Research Article

Effects of *Terminalia chebula* on Cisplatin induced Genotoxicity in Rat Bone Marrow Cells

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ABSTRACT

The study was with a view to investigate the phytochemical and therapeutic effects of *Terminalia chebula* on cisplatin induced genotoxicity activity in rat bone marrow cells. The fruit of *Terminalia chebula* was extracted with hydro alcoholic solvent methanol: water mixture. A presence of tannins, saponins, and flavonoids was identified in the extract. The aberration scored for extract plus cisplatin treated group was 30.99±1.376%which was found to be significantly less as compared to cisplatin treated group. Thus the results suggested that the extract contains some active principles which might be posed significant non-genotoxic activity.

Keywords: Genotoxicity cisplatin, chromosomal aberration, *Terminalia chebula.*

INTRODUCTION

Chemicals that exert their adverse effect through interaction with the genetic material (DNA) of cells were called genotoxic. Most human carcinogens were genotoxic in nature. The science of genotoxicity mainly concerned that chemicals which induce mutations in various experimental models, might conceive after the incidence of heritable mutations in man. Genotoxicity tests could be defined as *in vitro* or *in vivo* tests designed to detect drugs, which can induce genetic damage directly or indirectly by various mechanism of action. Genotoxicity tests enabled hazard identification with respect to DNA damage and its fixation in the form of gene mutations, large-scale chromosomal damage, recombination and numerical chromosome changes. Drugs that were positive in these tests that detected such kind of damage had the potential to be human carcinogens and /or mutagens [1].

In vitro Genotoxicity Assays[2]

In vitro Chromosomal Aberration Assays

Chemicals causing chromosomal aberrations may be identified with an in vitro cytogenetics assay. Cell cultures were treated with the test chemical and then mitosis was arrested in metaphase with an inhibitor, such as colchicines. The metaphase spreads was examined by light microscopy to detect chromosome or chromatid aberrations, or polyploidy cells. A biologically significant increase in the frequency of cells with structural or numerical aberrations compared with that of the concurrent control group indicates the chemical was clastogenic or aneugenic. A major drawback of this assay, in comparison with some other in vitro assays, was the subjectivity and cost of having the metaphase spreads scored by a highly trained observer.

In vitro Gene Mutation Assays

The *in vitro* assays commonly used in genetic toxicity testing include the Ames bacterial reverse mutation assays, which detected chemicals that cause point mutations or frame shift mutation in histidine auxotropic strains of *Salmonella Typhimurium* and reverse mutation assay using a tryptophan auxotropic strain of *E-Coli.* Mammalian forward mutation assays, such as thymidine kinase (*Tk*) assay or the hypoxanthine-guanine phosphor ribosyl transferase (*Hprt*) assay, detected mutations at the heterozygous *Tk* or hemizygous *Hprt*gene. Cells which had forward mutations at the TK or Hprt genes survive in the presence of the selective agent, while wild-type cells accumulate a toxic metabolite and did not proliferate. Comparison of the mutant frequency of the treatment groups with the concurrent negative group allows the identification of a mutagenic chemical.[3]

In vivo assay for somatic cell chromosomal aberrations.[3]

In vivo assay for chromosomal aberrations assess the potential of attest chemical to cause DNA damage that may affect chromosome structure or interfere with the mitotic apparatus causing changes in chromosome number. The *in vivo* assays took into account DNA repair processes and the effects of pharmacokinetic factors that do not play a role in *in vivo* systems. There were several short term assays that detected somatic cell chromosomal aberrations, these included the rodent erythrocyte micronucleus assay and the chromosomal aberration assays in bone marrow. These methods were described in the following sections.

Rodent Erythrocyte Micronucleus Assay

Because of its relative simplicity and sensitivity to clastogens, the rodent erythrocyte micronucleus assay had now become the most commonly conducted in *in*

vivo assay. It had achieved widespread acceptances and recommended test methods had been published as OECD Test Guideline 474. The micronucleus assay detected chromosome damage and whole chromosome loss in polychromatic erythrocytes and eventually in non-chromatic erythrocytes in peripheral blood as the red cells mature. Structural aberrations were believed to be result from direct or indirect interaction of the test chemical with DNA, while numerical aberrations were often a result of interferences with the mitotic apparatus prevented normal nuclear division. Bone marrow was the major hematopoietic tissue in the rodent.

