mutagen. There are several reports on the use of CP to evaluate the anticlastogenic/antigenotoxic effects of various natural compounds and other chemicals [87,61,28,92,32,33,18,70]. [42] reported the anticlastogenic effect of *Ricinus communis* extract against CP induced clastogenicity in mice bone marrow cells.

Since there are very few reports on the chemoprotective effects of septilin, the present study was undertaken to investigate its *in vivo* anticlastogenic effects against CP induced clastogenicity by using micronucleus assays in bone marrow and peripheral blood cells and sperm abnormality assay in germinal cells. Both test systems used in present study have the same sensitivity, specificity and accuracy and are being used as short term tests for the evaluation of carcinogenecity. Whereas, sperm morphology assay is useful in screening test for compounds that constitutes a potential genetic hazard for mammals.

2. Materials and methods

2.1. Chemicals

Septilin (The Himalayan drug company, Peenya Industrial Area, Bangalore, Batch No.-37300170B), containing the extracts of *Maharasanadi qoath* (130 mg), *Tinospora cordifolia* (98 mg), *Rubia cordifolia* (64 mg), *Emblica officinalis* (32 mg), *Moringa pterigosperma* (32 mg), *Glycyrriza glabra* (12 mg) and powders of *Balsamodendron mukul* (324 mg), *Shankha bhasma* (64 mg) was used.

Cyclophosphamide (CP-CAS No.-6055-19-2), Endoxan- N Baxter Oncology, Germany, Batch No.-JN1045 was used as the positive control. All other chemicals were obtained either from Merck, SRL and Hi-media, India.

2.2. Animals

Swiss albino mice belonging to *Mus musculus* species, bred and maintained in the institutional animal house were used for the experiment. They were housed in polypropylene shoe box type cages, bedded with rice husk and kept in air-conditioned room, at 23° C (±2° C) and RH 50 ± 5%, were fed with a pelleted diet (Amruth Feeds, India) and water *ad libitum*. 8–10 weeks old animals with average body weight of 25 ± 2 gms were used for the experiments. Five animals (3 females + 2 males) were used for each treatment and control group in micronucleus assays. In sperm abnormality assay, 8 week old male animals were used. All groups of animals were kept under an absolute hygienic condition as per the recommended procedures by fulfilling the necessary ethical standards. Care and experimental procedures were conducted as per the guidelines of CPCSEA, India. *In vivo* animal studies were conducted after obtaining the prior approval from Institutional Animal Ethics Committee (IAEC) of Mangalore University (MU/AZ/99/2013-14/IAEC dt: 2.04.2013).

2.3. Dose and treatment schedule

Scheme of an appropriate dosing schedule and regimen should be based on clinical use, exposure pattern, pharmacokinetics and practical consideration. If the substance is genotoxic highest dose

level used will show the evidence of adverse effects and maximum tolerated dose is normally used to set this dose level. Accordingly, doses of septilin selected for this study were 125, 250 and 500 mg/kg b.w. The LD50 value of septilin has been reported as 1250 mg/kg b.w. in mice [38]. Different doses of septilin prepared in 0.5% carboxymethyl cellulose (CMC) were orally administered to different groups of animals in 0.2 ml quantity for 5 days at 24 h interval. CP the positive control agent (50 mg/kg b.w.) dissolved in sterile distilled water was administered intraperitoneally in 0.1 ml quantity on the 5th day, one hour after the last treatment of septilin. After 24 h, peripheral blood was drawn from the tail vein from all different groups of animals and then they were euthanized by cervical dislocation; bone marrow cells were extracted and processed for micronucleus assay. Sperm abnormality assay, was done after 35th day of last treatment. 0.5% CMC and distilled water administered groups were maintained separately which formed the negative controls.

2.4. Bone marrow MN assay

Bone marrow MN preparations were made by employing the modified method of Schmid [81,83] and by following the standard guidelines of OECD [65]. Here, 5% bovine serum albumin (BSA) prepared in phosphate buffered saline (PBS; pH-7.2) was used as suspending medium. The bone marrow suspension was centrifuged at 1000 rpm and pellet was resuspended in required quantity of BSA mixed thoroughly and drop of suspension was smeared on clean slides and air dried. The dried slides were fixed in methanol and stained with buffered (pH 6.8) May-Grunwald-Giemsa. 2000 polychromatic erythrocytes (PCE) and normochromatic erythrocytes (NCE) in the corresponding field were screened from each animal to score the MN and to determine P/N ratio.

