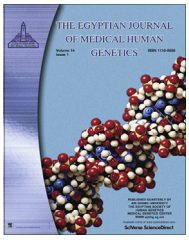
The Egyptian Journal of Medical Human Genetics (2013) **14**, 247–257

Ain Shams University

The Egyptian Journal of Medical Human Genetics

www.ejmhg.eg.net
www.sciencedirect.com



ORIGINAL ARTICLE

Does *Caesalpinia bonducella* ameliorate genotoxicity? An *in vitro* study in human lymphocyte culture and *in vivo* study in Albino mice

Md. Sultan Ahmad [a], Sheeba Ahmad [b], Afsar Ali [a], Mohammad Afzal [c,*]

[a] *Department of Zoology, S.N. (PG) College, Azamgarh, 276001 UP, India*
[b] *Department of Zoology, D.S. College, Aligarh, 202002 UP, India*
[c] *Section of Genetics, Department of Zoology, Faculty of Life Science, Aligarh Muslim University, Aligarh, 202002 UP, India*

Received 31 March 2013; accepted 21 April 2013
Available online 10 June 2013

KEYWORDS

Antitumor;
Ayurvedic medicine;
Caesalpinia bonducella;
Chromosomal aberration;
Sister chromatid exchange;
Replication index

Abstract Most of the world's populations residing in developing countries depend on alternative medicine and use of plant ingredients. The plant *Caesalpinia bonducella* belongs to the family of Caesalpiniaceae and it is commonly known as Natakaranja in Hindi. It contains bonducin and two phytosterols namely sitosterol and hepatsane. The twigs and young leaves of *C. bonducella* are rationally used for curing tumors, inflammation and liver disorders. In our present work alcoholic extracts of this ayurvedic plant were tested for their antimutagenic and anticarcinogenic properties. The aim of the study is to investigate the antimutagenic and antigenotoxic potential of alcoholic extracts of *C. bonducella* against methyl methane sulfonate (MMS) induced genotoxicity. In this experiment we have used *in vitro* method i.e., human lymphocyte culture and *in vivo* method in bone marrow cells of albino mice, while the parameters studied included chromosomal aberrations (CA), sister chromatid exchanges (SCEs) and cell growth kinetics (RI) both in the presence as well as in the absence of exogenous metabolic activation system for *in vitro* studies whereas total aberrant cells and the frequencies of aberrations were used for *in vivo* methods. Alcoholic extracts of *C. bonducella* significantly reduce chromosomal aberration from 42.75%, 44.25%, and 51.75% levels [at 24, 48, and 72 h due to methyl methane sulfonate (MMS)] to 28.50%, 30.25%, and

Abbreviations: MMS, methyl methane sulfonate, CA, chromosomal aberrations, SCE, sister chromatid exchange, RI, replication index, DMSO, dimethylsulfoxide, CE, alcoholic extracts of *Caesalpinia bonducella*.

* Corresponding author. Tel.: +91 571 2700920/3442.
E-mail address: afzal1235@rediffmail.com (M. Afzal).

Peer review under responsibility of Ain Shams University.

ELSEVIER Production and hosting by Elsevier

http://dx.doi.org/10.1016/j.ejmhg.2013.04.002

35.10%, respectively similarly sister chromatid exchanges were reduced from 7.70 ± 1.50 to 5.20 ± 1.50 at 48 h of treatment and replication index was enhanced *in vitro* for each concentration and duration of treatment and their ameliorating potential was dose and duration dependent. Similarly these extracts significantly reduced the number of aberrant cells and frequency of aberrations per cell in *in vivo*.

1. Introduction

It is estimated that about 85% of people living in developing countries depend on traditional medicine [1]. These are used not just in rural areas of developing countries, but also in developed countries where modern medicines are predominantly used [2]. Plants used in traditional medicine contain a wide range of ingredients that can be used to treat chronic as well as infectious diseases. The medicinal value of plants lies in certain chemical substances that produce a definite physiological action on the human body. The most important among these bioactive compounds of plants are alkaloids, flavonoids, tannins and phenolic compounds [3].

Caesalpinia bonducella belongs to the family of Caesalpiniaceae and is commonly known as Natakaranja in Hindi. It contains bonducin, two phytosterols, namely sitosterol and hepatsane along with alkaloids, flavonoids, glycosides, saponins, tannins and triterpenoids [4,5]. The twigs and young leaves of *C. bonducella* are tradionally used for the treatment of tumors, inflammation and liver disorders. In addition, various parts of this plant have been reported to possess multiple therapeutic properties like antiproliferatve [6], antidiabetic [7], hepato-protective, antitumor and antioxidant activities [8].

The rich and diverse plant sources of India are likely to provide the effective anticancer agents. The best approaches in searching for anticancer agents from plant resources are the selection of plants based on ethno-medical leads and testing the selected plants' efficacy and safety in the light of modern science. Exploration of traditional medical practices in Tamilnadu brought to light the ethnomedical use of young leaves of *C. bonducella* to treat certain tumors in a few remote villages in Kolli Hills, Nammakal district of Tamilnadu, India. This plant is also known as an antitumor agent in Ayurveda, an ancient system of medicine [9].

In Malaysia, the young leaves are used in the treatment of intermittent fevers, and for expelling intestinal worms. In Ceylon, they are applied for toothache, and also given for worms in children. In Guinea, the boiled leaves are used as a gargle for sore throat [10]. Finely powdered leaves are prescribed as a uterine tonic after child birth.

In studies done earlier [11], no significant variation in the body weight and weight of organs between the control and the treated group was observed following 28 days of treatment. Hematological analysis and clinical blood chemistry revealed no toxic effects of the extract. Pathologically, neither gross abnormalities nor histo-pathological changes were observed. Further no mortality was recorded in 28 days. In the present study, the acute toxicity studies of methanolic extract of *C. bonducella* were conducted in albino mice as per the organization for economic cooperation and development (OECD) guidelines 420 where the limited test dose of 2 gm/kg was used.

2. Materials and methods

The whole plants of *C. bonducella* were dried under shade at room temperature. The shade dried plants were powdered; around 60 gm of coarse powder was defatted with petroleum ether and were extracted exhaustively with 95% methanol at 60 °C. The extract was dried by vacuum evaporator. Methanolic extract of *C. bonducella* was dissolved in dimethyl sulfoxide (DMSO) to prepare different optimum concentration for studies.

2.1. In vivo method

Albino mice 8–10 weeks old (25–35 gm in weight) were exposed to different test chemicals by appropriate routes (intra peritoneal i.e., I.P injection) and were sacrificed at sequential intervals of 16, 24, and 32 h of stipulated treatment time. Animals were treated with each test substance as shown in the tables. The samples were taken separately after each treatment as mentioned above. The central sampling interval was 24 h, since cell cycle kinetics could be influenced by test substances. The early and late sampling intervals were adequately spaced within the range of 6–48 h. The additional dose levels were tested in subsequent experiments; these samples were taken at the predetermined duration.