Mammalian Bone Marrow Chromosomal Aberration Assay

Mammalian bone marrow chromosomal aberration assay could detect clastogenic effects of a test agent. However, in the chromosomal aberration assay, these effects were observed directly by examination of metaphase chromosome spreads. The recommended methodology had been published in OECD Test Guideline 475. The assay was based on the ability of a test agent to induce chromosome structural or numerical alterations that could be visualized microscopically. The target tissue for the chromosomal aberration assay was the bone marrow because it was a rapidly dividing, well vascularized tissue. The maximum tolerable dose should be identified in a pre miminary test as that producing toxicity such that higher doses was be expected to lead to mortality, or the dose producing evidence of bone marrow cytotoxicity.

Herbal drugs had been used since ancient times as medicines for the treatment of a range of diseases. Medicinal plants had played a key role in world health. In spite of the great advances observed in modern medicine in recent decades, plant still made an important contribution to health care[2.]. The *Termnalia chebula* belonging to the family combretaceae and it consisted of dried ripe and fully mature fruit of *Terminalia chebula.* It was also called myrobalan. It was rich in tannin. The chief constituent of tannin was chebulicacid, chebulagic acid, and gallic acid. The presence of tannins caused apoptosis in the damaged cell which was the main reason behind anticancer activity.

MATERIALS AND METHODS

Collection of plant material

The fruit of *Terminalia chebula* was collected from local market of Bhopal and powdered using grinder. The powdered drug packed in air tight container until use. And all other chemicals used in this study was of analytical reagent grade.

Authentification

The botanical identity was confirmed by Dr. Ziaul Hasan, Asst. Prof. Botany, Department of Botany, Safia College of sciences, Bhopal, where voucher specimen number (150/BOT/SAFIA/2010) has been deposited for further reference.

Preparation of plant extract

The powdered drug was extracted with hydro alcoholic solvent (methanol: water in the ratio 7:3) by soxhelation. After completion of extraction solvent was recovered and the saturated solvent was dried over water bath at 40-50ºc. The semi-solid paste formed was transferred to petri dish and kept in hot air oven at 60 ºc for further drying and stored in an air tight container and kept at 2-8 ºc for further use.

Phytochemical Screening[4]

The chemical tests were performed for different chemical groups present in extract.

Tests for Alkaloids

Evaporated the aq.alcoholic and chloroform extract separately. To the residue added dil.HCL shaken well and filter, with filtrate performed the following tests:

To the few ml filtrates, added few drops of following four reagents.

- Dragendorffs reagent : Orange brown ppt.
- Mayers reagent : Creamish ppt
- Hagers reagent : Yellow ppt
- Wagners reagent : Reddisg brown ppt
- Murexide test for purine alkaloids: To the 3-4 ml test solution, added 2-3 drops of conc.HNO_3. Evaporated to dryness. Cooled and added 2 drops of NH4OH. Purple color is observed.

Test for Tannins and Phenolic compounds

To the 2-3 ml of aq.or alcoholic extract, added few drops of the following reagents.

- 5% $FeCl_3$ solution: Deep blue –black color
- Lead acetate solution: White ppt
- Bromine water: Decolouration of bromine water
- Acetic acid: Red color solution.
- Gelatin solution: White ppt
- Potassium dichromate: Red ppt
- Dil.Iodine solution: Transient red color
- Dil HNO_3: Reddish to yellow color
- Dil. Potassium permanganate: Decolouration.

Test for flavonoids

- Shinoda test: To the dry powder or extract, added 5ml 95% ethanol, few drops conc.HCL and 0.5 g magnesium turnings pink colour was observed.
- To small quantity of residue added lead acetate solution, yellow coloured ppt is formed.
- Addition of increasing amount of NaOH to the residue shows yellow coloration which decolorizes after addition of acid.

Tests for steroids

- **Salkowaski reaction**: To 2ml of extract, added 2ml chloroform and 2ml conc.H2SO4.Shaken

well. Chloroform layer appeared red and acid layer shows greenish yellow fluorescence.

- **Libermann-Burchard reaction**: Mixed 2ml extract with chloroform, added 1-2 ml acetic anhydride and 2 drops conc. H_2SO_4 form side of test tube. First red then blue and finally green color appeared.
- **Libermann reaction**: Mixed 3ml extract with 3mlacetic anhydride. Heated and cooled. Added few drops conc.H_2SO_4. Blue color appeared.