2.5. Peripheral blood MN assay

The peripheral blood MN assay was done by using the method of Schlegel and MacGregor [82] by applying the standard guidelines of OECD [65]. The peripheral blood drawn from the tail vein were smeared on clean slides, dried and fixed in absolute methanol. The slides were stained with buffered Wright's-Giemsa (pH-6.8). About 2000 NCE per animal were scanned for the presence of MN and number of PCE present in the corresponding focus were scored from each animal to determine the effects of test agents on the erythropoiesis.

2.6. Sperm abnormality assay

The sperm abnormality assay was done according to the method of Wyrobek and Bruce [109] and Vega et al. [104] by following standard OECD guidelines OECD [66]. The doses of septilin and CP and treatment schedule were same as that of micronucleus tests. One post treatment sampling time *i.e.*, 35 days was used for this study. This is based on the principle that, the germ cells which are exposed at a late spermatogonial stage to the chemical, would reach the cauda epididymis after undergoing a series of changes during the course of development to give rise to sperms which are analysed for shape abnormalities and variations in the sperm count. Initial body weight of the animals was taken before the start of the experiments and final body weight was taken on 35th day just before euthanizing the animals. Animals were euthanized by cervical dislocation and the testes were dissected out and weighed. Both the cauda epididymis were removed and placed in a watch glass containing 1 ml phosphate buffered saline (pH = 7.2). The cauda epididymis was minced thoroughly and the suspension obtained was filtered through a fine mesh cloth to remove tissue debris and stained with 1% aqueous eosin for about 20 min. A drop of the sperm suspension

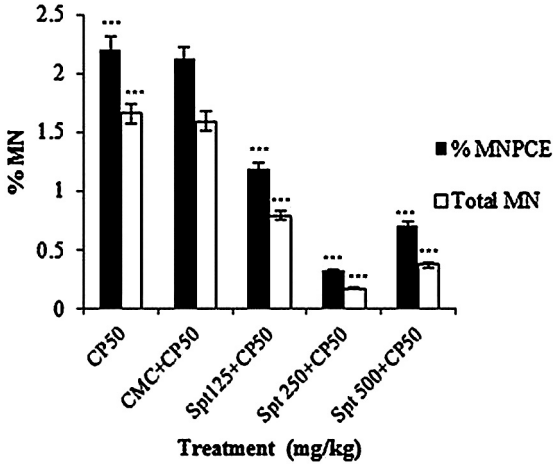

Fig. 1. Effect of septilin on MN PCE and Total MN induced by CP. ANOVA test, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

was smeared on a clean slide [105]. Sample is collected concurrently from all 5 animals, from each group. Two thousand sperms per animal were scored from each group for the presence of sperm shape abnormalities following the criteria of Wyrobek and Bruce [109]. For sperm count, an aliquot (0.05 ml) from the sperm suspension (1 ml) was diluted (1:40) with PBS and mixed thoroughly. Diluted sperm suspension was introduced into the Neubaur counting chamber and the total sperm count in 8 squares of 1 mm^2 was determined and multiplied by 5×10^4 to calculate the number of sperms per epididymis.

2.7. Statistical analysis

Statistical significance of the results was tested by comparing treatment groups with the respective control group by employing one way ANOVA and Dunnett's post hoc tests using Graph pad prism 5 (GraphPad Software, Inc., CA, USA). Differences with a P-value of 0.05 or lower were considered to be statistically significant.

3. Results

CP induced statistically significant MN in both bone marrow and peripheral blood cells (Tables 1 and 2). Septilin alone did not induce significant MN at any of the doses tested compared to CMC control. In the combined treatment group, septilin induced significant reduction in the MN frequencies in bone marrow cells at all doses tested (Fig. 1). In peripheral blood cells also in the CP + septilin treated groups, a significant reduction in MN in NCE (Fig. 3) and an increase in the mean percent PCE were observed (Fig. 5). Present study revealed the anticlastogenic potency of septilin against anticancer drug CP. All the three doses of septilin showed positive response as a protective agent against CP. Among these three doses 250 mg/kg b.w. dose was found to be more effective than other two doses (Figs. 2 and 4).