2.1.1. Preparation of slides

Immediately after the albino mice were sacrificed, the bone marrow contents were obtained by standard procedure and exposed to hypotonic solution and cells were fixed in Carnoy's fixative. Chromosome preparations were made from bone marrow cells following the standard procedure as described. The stained slides were examined and metaphase cells were scored for chromosomal aberrations. Prior to sacrifice, mice were further treated with colchicines, a spindle inhibitor to arrest the cells in C-metaphase. The slides were stained in 10% aqueous Giemsa solution and 100 bone marrow metaphase cells from each animal were scored under code. The types of chromosomal aberrations considered were chromatid and chromosome gaps, breaks, fragments, exchanges and pulverization (severely damaged cells). The reduction factors due to test chemical treatments were calculated using the formula:

$$\%\ \text{Reduction} = \frac{(\text{Aberrant cells in control} - \text{aberrant cells in MMS and test chemicals})}{(\text{Aberrant cells in control} - \text{aberrant cells in negative control})}$$

Control groups:

Concurrent positive and negative controls were included in this study. Whereas the positive control includes a single dose of MMS, a compound known to produce chromosomal aberrations *in vivo,* the negative control did not include any treatment.

2.2. In vitro lymphocyte culture method

Most of the cytogenetic studies were carried out involving the examination of metaphase chromosomes. The evaluation of chromosomal damage at metaphase stage gives a more precise and detailed picture of the clastogenic agent than anaphase or telophase stage. Human peripheral blood lymphocytes are extremely sensitive indicators of the *in vitro* assay system. The chromosomal changes (numerical and structural) were utilized for an investigation of the genotoxic as well as antigenotoxic potentiality of test chemicals. The parameters studied included chromosomal aberrations (CA), sister chromatid exchanges (SCEs) and cell growth kinetics (RI) both in the presence and in the absence of exogenous metabolic activation system.

2.2.1. Preparation of S_9 liver/microsome fraction

The S_9 liver/microsome fraction formed the exogenous metabolic activation system utilized for the present investigation and was prepared as follows.

2.2.1.1. Induction of rat liver enzyme. For preparing S_9 fraction, the standard procedures as recommended by Moron and Ames [12] were followed. Swiss albino healthy rats (Wister strain, obtained from animal house, Biotech, Varanasi, India) each weighing about 200 gm were given 0.1% (1 mg/ml) of phenobarbital in drinking water for one week for the induction of liver enzymatic activities.

2.2.1.2. Removal of livers from the rats. The rats were sacrificed through cervical dislocation. In order to ensure the sterility of S_9 preparation, the livers were removed aseptically using sterile surgical tools in a laminar flow cabinet. Utmost care was taken to avoid a cut across the esophagus or the intestine, which could otherwise cause the contamination of liver homogenate. The removed livers were immediately placed in 0.15 M KCl chilled solution in a culture tube.

2.2.1.3. Preparation of liver homogenate fraction. The whole procedure was carried out at 0–4 °C using sterilized solutions and glasswares. The livers were washed in chilled KCl several times to remove traces of hemoglobin, which inhibits enzyme activity. The washed livers were transferred to a beaker containing three volumes of 0.15 M KCl (3 ml/g wet liver) and after mincing with a sterile scissors, these were homogenized by a tissue homogenizer at 4 °C. The homogenate was centrifuged in a refrigerated centrifuge for 10 min at 9000 rpm. The supernatant (S_9 fraction) was decanted and saved as 1 ml aliquots in polypropylene storage vials and stored in liquid nitrogen till further use.

2.2.1.4. Preparation of S_9 mix. The S_9 fraction was prepared freshly every time for use in the culture. The S_9 fraction was complemented with 8 μM NADP, 100 μM Na_2HPO_4 of buffer at 7.4 pH. About 0.8 ml of S_9 mix was added every time along with the test chemicals to the cultures.

2.2.2. Chromosomal aberrations

2.2.2.1. Preparation of culture media. Tissue culture medium RPMI–1640 (flow laboratories) with L-glutamine and Hepes buffer without $NaHCO_3$ was prepared in advance and stored at 4 °C, but the storage period never lasted longer than a week. About 1.574 gm of medium was dissolved in 100 ml of double distilled water by gentle shaking. Antibiotics, penicillin (100 IU/ml) and streptomycin (100 IU/ml) (Hoechst) were also added and pH was adjusted at 6.8–7.2 with N/10 $NaHCO_3$ and HCl. The medium was filtered and sterilized using Millipore filtration assembly by 0.45 μm Millipore size. The filtered medium was then stored in sterilized tightly capped glass bottles.

2.2.2.2. Collection of blood samples. Peripheral blood from the healthy donors was taken fresh every time through veinal puncture under aseptic conditions (disposable needle and disposable syringes, Unitech) and Heparin (500 IU/ml; Micro Lab) was used as anticoagulant. The tightly capped glass vials were gently mixed and stored at 4 °C for half an hour to separate blood cells from plasma.

2.2.2.3. Setting of the cultures. Lymphocyte culture was carried out by adding 0.8 ml of plasma containing white blood cells (WBC) in 4.5 ml of culture medium supplemented with 0.1 ml phytohemagglutinin–P (PHA–P, Micro lab) and 15% fetal calf serum (Gibco). The culture vials were then tightly capped to avoid CO_2 loss and after gently mixing, culture tubes were incubated at 37 °C in dark and colchicine was added 2 h prior to harvesting for arresting the cells at metaphase stage.

2.2.2.4. Harvesting of the cultures. After appropriate durations, the cultures were taken out from the incubator and their contents, after gentle shaking, were transferred to a centrifuge tube, the cells were spun down by centrifugation for 10 min, at 1200 rpm, and pellets saved by discarding the supernatant. Hypotonic treatment (0.075 M KCl) was carried for 10–12 min at 37 °C and the cells were recollected by centrifugation. The cell pellet was suspended in 5 ml of freshly prepared chilled fixative (3:1 of methanol and acetic acid), which was added drop by drop with a pipette while continuously shaking the tube to avoid formation of clots. In order to ensure proper fixation, the cells were kept suspended in the fixative for a minimum period of one hour though preferably overnight. Three changes with fresh fixative were given before preparing the slides.

2.2.2.5. Slide preparation and staining. After giving final washing in the fixative, the cells were re-suspended in 0.2 ml of fresh fixative. Two or three drops of cell preparation were dropped over clean, grease free, pre-chilled and wet microscopic slides and air-dried. One-day-old slides were stained in Giemsa (Sigma) for 15 min and rinsed in 95% alcohol and finally in the absolute alcohol for proper differentiation. After proper air-drying these slides were dipped in xylene for 5 min before mounting in dibutyl phathalate xylene (DPX).