Test for glycosides

Cardiac glycoside

- Baljets test: A thick section showed yellow to orange colour with sodium picrate.
- Legals test: To the aq.or alcoholic extract, added 1ml pyridine and 1ml sodium nitropruside. Pink to red colour appeared.
- **Libermann reaction**: Mix 3ml extract with 3ml acetic anhydride. Heated and cooled .Added few drops conc.H2SO4.Blue colour appears.
- **Keller Killiani test**: To 2ml extract, added glacial acetic acid, one drop 5% Fecl3 and conc.H2SO4,reddish brown colour appeared at the junction of the 2 layer and upper layer appears bluish green.

Test for saponin glycoside

- Foam test: Shaken the drug extract or dry powder vigorously with water. Persistent form observed.
- Heamolytic test: Added drugs or dry power to one drop of blood placed on glass slide. Haemolytic zone appeared.

Test for anthraquinone glycoside

- Borntragers test: To 3ml of extract, added dil. H_2SO_4 boiled and filtered. To cold filtrate, added equal volume of benzene or chloroform. Shaken well. Separate the organic solvent. Added ammonia, ammonical layer turned pink or red.
- Modified Borntragers test: To 5ml of extract, added 5ml $FeCL_3$ and 5ml dil.HCL, heated for 5minin boiling water bath. Cooled and added benzene or any organic solvent, shaken well. Separated organic layer, added equal volume dil. ammonia. Ammonical layer showed pinkish red colour.

Effect on cisplatin induced genotoxicity in rat bone marrow

Bone marrow depression was the major side effect caused by anticancer drugs, for eg. cyclophosphamide, cisplatin etc. By this protocol chemopreventive/ genoprotective activity of the plant extract against anticancer drug (cisplatin) was studied. Bone marrow depression was induced by a single dose of cisplatin (5mg/kg body weight) in cisplatin treated group, and in extract treated group after 48 hours of the plant extract administration, Chemo preventive/genoprotective activity of the plant was evaluated by following methods:

- Micronucleus Assay
- Chromosomal Aberration test.

MICRONUCLEUS ASSAY[5]

The bone marrow micronucleus test was carry out according to the method of schmid. The femur bones was removed from rats and cleaned and the content was flushed into tube containing 1ml of calf serum and was centrifuged at 1000 rpm for 10 min. The obtained pellet was suspended with few drops of serum and slides were prepared and air dried for 18 hrs. After drying the slides will be stained by Giemsa stain.

Experimental Design

Young rats was selected and divided into four groups. Each group contained 5 animals. One group was treated with vehicle alone (5 % PEG) served as control. Second group was treated with double dose of plant extract (500 mg/kg body weight) at an interval of 24 hrs. Third group received double dose of plant extract at an interval of 24 hrs, then a single dose of cisplatin after 48 hrs of first treatment with extract. After 24 hrs of cisplatin administration animals was scarified by cervical dislocation. Fourth group received cisplatin alone (5 mg/kg body weight) served as positive control. (Table 1)

Procedure

- **Extraction of bone marrow:** Test substances applied intraperitoneally and animals was sacrified by cervical dislocation and bone marrow cells were harvested. From freshly killed animal bone marrow was removed from muscle by use of guaze and fingers. Bone marrow cell was aspirated by flushing with BSA solution with help of a syringe.
- **Preparation of smear:** The tube was centrifuged at 1000rpm for 5 min. The supernatant was removed from the cells in the sediment was carefully mixed by aspiration and a small drop of the viscous suspension was put on the end of slide and spread by pulling the material behind a polished cover glass held at an angle of 45 degree. The preparation was then dried and fixed for 2-5 min.
- **Staining:** Staining was carried out in ordinary vertical staining jars according to the following procedure. Stain for 10 min in Giemsa then rinsed in distilled water, blotted, clean back side with filter paper then dried on the slide warmer.
- **Analysis of slides:** PCEs were scored for micronuclei under the microscope at least 2000 PCEs per animal were scored for the incidence of micronuclei.

Grouping of animals

Table 1: Group of animals for micronucleus test.