In sperm abnormality assay CP induced significant frequency of abnormal sperms. Different types of abnormal sperms like amorphous, hookless, folded, banana, double headed and double tailed were observed (Table 3). Significant reduction in body weight, relative testes weight and sperm count were also observed (Table 4). Septilin alone did not induce any significant effects on all the above mentioned parameters. In the combined treatment groups, different doses of septilin significantly reduced the frequency of abnormal sperms induced by CP. Furthermore, septilin has shown its protective effect on germ cells by increasing the body weight, relative weight of testes, as well as sperm count significantly (Fig. 6).

Table 1
Frequency of micronucleus and total MN in bone marrow cells of animals treated with different doses of septilin and CP and their respective controls at 24 h time interval.

Treatment mg/kg	MNPCE ± SEM (%)	% inhibition	Total MN ± SEM (%)	% inhibition	P/N ± SEM
Dist. Water	0.09 ± 0.02	–	0.05 ± 0.02	–	1.10 ± 0.02
CMC 0.5%	0.10± 0.01	–	0.06 ± 0.01	–	1.10 ± 0.02
Spt 125	0.04 ± 0.02	–	0.02 ± 0.02	–	1.16 ± 0.02
Spt 250	0.01 ± 0.01	–	0.01 ± 0.01	–	1.30 ± 0.02
Spt 500	0.01 ± 0.01	–	0.01 ± 0.01	–	1.22 ± 0.02
CP50	2.20 ± 0.02[c]	–	1.66 ± 0.02[c]	–	0.58 ± 0.01[c]
CMC+ CP50	2.12 ± 0.05	3.64 ± 0.02	1.59 ± 0.02	4.22 ±0.01	0.59 ± 0.00
Spt 125+ CP50	1.18 ± 0.05[c]	46.4 ± 0.02[c]	0.79 ± 0.06[c]	52.4 ±0.02[c]	0.85 ± 0.01[c]
Spt 250 + CP 50	0.32 ± 0.05[c]	85.5 ±0.01[c]	0.17 ± 0.02[c]	89.8 ±0.01[c]	0.91 ± 0.02[c]
Spt 500 + CP 50	0.70 ± 0.07[c]	68.2 ±0.03[c]	0.37 ± 0.01[c]	77.7 ±0.03[c]	0.87 ± 0.02[c]

ANOVA test, [a] $p < 0.05$, [b] $p < 0.01$, [c] $p < 0.001$.

Table 2
Results of peripheral blood MN test in animals treated with different doses of septilin and CP and their respective controls at 24 h time interval.

Treatment mg/kg	Mean%NCE	Mean% PCE	Mean% of MN in NCE ± SEM	% Inhibition
Dist. Water	98.1 ± 0.10	1.90 ± 0.09	0.05 ± 0.05	–
CMC 0.5%	98.0 ± 0.07	2.00 ± 0.09	0.03 ± 0.01	–
Spt 125	97.6 ± 0.06	2.40 ± 0.03	0.02 ± 0.01	–
Spt 250	97.8 ± 0.01	2.20 ± 0.09	0.02 ± 0.01	–
Spt 500	97.8 ± 0.10	2.20 ± 0.01	0.02 ± 0.01	–
CP50	98.9 ± 0.02	1.10 ± 0.03[c]	0.53 ± 0.03[c]	–
CMC+ CP50	98.8 ± 0.02	1.20 ± 0.03	0.49 ± 0.02	7.50 ± 0.03
Spt 125 + CP50	98.8 ± 0.02	1.30 ± 0.04[a]	0.28 ± 0.03[c]	47.2 ± 0.03[c]
Spt 250 + CP50	98.3 ± 0.09	1.70 ± 0.10[c]	0.09 ± 0.02[c]	83.0 ± 0.04[a]
Spt 500 + CP50	98.7 ± 0.04	1.50 ± 0.03[b]	0.18 ± 0.03[c]	66.0 ± 0.06[c]

ANOVA test: [a] $p<0.05$, [b] $p < 0.01$, [c] $p < 0.001$.

Table 3
Different types and total abnormal sperms induced by CP (50 mg/kg b.w.) and the ameliorating effect of septilin (5 weeks post treatment period).