2.2.2.6. Analysis of the cells. In order to avoid the bias in scoring the chromosomal anomalies before and after treatment of different test chemicals, all slides were coded prior to scoring. A total of 300 well spread metaphase cells were analyzed for each concentration of the test chemicals and for each time of duration to analyze various chromosome and chromatid type aberrations using the method of Evans [13].

2.2.3. Sister chromatid exchange analysis

Sister chromatid exchange is a sensitive rapid and objective method of observing reciprocal exchange between sister chromatids. This method depends upon the phenomenon of 5-bromo-2-deoxyuridine (BrdU) incorporation into DNA in place of thymidine. After two rounds of cell division, the chromatids were labeled with BrdU and consequently differentially stained with Hoechst. The BrdU incorporation quenches the fluorescence of 33,258 Hoechst. Therefore, the light energy is absorbed but not emitted by such dyes, which results in the reduced staining of chromatid with Giemsa [14].

2.2.3.1. Labeling of chromosomes with BrdU. The sister chromatid exchange analysis was carried out following the standard procedure of Latt et al. [15]. The cells in the culture were exposed to nucleoside, BrdU (Sigma) after 24 h of culture initiation at the final concentration of 2 μg ml. The culture vials were tightly capped and covered with an aluminum foil to avoid light exposure and incubated at 37 °C for another 48 h in dark.

2.2.3.2. Slide preparation and staining. After 2 h of colchicine treatment, the cultures were harvested and processed following the same method as described for the chromosomal aberration analysis. For the differential staining of SCEs the methods of Latt et al. [15] with slight modifications were followed. One-day-old slides were dipped in 0.5 μg/ml of 33,258 Hoechst stain (Sigma) dissolved in double distilled water in a horizontal couplin jar. The slides were then put in a flat glass dish with the layer of cells facing upward. These were covered with a thick layer (2–3 cm) of phosphate buffer (pH 6.8) and exposed to UV lamp (15 W, 254 μm, Philips) from a distance of 10–15 cm for 30–45 min. The slides were taken out from the buffer, washed twice in double distilled water and air-dried. These were then incubated in 2× SSC (0.3 M NaCl, 0.03 M sodium citrate, pH 7.0) at 65 °C in water both for 90 min using vertical couplin jars. The slides were taken out and rinsed in distilled water. The air-dried slides were then stained in Giemsa for 20 min and rinsed in 90% alcohol followed by rinsing in absolute alcohol. The dried slides were dipped in xylene for 5 min and mounted in DPX.

2.2.3.3. Analyses of the cells. All slides were coded prior to scoring so as to avoid any ambiguity. Around 50 metaphases (25 metaphases/donor) with differentially stained chromatids were scored for each test chemical treatment in the absence of S_9 mix and 50 metaphases were scored for each treatment in the presence of S_9 mix. The interstitial exchanges between two sister chromatids were scored as two exchanges and the terminal exchanges were scored as a single exchange. Student's '*t*' test was applied for calculating the significance of difference between the treated and the controls.

2.2.4. Cell cycle kinetics analysis

The cells undergoing first (M_1) second (M_2) and third (M_3) divisions were detected by studying the BrdU labeled and differentially stained chromosomes, following the method of Crossen and Morgan [16]. The cells with both the chromatids being darkly stained were scored as M_1 cells, those with one dark and one lightly stained chromatid were taken as M_2 cells and those having a mixture of both the differentially stained and uniformly stained chromatids were scored as M_3 metaphase. Around 100 well spread metaphases were scored for each concentration and each treatment of duration from each donor in the absence as well as in the presence of S_9 mix. The replication index (RI) was calculated according to the formula of Tice et al. [17] as given below. The deviation from the controls was determined by using Chi-square (χ^2) test.

$$\text{R.I} = \frac{(M_1X1) + (M_2X2) + (M_3X3)}{100}$$

2.3. Statistical analysis

Standard deviation (SD) and standard error (SE) were calculated using the following formula.

$$\text{Mean } (\bar{X}) = \frac{\Sigma X}{n}$$

$$\text{Variance } \delta^2 = \frac{\Sigma(X^2 n.X^2)}{n-1}$$

$$\text{S.D.}(\delta) = \sqrt{\frac{\Sigma X^2 - nX^2}{n-1}}$$

$$\text{SE} = \frac{\text{SD}}{\sqrt{n}}$$

where,

X = variable.
N = number of observation.
ΣX^2 = sum of square of individual variables.
$\bar{X}$ = mean of variables.
δ = standard deviation.

Chi-square (χ^2) test for homogeneity test of variance was used to analyze the cell growth kinetics exchange with the normal control. The level of significance was tested from standard statistical tables of Fisher and Yates [18].

Student's two-tailed '*t*' test was used for calculating the statistical significance in SCE and chromosomal aberrations by comparing the effect induced by different test chemicals with the respective control. The following formula was used for this purpose.

$$t = \frac{\bar{X}(\text{control}) - \bar{X}(\text{treated})}{\sqrt{(\text{SE of control})^2 + \sqrt{(\text{SE of treated})^2}}}$$

The statistical significance was calculated from Fisher and Yates table at $(n_1 + n_2 - 2)$ degree of freedom (df) at 0.05% level of significance.

2.4. Table of chemical concentration

(A) *Control*

Positive and negative control	Concentrations
MMS	5 μg/ml
Dimethyl sulfoxide (DMSO)	5 μg/ml

(B) *In vivo concentrations of phyto-chemicals*

Phytoproducts (mg/kg bw)	1st dose	2nd dose	3rd dose	4th dose	5th dose
Caesalpinia extracts	CE_1 200	CE_2 250	CE_3 300	CE_4 350	CE_5 400

(C) *In vitro concentrations of phyto-chemicals*

Phyto-products (μg/ml)	1st dose	2nd dose	3rd dose	4th dose
Caesalpinia extracts	CE_1 150	CE_2 200	CE_3 250	CE_4 300

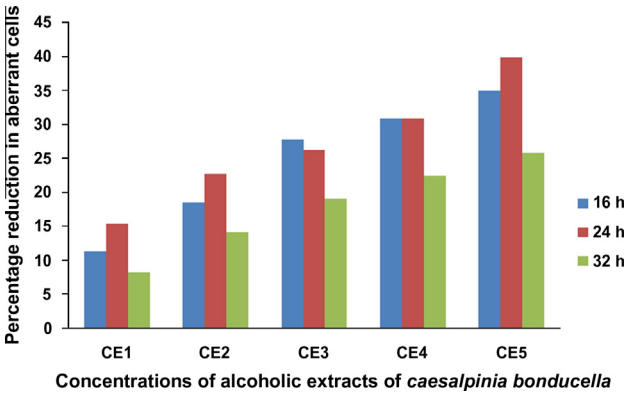


Figure 1 Shows effects of five increasing concentrations of alcoholic extracts of *Caesalpinia bonducella* (CE) at 16, 24, and 32 h of treatment durations in the bone marrow cells of albino mice *in vivo* against methyl methane (MMS) induced clastogeny.