Group	Treatment	No. of Animals
1	Vehicle Alone	5
2	T.C.E (250 mg/kg)ALONE	5
3	T.C.E (500 mg/kg)+CISPLATIN 5MG/KG	5
4	CISPLATIN 5MG/KG	5

❖ **Parameter studied**

- Micronucleus
- PCE Count

CHROMOSOMAL ABERRATION ASSAY[6, 7]

For chromosome analysis, the slides was prepared essentially as per the slightly modified method of Preston .In brief, the harvested cells were incubated in 0.075 M KCL for 30 min at 37ºc and then centrifuged for 15 min at 1000 rpm. Cells were fixed in cold glacial acetic acid-methanol (1:3 v/v) and burst opened on a clean slide to release chromosomes. The slides were stained with Giemsa and cooled before observation. Fifty well spread metaphases per rat (250 metaphase for each group) was scored to study the incidence of CA. The cell cycle was arrested in metaphase by the treatment of colchinine (3-5 mg/kg I.P) (Table 2).

Procedure

- Administed drug *Terminalia chebula* fruit extract orally for two days and after 48 hrs of first dose cisplatin was injected intraperitonially.
- Injected was sacrified I.P after 24 hr of cisplatin.
- Animal was sacrified by cervical dislocation after 90 min of colchines injection.
- Animal was dissected and femur bone was excised.
- Bone marrow was aspirated by flushing with normal saline in the centrifuge tube.
- Flush the suspension in the tube properly to get good cell suspension.
- The suspension was centrifuged for 15 min at 1000 rpm.
- Supernatant was discarded.
- Pellet was treated with pre-warmed (37ºc) 0.56 % KCL on cyclomixer to get a uniform suspension.
- Kept the above suspension in a water bath for 30min.
- Then it was centrifuged for 15 min at 1000 rpm and the supernatant was discarded.
- Pellet was dispersed with freshly prepared cornoys fixation on cyclo mixer.
- Again it was centrifuged for 15 min at 1000 rpm and the supernatant was discarded.
- The treatment with Cornoys fixation was repeated at least for 3 times to get Debris free white pellet.
- To the debris free pellet Cornoys Fixation (quantity sufficient) was added to get a good cell suspension.
- Slides were prepared by Air Drop Method.
- Slides was stained and then dried.
- Observe through dried slides under microscope in 40 X and then in 100x magnification.
- No. of cells having aberration and particular were scored (Total 50 metaphase plates were counted).
- Data was analysed by ANOVA.

Grouping of animals

Table 2: Group of animals for chromosomal aberration test.

Group	Treatment	No. of Animals
1	500MG/KG T.C.E +Cisplatin 5 mg/kg + Colchicines 3-5 mg/kg	5
2	Cisplatin 5 mg/kg +Colchicines 3-5 mg/kg body wt	5
3	500mg/kg T.C.E alone +Colchicines 3-5 mg/kg body wt.	5
4	Vehicle +Colchicines' 3-5 mg/kg body weight	5

Parameter Studied:

The prepared slides was observed and studied for presence or absence of particular aberration such as ring, gap, fragments, pulverized, break etc.

Statistical Analysis

Statistical Analysis was carried out using Primer of biostaticals software. All the results were expressed as Mean ±standard error of the mean (SEM). Data was analysed using one way ANOVA followed by Bonferroni t – test. In the entire test, the criterion for statistical significance was p≤0.05.

RESULTS AND DISCUSSION

Result for extraction process:

In the present study the hydro alcoholic extract of *Terminalia chebula* obtained shows dark brown in color and stick consistency and the yield was found to be 9.43% and details was given below.

Parameter	Inference
Colour	Dark brown
Consistency	Sticky paste
Odour	Characteristic
Taste	Astringent
%Age yield	9.43%

Result of phytochemical tests

Phytochemical analysis of the extract indicated the presence of flavonoids, tannins and saponins. Other secondary metabolite such as steroids and glycosides, alkaloids was not detected. (Table 3)

Table 3: Table showing presence or absence of phyto constituents.