Treatment* mg/kg	A	H	F	B	DH	DT	Total	% Abnormal sperms ± SEM
Dist. Water	76	44	2	0.	0	0	122	1.22 ± 0.05
CMC 0.5%	76	43	2	0	0	0	121	1.21 ± 0.05
Spt 125	55	48	0	0	0	0	103	1.03 ± 0.04
Spt 250	28	15	0	0	0	0	43	0.43 ± 0.03
Spt 500	41	17	0	0	0	0	58	0.58 ± 0.03
CP50	250	136	88	38	28	23	563	5.63 ± 0.24
CMC+ CP50	247	135	87	36	25	20	550	5.50 ± 0.22
Spt 125 + CP50	189	130	71	30	07	08	435	4.35 ± 0.03[c]
Spt 250 + CP 50	76	46	53	16	04	04	199	1.99 ± 0.20[c]
Spt 500 + CP 50	87	58	50	25	07	05	232	2.32 ± 0.03[c]

A—Amorphous; H—hookless; F—folded; B—Banana shaped; DH—Double Headed; DT—Double Tailed.
* Five animals in each group; 2000 sperms/animal; ANOVA test, [a] $p < 0.05$, [b] $p < 0.01$, [c] $p < 0.001$.

Table 4
Effect of different doses of septilin and controls on body weight, testes weight and sperm count in mice.

Treatment* mg/kg	Initial b.w(gms)	Final b.w(gms)	Testes wt. (gms) ± SEM	Relative wt. of testes ± SEM	Sperm count/epididymis ($\times 10^6$) ± SEM
Dist. Water	27.91	32.88	0.31 ± 0.01	0.94 ± 0.01	7.95 ± 0.15
CMC 0.5%	27.33	32.13	0.32 ± 0.02	0.99 ± 0.01	7.75 ± 0.42
Spt 125	26.14	31.60	0.32 ± 0.02	1.01 ± 0.01	8.00 ± 0.23
Spt 250	26.94	32.08	0.33 ± 0.04	1.03 ± 0.02	8.63 ± 0.23
Spt 500	26.78	31.22	0.32 ± 0.02	1.02 ± 0.01	8.13 ± 0.40
CP50	25.46	29.58	0.24 ± 0.01	0.81 ± 0.03	5.25 ± 0.38
CMC+ CP50	26.66	30.94	0.24 ± 0.01	0.78 ± 0.03	5.38 ± 0.40
Spt 125+ CP50	23.78	28.48	0.25 ± 0.02[a]	0.88 ± 0.06[a]	6.75 ± 0.23[c]
Spt 250 + CP 50	25.44	30.12	0.33 ± 0.01[a]	1.10 ± 0.03[a]	8.00 ± 0.23[c]
Spt 500 + CP 50	27.68	31.80	0.32 ± 0.01[a]	1.00 ± 0.03[a]	7.70 ± 0.15[c]

* Five animals in each group, ANOVA test, [a] $p < 0.05$, [b] $p < 0.01$, [c] $p < 0.001$.

4. Discussion

CP is a well known cytotoxic genotoxin. One of the useful strategies for the protection against chromosomal damage induced by this type of agents is using the compounds which are capable of preventing the future damages or repairing an already made damage [18]. In the present investigation, septilin was used to study its effect on the cytogenetic damage induced by CP.

Septilin is an ayurvedic herbomineral formulation which has a number of beneficial health effects. It consists of six different medicinal plant extracts. These plants in their individual capacities also have medicinal properties. In view of this, septilin was selected to study its protective effects against CP induced genotoxicity. In the present study, it demonstrated effective chemoprotective properties against the CP induced clastogenecity in *in vivo* mouse and

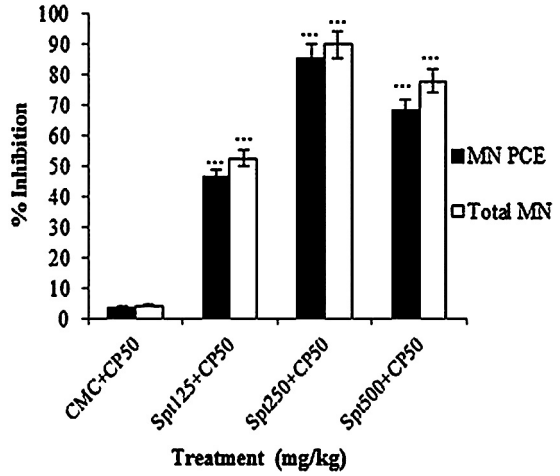

Fig. 2. % inhibition of CP induced MN by septilin. ANOVA test, *p < 0.05, **p < 0.01, ***p < 0.001.