3. Results

3.1. *In vivo* effects

In these *in vivo* experiments we have selected five most optimum concentrations of alcoholic extract of Caesalpinia and their effects on chromosomal aberrations given at three different durations were calculated and are presented in the tables.

As shown in Table 1 for 16 h of treatment duration, the percentage of aberrant cells were 11.0% due to treatment with MMS which were reduced to 8.6%, 7.9%, 7.0%, 6.7%, and 6.3% respectively for five consecutive concentrations of Caesalpinia extract. The fragment types of aberrations were most prominent followed by breaks and gaps. Similarly reduction in the frequencies of aberrations put the values at 11.34%, 18.55%, 27.83%, 30.92%, and 35.05% against five different concentrations of Caesalpinia extract respectively. The trend of effects was linear i.e., as concentration of Caesalpinia extract increases the effect also increases (Fig. 1). The gross effect on the total number of frequencies per thousand cells was 155, 132, 115, 105 and 92 of five different concentrations of Caesalpinia extract against 199 of MMS alone. The normal values were 23 for distilled water treatment and 35 and 47 for DMSO and Caesalpinia extract treatment alone (Table 2).

When the treatment duration was increased to 24 h, the effects were similar, with increasing values. The observed values were 9.3%, 8.5%, 8.1%, 7.6%, and 6.9% due to five increasing concentrations of Caesalpinia extract, against 11.0% of MMS alone. The normal values were 3.0%, 3.5%, and 3.5% for pure water, DMSO and Caesalpinia extract alone respectively. It also shows dose–response relationship (Table 3). Effects of Caesalpinia extract on the frequency of aberrations per cell and total aberrations noticed were not different statistically. The total aberrations per thousand cells were 182, 152, 157, 153, and 135 for Caesalpinia extract against the value of 230 with MMS alone (Table 4).

At 32 h of treatment, we observed the percentage of aberrant cells as 12.0% for MMS alone, and 11.0%, 10.3%, 9.7%, 9.3%, and 8.9% respectively for five different concentrations of Caesalpinia extract given with MMS, whereas the values for normal control was 3.1%, and the same for DMSO and Caesalpinia extract alone (respectively) were 3.2% and 3.2%. In terms of the effects on the percent reduction in aberrant cells, the values were 8.33%, 14.16%, 19.16%, 22.50%, and 25.83% respectively. These values were statistically significant at <0.05 probability level due to Caesalpinia extract on the number and percentage of aberrant cells. It also showed

Table 1 Effect of alcoholic extracts of *Caesalpinia bonducella* on the frequency of cells with chromosome aberrations induced by methyl methane sulfonate.

Treatment	Caesalpinia (Y/kg bw)	Cell with pulverized chromosome	Types of chromatic aberrations				Aberrant cell no. percentage (%)	(%) Reduction
			Gaps	Breaks	Fragments	Exchange		
DH_2O	0	00	01	01	21	00	22 (2.2)	
DMSO	0	00	03	02	30	00	32 (3.2)	
MMS	0	14	49	50	41	05	110 (11.0)	
Caesalpinia extract	CE_5	00	04	03	40	00	43 (4.3)	
MMS + Caesalpinia extract	CE_1	11	44	38	32	05	86 (8.6)	11.34
	CE_2	09	43	35	31	04	79 (7.9)	18.55
	CE_3	07	36	30	32	01	70 (7.0)	27.83*
	CE_4	08	31	27	30	02	67 (6.7)	30.92*
	CE_5	09	28	23	28	03	63 (6.3)	35.05*

Note: CE, concentrations of alcoholic extracts of *Caesalpinia bonducella*; MMS, methyl methane sulfonate 5 μg/ml/kg body weight; DMSO, dimethylsulfoxide; DH_2O, distilled water. Calculations were made at 16 h of treatment, excluding the gaps type of aberration.

* Significant at <0.05 probability. Y/kg bw is the concentration of alcoholic extracts of *Caesalpinia bonducella*.

Table 2 Effect of alcoholic extracts of *Caesalpinia bonducella* on the total number and types of frequency of cells with chromosome aberrations induced by MMS.

Treatment	Caesalpinia (Y/kg bw)	Cell with aberration							Total number of aberration
		0	1	2	3	4	5	6–9	
DH_2O	0	988	19	02	00	00	00	00	23
DMSO	0	978	29	03	00	00	00	00	35
MMS	0	890	61	30	08	06	03	02	199
Caesalpinia	CE_5	957	39	04	00	00	00	00	47
MMS + Caesalpinia extract	CE_1	914	48	25	05	04	02	02	155
	CE_2	921	50	18	04	04	02	01	132*
	CE_3	930	45	17	02	03	02	01	115*
	CE_4	933	44	16	03	02	01	01	105*
	CE_5	937	43	15	02	02	01	00	92*

Note: CE, concentrations of alcoholic extracts of *Caesalpinia bonducella*; MMS, methyl methane sulfonate 5 µg/ml/kg bw; DMSO, dimethylsulfoxide; DH_2O, distilled water. Calculations were made at 16 h of treatment, excluding the gaps type of aberration.
* Significant at <0.05 probability. The animals were sacrificed 16 h after MMS treatment 1000 cells from 10 animals were analyzed for each point. Y/kg bw is the concentration of alcoholic extracts of *Caesalpinia bonducella*.

Table 3 Effect of alcoholic extracts of *Caesalpinia bonducella* on the frequency of cells with chromosome aberrations induced by methyl methane sulfonate at 24 h of treatment.

Treatment	Caesalpinia conc. (Y/kg bw)	Cell with pulverized chromosome	Types of chromatic aberrations				Aberrant cell no. (%)	(%) Reduction
			Gaps	Breaks	Fragments	Exchange		
DH_2O	0	0	2	3	27	0	30 (3.0)	
DH_2O + DMSO	0	0	3	3	32	0	35 (3.5)	
MMS	0	11	32	51	45	3	110 (11.0)	
Caesalpinia	CE_5	0	1	2	32	0	35 (3.5)	
MMS + Caesalpinia extract	CE_1	10	38	37	42	4	93 (9.3)	15.45
	CE_2	8	35	30	43	4	85 (8.5)	22.72
	CE_3	7	40	36	35	3	81 (8.1)	26.36*
	CE_4	7	35	39	27	3	76 (7.6)	30.90*
	CE_5	7	29	32	30	0	69 (6.9)	40.00*

Note: CE, concentrations of alcoholic extracts of *Caesalpinia bonducella*; MMS, methyl methane sulfonate 5 µg/ml/kg body weight; DMSO, dimethylsulfoxide, DH_2O, distilled water. Calculations were made at 24 h of treatment, excluding the gaps type of aberration.
* Significant at <0.05 probability. Y/kg bw is the concentration of alcoholic extracts of *Caesalpinia bonducella*.