S. No.	Tests	Inference
1	Test For Carbohydrates	Absent
2	Test For Alkaloid	Absent
3	Test For Saponin	Present
4	Test For Tannins	Present
5	Test Foe Proteins	Absent
6	Test For Steroids	Absent
7	Test For Flavonoids	Present

Effect on cisplatin induced genotoxicity

Micronucleus test

Table no 4 showed the result of micronucleus test for rats treated with vehicle only, cisplatin only, extract treated (500mg/kg) and extract plus cisplatin treated. An increase in the frequency of micronucleus in PCE is observed in extract treated group when compared to the vehicle but the increase was not significant. Cisplatin, a mutagen used as a positive control, induce a significant increase in the frequency of MNPCEs. Meanwhile the treatment of extract con currently with cisplatin showed a very significant decrease in the frequency of MNPCEs. The test indicated the protective action of *Terminalia chebula* extract against cisplatin induced micronuclei formation.

Table4: Effect of extract against cisplatin induced geno toxicity by micronucleus test

MICRONUCLEUS ESTIMATION **		
GROUP *	**MN-PCEs**	**TOTAL PCEs**
VEHICLE ONLY	4.2±0.8602	2000
T.C.E (500 mg/kg) b.w.t	7.6±0.9274[a]	2000
CISPLATIN ONLY	28.8±1.393[b,c]	2000
T.C.E. +CISPLATIN	11±0.9487[d,e]	2000

*-Each group consists of 5 animals

**-Data is in Mean ±SEM

a- Non-Significant effect on PCEs as compared to that of vehicle treated group.
b- Significant effect on PCEs as compared to that of vehicle treated group.
c- Significant effect on PCEs as compared to that of extract treated group
d- Non-Significant difference in MNPCEs as compared to that of extract treated group.
e- Significant reduction in MNPCEs as compared to that of Cisplatin treated group.

Data analysed by ONE WAY ANOVA FOLLOWED BY Bonferroni test.

Chromosomal Aberration Test

Table no 5 and 6 shows the results of chromosomal aberration test in rats treated with vehicle only, cisplatin only, extract treated(500 mg/kg)and extract concurrently with cisplatin. %aberration in all groups was tabulated in table no:7. In the present experiment control group shows 2.0175±0.4899 % aberration which consisted of break, fragments, and gap (fig 1 a)(table 5,6 and 7).The group treated with extract only had 2.2125±0.56 %aberration which was almost same as to that of control treated group (fig 1 b). The group treated with cisplatin only showed significant induction of aberration 46.443±6.623%.Particular aberration ring, fragment, gap and break were noticed in the cisplatin only treated group (fig 1 c). The group treated with extract (500 mg/kg body wt) and cisplatin concurrently showed a significant decrease in aberration (fig.1d,e ,f). The aberration scored for extract plus cisplatin treated group was 30.99±1.376% which was found to be significantly less as compared to cisplatin treated group (table no :6). The significant decrease in chromosomal aberration showed genoprotective activity of the extract against cisplatin induced genotoxicity (Graph 1).

Table 5: Mean values of chromosomal aberration.

Chromosomal Aberration (Mean)					
Group	**Break**	**Fragments**	**Ring**	**GAP**	**Total % aberration**
Vehicle only	0.725	0.5175	0	2.02	2.0175
Cisplatin	11.29	16.8925	5.845	10.86	46.4425
T.C.E	0.64	0.62	0	1.755	2.215
Extract +Cisplatin	6.09	11.445	3.7975	7.448	30.9975

Table 6: SD values of chromosomal aberration

Chromosomal Aberration (SD)					
Group	**Break**	**Fragments**	**Ring**	**Gap**	**Total % aberration**
Vehicle only	0.086987	0.1826426	0	0.288906	0.489923463
Cisplatin	2.203845	1.3775673	1.5046	1.133652	6.623646403
T.C.E	0.279091	0.3007075	0	0.202875	0.5610407
Extract + Cisplatin	1.508951	1.6661633	0.5131	1.199205	1.376332687

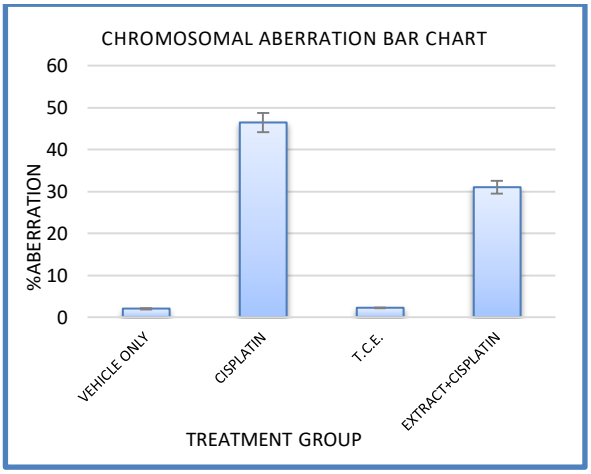
Table7: Data for %Chromosomal Aberration