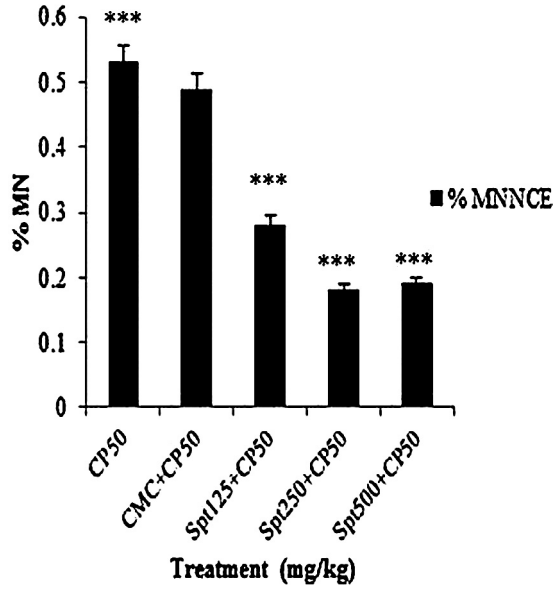

Fig. 3. Effect of septilin on MN NCE induced by CP. ANOVA test, *p < 0.05, **p < 0.01, *** p < 0.001.

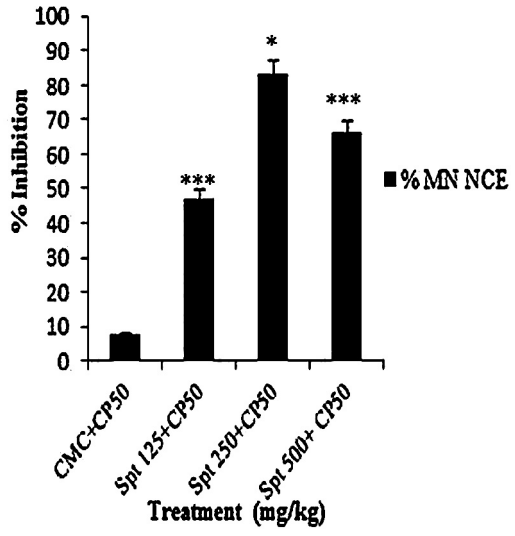

Fig. 4. % inhibition of MN NCE in peripheral blood by septilin in combined treatment. ANOVA test, *p < 0.05, **p < 0.01, *** p < 0.001.

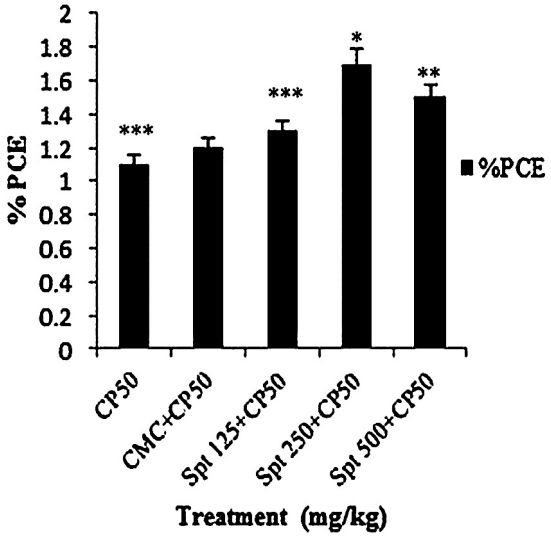

Fig. 5. Effect of septilin on% of PCE in peripheral blood with CP alone and with combination of septilin. ANOVA test, *p < 0.05, **p < 0.01, *** p < 0.001.

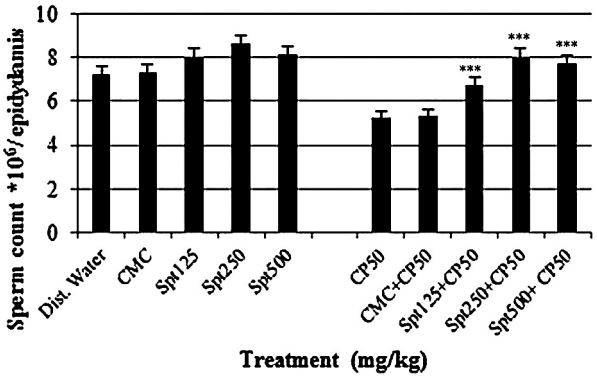

Fig. 6. Effect of septilin on sperm count with CP alone and with combination of septilin. ANOVA test, *p < 0.05, **p < 0.01, *** p < 0.001.

it did not induce any cytotoxic effects. The results revealed the anticlastogenic and genoprotective effects of septilin.