Table 4 Effect of alcoholic extracts of *Caesalpinia bonducella* on the total number and types of frequency of cells with chromosome aberrations induced by MMS.

Treatment	Caesalpinia (Y/kg bw)	Cell with aberration							Total number of aberration
		0	1	2	3	4	5	6–9	
DH_2O	00	970	28	02	00	00	00	00	32
DH_2O + DMSO	00	965	32	03	00	00	00	00	38
MMS	00	890	69	16	09	07	03	06	230
Caesalpinia	CE_5	965	31	04	00	00	00	00	39
MMS + Caesalpinia extract	CE_1	907	58	15	08	04	03	05	182
	CE_2	915	57	12	06	04	03	03	152*
	CE_3	919	53	13	04	03	03	05	157*
	CE_4	924	45	14	06	05	02	04	153*
	CE_5	931	40	11	06	04	03	05	135*

Note: CE, concentrations of alcoholic extracts of *Caesalpinia bonducella*; MMS, methyl methane sulfonate 5 µg/ml/kg body weight; DMSO, dimethylsulfoxide; DH_2O, distilled water. Calculations were made at 24 h of treatment, excluding the gaps type of aberration.
* Significant at <0.05 probability level. The animals were sacrificed 24 h after MMS treatment 1000 cells from 10 animals were analyzed for each point. Y/kg bw is the concentration of alcoholic extracts of *Caesalpinia bonducella*.

almost dose dependent relationship, though the highest doses were not much effective in comparison with the preceding dose. More of chromosomal exchange types of aberrations were seen in contrast to the previous two durations of treatment (Table 5 and Fig. 1). The total frequencies per thousand cells were 233 for MMS only and 202, 179, 158, 154, and 149

Table 5 Effect of alcoholic extracts of *Caesalpinia bonducella* on the frequency of cells with chromosome aberrations induced by methyl methane sulfonate at 32 h of treatment.

Treatment	Caesalpinia (Y/kg bw)	Cell with pulverized chromosome	Types of chromatic aberrations				Aberrant cell no. (%)	% Reductions (%) Red
			Gaps	Breaks	Fragments	Exchange		
DH_2O	00	00	02	03	28	00	31 (3.1)	
DH_2O + DMSO	00	00	04	02	30	00	32 (3.2)	
MMS	00	13	37	54	48	05	120 (12.0)	
Caesalpinia	CE_5	00	03	03	29	00	32 (3.2)	
MMS + Caesalpinia extract	CE_1	11	38	46	48	05	110 (11.0)	8.33
	CE_2	09	36	41	48	05	103 (10.3)	14.16
	CE_3	10	31	37	46	04	97 (9.7)	19.16
	CE_4	08	29	33	50	02	93 (9.3)	22.50*
	CE_5	07	24	30	52	00	89 (8.9)	25.83*

Note: CE, concentrations of alcoholic extracts of *Caesalpinia bonducella*; MMS, methyl methane sulfonate 5 µg/ml/kg body weight; DMSO, dimethylsulfoxide; DH_2O, distilled water. Calculations were made at 32 h of treatment, excluding the gaps type of aberration.
* Significant at <0.05 probability level. Y/kg bw is the concentration of alcoholic extracts of *Caesalpinia bonducella*.

Table 6 Effect of alcoholic extracts of *Caesalpinia bonducella* on the total number and types of frequency of cells with chromosome aberrations induced by MMS.

Treatment	Caesalpinia conc. (Y/kg bw)	Cell with aberration							Total number of aberration
		0	1	2	3	4	5	6–9	
DH_2O	00	969	25	06	00	00	00	00	37
DH_2O + DMSO	00	968	26	06	00	00	00	00	38
MMS	00	880	77	20	05	07	05	06	233
Caesalpinia	CE_5	968	28	04	00	00	00	00	36
MMS + Caesalpinia extract	CE_1	890	72	19	05	05	05	04	202
	CE_2	897	68	20	05	04	03	03	179
	CE_3	903	67	18	04	03	03	02	158*
	CE_4	907	65	17	03	03	02	03	154*
	CE_5	911	60	19	02	03	04	01	149*

Note: CE, concentrations of alcoholic extracts of *Caesalpinia bonducella*; MMS, methyl methane sulfonate 5 µg/ml/kg body weight; DMSO, dimethylsulfoxide; DH_2O, distilled water. Calculations were made at 32 h of treatment, excluding the gaps type of aberration.
* Significant at <0.05 probability. The animals were sacrificed 32 h after MMS treatment 1000 cells from 10 animals were analyzed for each point. Y/kg bw is the concentration of alcoholic extracts of *Caesalpinia bonducella*.

respectively for Caesalpinia extract along with MMS. These significantly reduced values show the effects of Caesalpinia extract on the total aberrations as well as aberrations per cell as shown in Table 6.

3.2. In vitro effects

Here the culture treated with methyl methane sulfonate (MMS) resulted in clastogenic abnormalities as observed in percent metaphase aberration, types of aberrations and aberration per cell (42.75%, 44.25%, and 51.75% or 0.43, 0.44 and 0.52 aberration per cell) at single standard dose and three durations viz., 24, 48, and 72 h respectively whereas, with control, the normal and DMSO plus Caesalpinia extract, these values were (0.05, 0.05) per cell. Caesalpinia extracts bring down aberrations from 42.75% to 37.75%, 33.75%, 31.25%, and 28.50% with four consecutive doses of the extract at 24 h of duration, whereas at 48 h, it get lowered from 44.25% to 41.00%, 35.85%, 33.00%, and 30.25% by administration of 1st to 4th concentrations of Caesalpinia extract respectively. The same trends were noticed, when the treatment durations were increased to 72 h. These values showed linearly increasing trends with doses, but it does not depend on durations. The maximum percentage reductions in the aberrations were 33.33 for 24 h and 31.63 and 32.17 respectively for 48 and 72 h that were caused by highest doses of Caesalpinia extract (Table 7 Fig. 2).

Similarly when the experiment was setup along with metabolic activation system ($+S_9$ mix) the effect of MMS got increased. The effect of Caesalpinia extract also showed a similar trend; they lower the clastogenic activity of MMS. These values show linear increases with doses (Table 8). The maximum effective percentage reductions were 32.65%, 33.11%, and 33.07% for 24, 48 and 72 h respectively. When sister chromatid exchanges were counted (Tables 9 and 10) the reduction was evident both in the absence as well as in the presence of metabolic activation; there being a lowering of the mean range and the total SCEs and SCE per cell from 07.70 to 05.20 and from 07.00 to 05.10. For the analysis of SCE, only 48 h of cultures were used and 50 metaphases were scanned. The effects of Caesalpinia extract on replication index (Tables 11 and 12) showed an elevated level when compared with the MMS treatment alone i.e., from 1.51 to 1.66, though still being lower than the normal level of 1.76. The effect, after

Table 7 Analysis of chromosomal aberrations after treatment with methyl methane sulfonate along with alcoholic extracts of *Caesalpinia bonducella in vitro* in the absence of metabolic activation ($-S_9$ mix.).