Chromosomal Aberration Test **	
Group *	**%Chromosomal Aberration**
Vehicle only	2.0175±0.4899
Cisplatin	46.4425±6.623[a,c,d]
T.C.E.	2.2125±0.56[b]
Extract + Cisplatin	30.99±1.376[a,c]

*-Each group consists of 4 animals

**-Data is in Mean ±SD

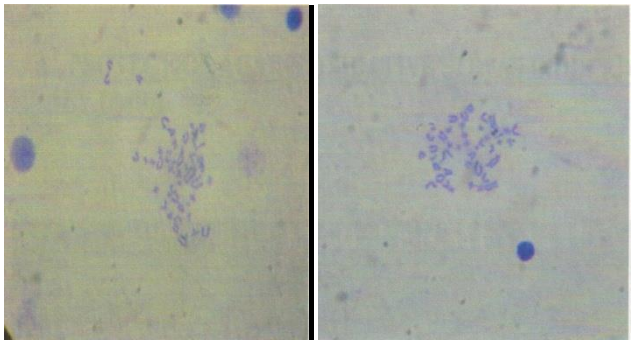
a- Significant effect on chromosome with respect to that of vehicle treated group.
b- Non-Significant effect on chromosome with respect to that of vehicle treated group.
c- Significant effect on PCEs as compared to that of extract treated group
d- Significant effect on chromosomes as compared to that of extract treated group.
e- Significant effect on chromosomes as compared to extract +Cisplatin treated group.

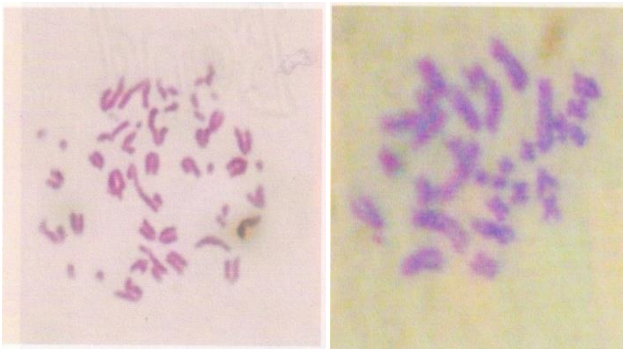


Graph 1: Effect of TerminaliaChebula treatment on chromosomal aberration

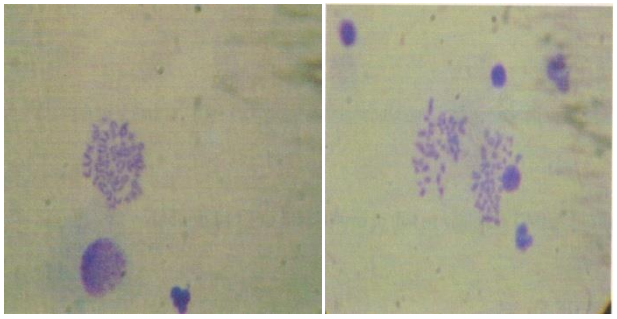
HEALTHY AND ABERRATED METAPHASE

A **B**





C **D**



E **F**

Figure 1: In the figure A, B represents the vehicle treated and T.C.E. treated group. Fig c represents cisplatin treated group and represents aberrated metaphase. Fig D, E and F represents T.C.E and cisplatin treatment concurrently.

CONCLUSION

The present study was undertaken to evaluate the genoprotective activity of *Terminalia chebula. Terminalia chebula* extract was found to be effective against cisplatin induced genotoxicity at dose 500 mg/kg body weight. The groups of rats pre-treated with extract prior to cisplatin showed significant decrease in frequency of micronucleus and chromosomal damage when compared to the cisplatin treated group. The possible mechanism for the protective effect of T*erminalia chebula* fruit extract includes enhancing the antioxidant defence mechanism to neutralize the toxic effect of reactive oxygen species generated by cisplatin. Moreover the presence of tannins caused apoptosis in the damaged cells which was the main reason behind the activity of *Terminalia chebula.*

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