There are some reports on the beneficial cellular effects of septilin. Kumar et al. [47] reported the chemoprotective action of septilin against CP induced toxicity in mouse. Septilin improved the total WBC count, haemoglobin levels and bone marrow cellularity in the CP treated animals. In another study, they showed the immunopotentiating effects of septilin [46]. It augmented the cellular immune responses and activated the humoral responses [48].

Ameliorative effect of septilin has been proved against gamma-irradiation induced oxidative stress and tissue injury in rats. In this study septilin administration for five consecutive days prior to gamma irradiation restored the depleted levels of cellular antioxidants and lowered the radiation induced lipid peroxidation [55]. Septilin has been reported as an antioxidant against radiation induced sickness and mortality and also increased the GSH level and reduced the lipid peroxidation. Septilin also showed anti-inflammatory and analgesic effects [45].

In the present study solvent controls dist. water, 0.5% CMC and septilin alone groups did not induce any significant effects on different parameters used. In turn, different doses of septilin induced protective effects against the CP induced genotoxic effects.

There are many reports on the protective effects of individual plants present in the septilin. Parvaiz et al. [68], Roshan et al. [76] and Saxena [80] reviewed the medicinal properties of *Glycyrrhiza*

glabra including anticancer and antioxidant effects. There are several review articles on the pharmacological properties of *Glycyrrhiza glabra* and its bioactive compounds [16,2,5,30,43,102,34].

Tripathi et al. [101], Damle [16] and Devprakash et al. [19] reviewed the phytochemical and pharmacological profile of *Tinospora cordifolia* (TCE). Sharma et al. [88] reported the radiation induced testicular injury and its amelioration by *Tinospora cordifolia* extract. Pretreatment with TCE protected the testicular cells from radiation damage. The effect is attributed to various factors especially efficient free radical scavenging. In the present study, septilin showed the protective effects against CP induced chromosomal damage. Probably the mechanism of action may be similar to the radiation protection events. *Tinospora cordifolia* also showed protective action against radiation induced biochemical alterations in liver. Irradiation of mice resulted in the elevation of biochemical parameters whereas; pretreatment with *Tinospora cordifolia* restored such alteration compared with radiation alone treated group [85]. Pretreatment with the extract of *Tinospora cordifolia* ameliorated the irradiation induced histopathological changes in mice ovarian tissue showing its protective effect against gamma radiation induced tissue damage [84].

Deoda et al. [20], evaluated the pharmacognostic, phytochemical and pharmacological properties of *Rubia cordifolia,* another plant component of septilin. Priya and Siril [71], also studied the pharmacognostical and phytochemical characters of *Rubia cordifolia* and further they reviewed the different medicinal properties of *Rubia cordifolia* in the treatment of various ailments. [41], suggested that *Rubia cordifolia* can be used as an adjuvant with chemotherapeutic drugs since, in their study it showed the reduction in the renal damage induced by chemotherapeutic drug cisplatin. As stated by them, antioxidant and free radical scavenging properties of *Rubia cordifolia* may be exerted a protective effect against cisplatin induced nephrotoxic effects. Tripathi and Singh [100] observed the protective effects of alcoholic root extract of *Rubia cordifolia* against radiation induced lipid peroxidation, hemopoietic injury and genotoxicity. Tripathi and Singh [100] observed the radioprotective effects of alcoholic root extract of *Rubia cordifolia*. In this study, he showed the increased rate of animal survival and decreased level lipid peroxidation and also micronuclei in extract supplemented groups there by protecting against radiation induced lipid peroxidation, hemopoietic injury and genotoxicity. [52] evaluated the *in vivo* antioxidant effects of alcoholic extracts of *Rubia cordifolia* against lead nitrate induced free radical mediated toxicity simultaneously by increasing the activity of SOD, CAT, and GSH in liver and testis of mice and also by decreasing the lipid peroxidation.