Treatments	Durations (h)	Metaphase scored	Percent aberration metaphase		Types of aberration (%)			Aberration/ cell ± SE
			Including gaps	Excluding gaps	Chromatid	Chromosome	Total	
MMS	24	200	29.25	26.75	27.50	15.25	42.75	0.43 ± 0.04
	48	200	30.35	22.25	30.25	14.00	44.25	0.44 ± 0.05
	72	200	39.25	33.50	31.50	20.25	51.75	0.52 ± 0.06
MMS + CE_1	24	200	27.35	23.50	23.50	14.25	37.75	0.38 ± 0.04
	48	200	29.50	21.25	30.50	10.50	41.00	0.41 ± 0.05
	72	200	37.00	30.50	31.00	17.25	48.25	0.48 ± 0.05
MMS + CE_2	24	200	25.25	21.50	24.25	13.50	33.75	0.34 ± 0.03
	48	200	27.50	19.50	28.75	12.25	35.85	0.36 ± 0.04
	72	200	32.75	29.25	29.50	18.75	43.50	0.44 ± 0.05*
MMS + CE_3	24	200	23.00	19.25	20.75	10.50	31.25	0.31 ± 0.03*
	48	200	25.50	17.50	23.25	9.75	33.00	0.33 ± 0.03*
	72	200	30.25	26.50	25.50	14.25	39.75	0.40 ± 0.04*
MMS + CE_4	24	200	21.50	17.75	19.25	9.25	28.50	0.29 ± 0.03*
	48	200	23.25	15.25	21.50	8.75	30.25	0.30 ± 0.03*
	72	200	29.50	23.50	22.75	12.35	35.10	0.35 ± 0.04*
Control								
Normal	72	200	4.00	1.50	3.50	1.00	4.50	0.05 ± 0.01
DMSO + CE_2	72	200	4.50	1.50	3.25	1.50	4.75	0.05 ± 0.01

Note: SE, standard error; CE, concentrations of alcoholic extracts of *Caesalpinia bonducella*; DMSO, dimethylsulfoxide; DH_2O, distilled water; MMS, methyl methane sulfonate 5 μg/ml/culture. Calculations were made excluding the gaps type of aberration.
* Significant at <0.05 probability level.

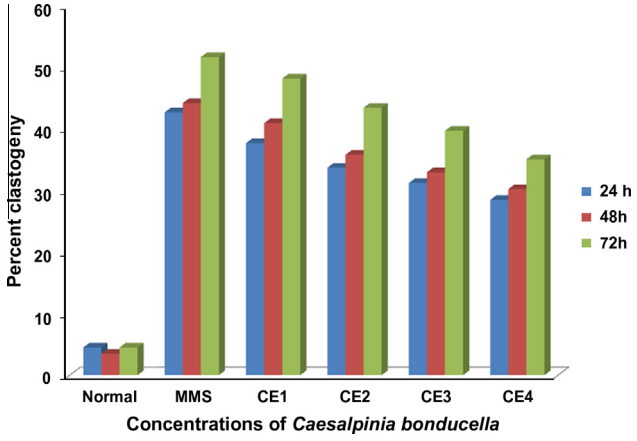


Figure 2 Shows effects of four increasing concentrations of alcoholic extracts of *Caesalpinia bonducella* (CE) at 24, 48, and 72 h of treatment durations in the human lymphocytes *in vitro* against methyl methane (MMS) induced clastogeny.

treatment with metabolic activation system, shows the level from 1.36 to 1.56, i.e., again being much effective in comparison to that without metabolic activation system.

4. Discussion

Methanolic extract of *C. bonducella* (MECB) significantly reduces the elevated levels of lipid peroxidation and increases the glutathione content in Ehrlich ascites carcinoma (EAC)-treated mice, the anti-tumerogenic effect of MECB may in fact be due to the antioxidant status and the free radical quenching property of the phyto-constituents of MECB.

The nitric oxide scavenging activity of ethanol extracts of *C. bonducella* showed the percentage inhibition of 58.16 ± 0.37 when compared with the standard ascorbic acid whose effect seems to be as effective as 36.71 ± 3.21 at a concentration of 25 μg/ml. The seed extracts of *C. bonducella* present the result as 102.65 gm/ml [19]. The IC50 value of nitric oxide scavenging activity of the methanol leaf extract of *C. bonducella* was found to be as good as 102.8 gm/ml, when compared with the standard curcumin value of 20.4 gm/ml [20].

The methanol extract of *C. bonducella* (Caesalpiniaceae) leaves (MECB) were evaluated for antitumor activity against Ehrlich ascites carcinoma (EAC)-bearing Swiss albino mice. MECB caused a significant ($P < 0.01$) decrease in tumor volume, packed cell volume, and viable cell count; and it prolonged the life span of EAC-tumor bearing mice. Hematological profile was converted to more or less normal levels in the mice treated with extract. MECB significantly ($P < 0.05$) decreased the levels of lipid peroxidation and significantly ($P < 0.05$) increased the levels of glutathione reductase, Superoxide dismutase and catalase. The results indicate that MECB exhibited a significant antitumor and antioxidant activity in EAC-bearing mice [20].

The ethanolic extract also inhibited the hydroxyl radical, nitric oxide, superoxide anions with IC50 (half maximal inhibitory concentration) values of 109.85, 102.65, and 89.84l gm/ml, respectively. However, the IC values for the standard ascorbic acid were noted to be 70.79, 65.98, and 36.68 gm/ml respectively. The results obtained in our study may indicate that *C. bonducella* has a significant potential to be used as a natural antioxidant agent that decreases the number and frequencies of chromosomal aberrations and sister chromatid exchange and enhances the replication indices.

These studies are conducted in the mice evaluating the *in vivo* immunomodulatory activities of the aqueous extract

Table 8 Analysis of chromosomal aberrations after treatment with methyl methane sulfonate along with alcoholic extracts of *Caesalpinia bonducella in vitro* in the presence of metabolic activation ($+S_9$ mix.).