There are several reports in the genoprotective effects of *Emblica officinalis*. *In vivo* protective effect of *Emblica officinalis* extract against the genotoxicity of benzo[*a*]pyrene and CP was studied by using bone marrow chromosomal aberration and micronucleus assays in mice [86]. Banu et al. [8] noticed the protective effect of ethanolic extract of *Emblica officinalis* fruit extract in Swiss albino mice thereby reducing the frequency of bone marrow micronuclei and increased the liver antioxidants induced by 7,12-dimethylbenz(*a*)anthracene (DMBA) a rodent carcinogen which inturn showed its antigenotoxic effect. Dasaroju and Gottumukkala [17] reviewed the pharmacological profile of *Emblica officinalis*. Chakraborty and Verma [13] evaluated the ameliorative effect of aqueous extract of *Emblica officinalis* against ochratoxin induced spermatotoxic effects in mice. Protective effect of *Emblica officinalis* is further encouraged by Chakraborty and Verma [13]. In their study, plant extract alleviated the ochratoxin-induced reproductive alterations such as decreased sperm count, motility and viabilty in mice. This possible effect is mainly due to the free radical scavenging property of this plant.

Sathya et al. [79] demonstrated that phytoconstituents present in the of *Moringa oleifera* Lam./*Moringa pterygosperma* leaves rendered protection against cyclophosphamide induced micronucleus and DNA damage in the bone marrow and liver tissue of mice. Tejas et al. [97] and Anwar et al. [6] reviewed the pharmacognostic, pharmacological, nutritional and therapeutic properties of *Moringa pterygosperma.* Also, Luqman et al. [54], studied the antistress, antioxidant, and scavenging potential of *Moringa pterygosperma* by using *in vitro* and *in vivo* assays. As a result, the ethanolic fruit extract showed strong free radical scavenging capacity in both *in vitro* and *in vivo* conditions.

Rout et al. [78] reviewed the medicinal and therapeutic properties of *Blasmodendron mukul* (guggul). Xiao and Xiao [112] reported the potent chemopreventive and chemotherapeutic properties of guggul on human prostate cancer cell lines.

In Indian traditional system of medicine, Shankha bhasma is used for therapeutic purposes. It is a powder prepared by incinerating the conch shell containing different calcium salts [31,96]. Also, Vardini et al. [103] proved the nongenotoxic properties of bhasmas by using *in vivo* bone marrow micronucleus assay and alkaline comet assay to assess clastogenic and aneugenic effects and also to detect the intensity of DNA damage in Wistar rats.

CP is a well-known anticancer, chemotherapeutic and immunosuppressive drug, which was used as a clastogenic agent in the present study. The results obtained in this study revealed the protective effects of the septilin against the genotoxicity of CP in mouse. Administration of CP alone induced significant micronuclei in both bone marrow and peripheral blood cells and also it reduced the P/N ratio in bone marrow cells. Although CP has potent therapeutic value it also shows wide spectrum of cytotoxicity against normal cells which is a hindrance for its usage. The cytotoxicity of drug is mainly due to its ability to damage DNA. CP is an alkylating agent and its alkyl group can bond with nucleic acids resulting in DNA strand breaks, micronuclei formation and ultimately by the cell death [37]. CP showed significant effects in all the parameters used. In the combined treatment groups, septilin significantly reduced MN induced by CP and it enhanced the P/N ratio significantly. In the peripheral blood also CP significantly reduced the mean percent of PCE, and also it reduced the frequency of MN in NCE induced by CP.

The sperm morphology assay, is one of the most widely used genetic toxicology assays, has potential in identifying chemicals that induce spermatogenic dysfunction and perhaps heritable mutations Wyrobek [107]. Furthermore, the development of sperm head abnormality has been used as a reliable short-term biological indicator in the evaluation of chemical genotoxicity [53,29]. In the present findings, significant increase in the percentage of sperm head abnormalities, reduced relative testes weight and sperm count were observed in the CP treated group. Pretreatment of animals with septilin significantly decreased the percentage of abnormal sperms and there was an increase in the sperm count and relative testes weight, which demonstrate the protective effect of septilin on germinal cells.

Although it is difficult to elucidate the exact mechanism of antigenotoxic effect of septilin, it could be possible to explain the reasons for these effects based on some of the related studies conducted by different investigators. Vilar et al. [106] studied the antimutagenecity protection of *Ginkgo biloba* extract against mitomycin C and CP in mouse bone marrow. According to them there are some common mechanism of action of plant extracts such as antioxidant activity, free radical scavenging and even the gene regulation which contributes its direct or indirect anti mutagenic effects. CP is an alkylating anticancer drug which is activated *in vivo* by passing through various metabolic steps producing reactive molecules. Scavenging of these reactive molecules is one of the important techniques in the armoury of antimutagenesis. This may

be one reason for the protective effect of septilin. Similar hypothesis has been given by Hosseinimehr and Karami [37] while reporting the chemoprotective effects of captopril against CP induced genotoxicity. They reported the reduction of CP induced genotoxicity in bone marrow cells and they opine that this effect is due to the antioxidant activity of thiols in captopril. In our own study, we observed the antioxidant effects of septilin against CP, as revealed by significant enhancement of liver SOD and GSH in the combined treatment group (to be published elsewhere). Alija et al. [3] studied the cytotoxic and genotoxic effects of β- carotene and its cleavage products (CP) in rat hepatocytes. They opine that the genotoxic effects may be due to the prooxidant properties of CP or direct or indirect action on DNA. Delarmelina reported the antimutagenic activity of ipriflavone against DNA damage induced by CP in mice. Probable mechanisms proposed by them are desmutagenesis or bio antimutagenesis. Although in the present study it is difficult to elucidate the mechanisms, septilin is containing extracts of various plants which possess flavonoids thereby eliciting protective effects. There are several reports on such effects of plant extracts, bioactive constituents and natural compounds. Flavonoids are the phenolic compounds which are naturally found in fruits, vegetables and other plant parts. They have many favourable biological effects due to their antioxidant and free radical scavenging abilities. However, at higher doses they act as prooxidants. Prooxidants show cytotoxic and clastogenic properties [113]. Tourino et al. [99] reported the antioxidant/prooxidant effects of bioactive polyphenolics. According to them the most effective antioxidants are also the most cytotoxic and effective anti-proliferative agents, may be due to the dual antioxidant/prooxidant effect of polyphenols. Although, various doses of septilin showed significant genoprotective effect against CP, maximum effect was observed at middle dose (250 mg/kg b.w.) and there was no increased effect at highest dose. There are several reports on the phytocomponents of individual plants extract present in septilin, which are known biological protective agents. Sharma and Agrawal [87] studied the antigenotoxic effects of *Glycyrrhiza glabra* root extract against CP induced chromosomal aberration in Swiss albino mice. According to them the protective effect is due to the reduced immunosuppressant effects of CP by *Glycyrrhiza glabra* and presence of the phytotherapeutic molecules such as flavonoids, tannins, saponins, triterpinoids in this plant. Parvaiz et al. [68] also reported the presence of these compounds in a review on this plant. Pendli et al. [69] carried out phytochemical analysis of extracts of various plant parts of *Rubia cordifolia* Promkum et al. [72] studied anticlastogenic effects of *Moringa oleifera* against mitimycin C (MMC) and 7, 12-dimethyl benz(*a*)anthracene, potent clastogens. They are of the view that the protective effect of this plant due to the presence of various nutrients and phytochemical compounds such as β-carotene, vitamins and various phenolics. Torres-Castillo et al. [98] reported the presence of polyphenols, alkaloids and saponins in *Moringa oleifera*.

Our study revealed the protective effects of septilin against CP induced damage in both somatic and germinal cells which may be due to the synergistic effect of different compounds and phytochemicals present in septilin. [60] reported the synergies in the inhibition of cancer cell proliferation by tea catechin.

As already described septilin is a combination drug, containing some medicinal plant extracts along with powdered shankha bhasma and *Blasmodendron mukul*. The positive responses obtained in the present study, may be due to the synergic effects of these various compounds.

Synergy is the combined action of different components of a medicine, leading to potentiating effects which means the effect of combination is greater than the sum of individual effects. One such example in natural products is the synergy between Cinchona alkaloids as reviewed by Rasoanaivo et al. [77]. In antimutagenicity/anticlastogenicity studies also several authors proposed the synergistic effects of individual plants/their constituents in bringing out the protective effects. Cevallos et al. [12] observed the chemo protective effects of spirulina against CP induced mutagenicity in mouse test system. The positive response obtained in this study is hypothesised as due to the synergistic action of wide spectrum of antioxidants present in the algal extract. In the present context anticlastogenic effects shown by the septilin may be due to the synergistic effects of bioactive photochemical components present in the plants.

In conclusion, our study revealed that septilin, a natural drug formulation has a potential to inhibit the clastogenic damage induced by an anticancer drug, cyclophosphamide. Hence, it could be used as an adjuvant with toxic chemotherapeutic drugs to enhance their effectiveness.

Conflict of interest

The authors declare that there are no conflicts of interest.

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