Treatments	Durations (h)	Metaphase scored	Percent aberration metaphase		Types of aberration (%)			Aberration/cell ± SE
			Including gaps	Excluding gaps	Chromatid	Chromosome	Total	
MMS	24	200	25.50	22.50	23.50	13.25	36.75	0.37 ± 0.04
	48	200	26.25	18.75	27.25	11.25	38.50	0.39 ± 0.04
	72	200	35.50	29.25	28.50	16.25	44.75	0.45 ± 0.05
MMS + CE_1	24	200	24.35	20.50	21.25	11.25	32.50	0.33 ± 0.03
	48	200	25.50	18.25	24.75	10.50	35.25	0.35 ± 0.03
	72	200	34.75	27.25	25.50	14.35	39.85	0.40 ± 0.04
MMS + CE_2	24	200	22.50	18.25	18.75	10.50	29.25	0.29 ± 0.03*
	48	200	24.25	16.50	22.25	9.25	31.50	0.32 ± 0.03*
	72	200	29.75	25.75	24.50	12.25	36.75	0.37 ± 0.04*
MMS + CE_3	24	200	19.25	16.25	17.25	9.25	26.50	0.27 ± 0.03*
	48	200	21.50	13.50	19.35	8.75	28.10	0.28 ± 0.03*
	72	200	26.75	23.25	21.50	11.75	33.25	0.33 ± 0.03*
MMS + CE_4	24	200	14.75	15.25	16.25	8.50	24.75	0.25 ± 0.03*
	48	200	12.25	18.50	18.50	7.25	25.75	0.26 ± 0.03*
	72	200	19.75	19.75	19.75	10.25	29.95	0.30 ± 0.03*
Control								
Normal	72	200	3.50	2.00	2.00	1.50	3.50	0.04 ± 0.01
DMSO + CE_2	72	200	4.00	1.50	2.25	1.75	4.00	0.04 ± 0.01

Note: SE, standard error; CE, concentrations of alcoholic extracts of *Caesalpinia bonducella*; DMSO, dimethylsulfoxide; DH_2O, distilled water; MMS, methyl methane sulfonate 5 μg/ml/culture. Calculations were made excluding the gaps type of aberration.
* Significant at <0.05 probability level.

Table 9 Analysis of sister chromatid exchange after treatment with methyl methane sulfonate along with alcoholic extracts of *Caesalpinia bonducella in vitro*, in the absence of metabolic activation ($-S_9$ mix.).

Treatment	Duration(h)	Metaphase scored	Total	Range	SCE/cell ± SE
MMS	48	50	385	2–12	7.70 ± 1.50
MMS + CE_1	48	50	350	1–11	7.00 ± 1.50
MMS + CE_2	48	50	325	1–11	6.50 ± 1.40
MMS + CE_3	48	50	295	1–10	5.90 ± 1.40*
MMS + CE_4	48	50	260	2–9	5.20 ± 1.20*
Control					
Normal	48	50	94	0–4	1.88 ± 0.50
DMSO	48	50	96	0–5	1.92 ± 0.50
DMSO + CE_2	48	50	95	0–5	1.90 ± 0.50

Note: SCE, sister chromatid exchange; SE, standard error; CE, concentrations of alcoholic extracts of *Caesalpinia bonducella*; DMSO, dimethylsulfoxide; DH_2O, distilled water; MMS, methyl methane sulfonate 5 μg/ml/culture.
* Significant at <0.05 probability level.

Table 10 Analysis of sister chromatid exchange after treatment with methyl methane sulfonate along with alcoholic extracts of *Caesalpinia bonducella in vitro*, in the presence of $+S_9$ mix.

Treatment	Duration (h)	Metaphase scored	Total	Range	SCE/cell ± SE
MMS	48	50	350	1–11	7.00 ± 1.50
MMS + CE_1	48	50	315	1–10	6.30 ± 1.50
MMS + CE_2	48	50	285	1–10	5.70 ± 1.40*
MMS + CE_3	48	50	270	1–10	5.40 ± 1.40*
MMS + CE_4	48	50	255	1–11	5.10 ± 1.20*
Control					
Normal	48	50	92	0–5	1.84 ± 0.50
DMSO	48	50	94	0–5	1.88 ± 0.50
DMSO + CE_2	48	50	93	0–5	1.86 ± 0.50

Note: SCE, sister chromatid exchange; SE, Standard error; CE, concentrations of alcoholic extracts of *Caesalpinia bonducella*; DMSO, dimethylsulfoxide; DH_2O, distilled water; MMS, methyl methane sulfonate 5 μg/ml/culture.
* Significant at <0.05 probability level.

Table 11 Analysis of cell cycle kinetics after treatment with methyl methane sulfonate along with alcoholic extracts of *Caesalpinia bonducella*, *in vitro*, in the absence of metabolic activation ($-S_9$ mix.).

Treatment	Cell scored	(%) Cell in			Replication index	2×3 Chi square test
		M_1	M_2	M_3		
MMS	200	68	26	06	1.38	
MMS + CE_1	200	63	32	05	1.42	
MMS + CE_2	200	53	37	10	1.57	*Significant
MMS + CE_3	200	49	38	13	1.64	*Significant
MMS + CE_4	200	46	40	14	1.68	*Significant
Control						
Normal	200	40	44	16	1.76	
DMSO	200	40	43	17	1.77	
DMSO + CE_2	200	39	43	18	1.79	

Note: 2×3 Chi square test were conducted, CE, concentrations of alcoholic extracts of *Caesalpinia bonducella*; DMSO, dimethylsulfoxide; DH_2O, distilled water; MMS, methyl methane sulfonate 5 μg/ml/culture.
* Significant at < 0.05 probability level.

Table 12 Analysis of cell cycle kinetics after treatment with methyl methane sulfonate along with alcoholic extracts of *Caesalpinia bonducella*, *in vitro*, in the presence of $+S_9$ mix.

Treatment	Cell scored	(%) Cell in			Replication index	2×3 Chi square test
		M_1	M_2	M_3		
MMS	200	70	24	06	1.36	
MMS + CE1	200	66	27	07	1.41	
MMS + CE2	200	56	33	11	1.55	*Significant
MMS + CE3	200	53	35	12	1.59	*Significant
MMS + CE4	200	50	36	14	1.64	*Significant
Control						
Normal	200	42	43	15	1.73	
DMSO	200	43	40	17	1.74	
DMSO + CE2	200	45	38	17	1.72	

Note: 2×3 Chi square test were conducted, CE, concentrations of alcoholic extracts of *Caesalpinia bonducella*; DMSO, dimethylsulfoxide; DH_2O, distilled water; MMS, methyl methane sulfonate 5 μg/ml/culture.
* Significant at < 0.05 probability level.

of *C. bonducella* seeds. The aqueous extract of *C. bonducella* seeds was tested for their effect on cell mediated and humoral components of the immune system in rats and reported antioxidant activity in the chloroform extract of *C. bonducella* seeds [19,21].

Mandal et al. [22], give the assessment of the antioxidant role and reactive oxygen species scavenging activity of methanolic extract of *Caesalpinia* leaf. It may be concluded that 70% of the methanol extract of *Caesalpinia crista* leaves acts as an antioxidant and reactive oxygen species (ROS) scavenger, which may be due to the presence of phenolic and flavonoid compounds. It has been reported that antioxidant activity and total phenolic content of ethanolic extract of *C. bonducella* seeds are quite good. The results obtained in this study clearly support our findings and indicate that *C. bonducella* has a significant potential to be used as a natural antioxidant as well as antimutagenic and anticarcinogenic agent.

Compounds of cassane diterpenes were isolated from *C. bonducella* and the isolated compounds were tested for their antiproliferative activity against MCF-7 (breast adenocarcinoma), DU145 (prostate carcinoma), C33A (Cervical carcinoma) and Vero (African green monkey kidney fibroblast) cells [6]. Gupta et al. [20], reported antitumor activity and antioxidant status of *C. bonducella* against Ehrlich ascites carcinoma in swiss albino mice. The methanol extract of *C. bonducella* leaves (MECB) were evaluated for antitumor activity against Ehrlich ascites carcinoma (EAC)-bearing swiss albino mice and reports indicate that MECB do exhibit significant antitumor and antioxidant activity in EAC-bearing mice.

Herbal medicines have received greater attention as an alternative to clinical therapy and the demand for these remedies has currently increased. An experimental screening method is important in order to ascertain the safety and efficacy of traditional and herbal products [23].

5. Conclusion

To conclude Caesalpinia extract acts as an effective anticlastogenic agent as it significantly reduces the chromosomal aberrations and sister chromatid exchange and enhances cell life as indicated by replication indices in *in vitro* experiments. Similarly, it reduces total aberrant cells and frequency of aberrations in the albino mouse bone marrow cells in *in vivo* experiments using MMS induced assay of clastogeny.

Conflict of interest

The authors declare that there is no conflict of interest.

Acknowledgments

The author is highly thankful to Department of Science and Technology (DST No. SR/FT/L-135/2005) New Delhi and University Grant Commission (UGC), New Delhi for providing major research projects. Help from research scholars of human genetics and toxicology laboratory, Department of zoology, AMU, Aligarh is also acknowledged.

References

[1] Mukherjee PK. Quality control of herbal drugs: an approach to evaluation of botanicals. 1st ed. New Delhi: Business Horizon; 2002.
[2] Mythilpriya R, Shanthi P, Sachdanandam P. Oral acute and sub acute toxicity studies with Kalpaamurthaa, a modified indigenous preparation on Rats. J Health Sci 2007;53(4):351–8.
[3] Edeoga HO, Okwu DE, Mbaebie BO. Phytochemical constituents of some Nigerian medicinal plants. Afr J Biotechnol 2005:685–8.
[4] Gaur RL, Sahoo MK, Fatma N, Rastogi S, Murthy PK. Antifilarial activity of *Caesalpinia bonducella* against experimental filarial; infections. Indian J Med Res 2008;28:65–70.
[5] Gurunath HM, Sidramayya MS, Patil KS, Wadkar GH. Effect of *Caesalpinia bonducella* seed extracts on human neutrophil. J Pharm Res 2010;3(3):467–9.
[6] Yadav PP, Maurya R, Sarkar J, Arora A, Kanojiya S, Sinha S, et al. Cassane diterpenes from *Caesalpinia bonduc*. Phytochemistry 2009;70(2):256–61.
[7] Aswar PB, Khadabadi SS, Deokate UA, Jain SP, Biyani KR. Evaluation of antidiabetic activity of *Caesalpinia bonducella* (L) Roxb leaves in alloxan induced diabetic mice. Indian J Pharm Edu Res 2009;43(3):280–3.
[8] Ahsan RMD, Islam MKM, Haque MD, Mossaddik AMD. Hepato-protective activity of methanol extract of some medicinal plants against carbon tetra chloride induced hepatoprotective in rats. Eur J Sci Res 2009;37(2):302–10.
[9] Kirtikar Basu. Indian medicinal plants. 2nd ed. New Delhi: BSMP Singh, Periodical Experts; 1975.
[10] Kirtikar Basu. Indian medicinal plants. 2nd ed. Dehra Dun: BSMP Singh, Periodical Experts; 1993.
[11] Preeja Pillaia G, Suresh P. Evaluation of the acute and sub acute toxicity of the methanolic leaf extract of *Caesalpinia bonducella* in Balb C mice. Eur J Sci Res 2011;53:462–9.
[12] Maron DM, Ames BN. Revised method for the *Salmonella* mutagenicity test. Mutat Res 1983;113:173–215.
[13] Evans HJ. Human peripheral blood lymphocytes for the analysis of chromosome aberrations in mutagen tests. In: Kilbey BJ, Legator M, Nichols W, Ramel C, editors. Hand book of mutegenicity test procedures. Amsterdam: Elsevier; 1984. p. 40–427.
[14] Latt SA, Wohllel JC. Optical studies of the induction of 33,258 Hoechst with DNA, chromatin and metaphase chromosomes. Chromosome 1975;52:297–316.
[15] Latt SA, Allen J, Bloom SE, Carrono AV, Falke E, Kram D, et al. Sister chromatid exchange; a report of the sister chromatid exchange; a report of the genotoxic programme. Mutat Res 1981;87:1777.
[16] Crossen PE, Morgan WF. Analysis of human lymphocyte cell cycle time in culture measured by sister chromatid differential staining. Exp Cell Res 1977;104:453–7.
[17] Tice R, Schneider EL, Rary JM. The utilization of BrdU incorporation into DNA for the analysis of cellular kinetics. Exp Cell Res 1976;102:232–6.
[18] Fisher A, Yates F. Statistical tables for biological agricultural and medical research. 6th ed.. New York: Hafner Pub Co.; 1963.
[19] Shukla S, Mehta A, John J, Singh S, Mehta P, Vyas SP. Antioxidant activity and total phenolic content of ethanolic extract of *Caesalpinia bonducella* seeds. Food Chem. Toxicol. 2009;47:1848–51.
[20] Gupta M, Mazumdar UK, Sambath KR, Gomathi P, Rajeshwar Y, Sivakumar T. Screening of antioxidant and antimicrobial activities of *Caesalpinia bonducella* Flem, leaves (Caesalpiniaceae). J Orient Pharm Exp Med 2004;4:194–206.
[21] Sachan NK, Verma S, Sachan AK, Arshad H. Annal Pharm Pharm Sci 2010;12010:88–91.
[22] Mandal S, Hazra B, Sarkar R, Biswas S, Mandal N. Assessment of the antioxidant and reactive oxygen species scavenging activity of methanolic extract of *Caesalpinia crista* leaf. Evid Based Complement Alternat Med 2011;13: PMCID: PMC3136223.
[23] Sim KS, Nurestri AM, Kim KH. Acute oral toxicity of Pereskiableo and Pereskia grandifolia in mice. Phcognosy Magazine 2010;6(21):67–70.