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A study on genotoxic effect of *Calotropis* on human chromosomes

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Abstract
Introduction: *Calotropis* is one of the most common medicinal plants in India, also called Arka. Even though it is very much toxic. There are several harmful effects were reported in the use of Arka.
Materials and Methods: The present study analyzed the cytotoxic effect of *Calotropis procera* and *Calotropis gigantea* on human chromosomes by treating the blood cells with methnolic, chloroform and aqueous (water) extract of roots and leaves of both plants.
Results: The results of present study suggest a genotoxic effect in the presence of bovine liver homogenate at 2.5 mg/ml in human peripheral blood culture under *in vitro* condition altered morphology of chromosome as compared to control. However, further assay needed to characterize morphology with respect to various types of structural chromosomal aberrations.
Conclusion: It is advisable to use optimal, acceptable and lowest possible doses of *Calotrophis* extracts as medicine to minimize any risk, otherwise lead to mutation.

Keywords: *Calotropis procera, Calotropis gigantea,* Chromosomal analysis

1. Introduction
In addition to forest and rural people, maximum urban people use natural preparation derived from plant materials for the primary treatment of different diseases. Mostly in present scenario, the herbal drugs which are prescribed by physicians are either modified version of natural products or isolated from plants (Wang et al., 2007) [1].
In comparison to allopathic medicine the medicine made from plants are less cost effective and has lesser side effect, hence people draw their attention towards Ayurvedic treatment. Same way *Calotropis* is one of the herbal plants used to cure different ailments. It is one of the most common medicinal plants in India, also known as Arka (Hadiman and Anitha, 2015) [2]. In India, Hindu people use the flower and leaves of it for worshiping lord Hanuman. There are four species of *Calotropis* reported till now, however two species (*Calotropis procera* and *Calotropis gigantea*), which are most common and these species are also described by Sanskrit writer (Yelne et al., 2000) [3]. But both penetrate same effect when use in proper dosage to cure disorders. This plant is salt tolerant and has high degree drought resistant. (Sharma et al., 2011) [4].
Calotropis is utilized to treat different ailments from common cold to paralysis. *Calotropis procera* is used in many and varied way as similar to *Calotropis gigantea*. Powder of *Calotropis procera* bark is used in the treatment of diarrhea, dysentery, stomachache, fever, cardiovascular problem and leprosy (Dewan et al., 2009) [5]. Dried root powder of *Calotropis procera* after mixing with water can be used to increase milk flow to feed child and also helps in easy child birth. Leaf pulp is taken as antidote at the time of snake bite to stop the spread of poison. Flower are used to treat cold, astringent, improve digestive system and also treat impotence (Maroyi, 2012) [6].
Different parts of *Calotropis gigantea* including latex are used as medicine to treat various ailments at appropriate dosage. It also play an important role to cure skin disease by astringent action. Latex and leaves juice are used as antidote for snake poison and also cure diarrhea (Dewan et al., 2009) [5]. Dried leaf powder can be used to treat the wounds during injury. Jaggery and flower mixture help during cough and in improvement of appetite (Oudhia et al., 1997) [7].
Even though plethora of medicinal uses and benefits of *Calotropis*, it is very much toxic also. Hence, before using it, a person should have to consult doctor. If it is taken in excess amount it may causes vomiting and diarrhea.

In pregnancy and lactation time it can be harmful if used in higher amount (Greim and Synder, 2008) [8]. The use of *Calotropis procera* and *Calotropis gigantea* is increasing day by day in the treatment of many disease due to belief that it is natural, safe to use and easily available and inexpensive. Many articles are published on direct or indirect medicinal uses of *Calotropis* plants (Poonam and Punia, 2013) [9]. However, there are only very few studies which showed its toxicological potential (Palejkar et al., 2012) [10]. The toxicity of this plant is reversible which induced permanent loss of endothelial cells and also damages retina of eye. Person become blind if his/her eye directly or indirectly come in contact with milk of *Calotropis*. The over dosage may lead to death of person as it is very poisonous. Evaluation of toxicological potential of *Calotropis* is very much essential, as plant have so many toxic effects, if used in inappropriate dosage and without the guidance of the expert physicians (Singh, 2012) [11].

Looking in to medicinal value of *Calotropis* present study designed to evaluate genotoxicity of various plant extracts of *Calotropis procera* and *Calotropis gigantea* on peripheral blood lymphocytes of human.

2. Materials and Methodology

2.1. Sample collection

Root and leaves samples of *Calotropis procera* and *Calotropis gigantea* were collected from New Vallabh Vidyanagar, Vadtal and Bardoli regions of Gujarat. Blood samples from disease free, non-smoker, healthy adult male and female volunteers were collected after signing the written consent.

2.2. Preparation of plant extracts

Leaves and root of selected plants were dried and grinded with the help of household mixture. The resultant powder sequentially extracted with solvents (Chloroform, Methanol and distilled water). The solvents from the extracts were evaporated aseptically to get final dried matter. The materials were scrapped out and dissolved in RPMI 1640 to make final concentration of 100 mg/ml.

2.3. Liver homogenate preparation

The substitute of S9 mixture (Hakura et al., 2004) was prepared by taking 1 gm of liver tissue of male buffalo which was collected from slaughter house. Liver tissue was washed with phosphate buffer (P^H 7.4), cut into small pieces and homogenate was prepared by grinding it into mortar and pestle. Metabolic activation of all three solvent extracts of *Calotropis procera* and *Calotropis gigantea* roots and leaves were carried out by treating 100 mg of extracts per 1 ml of liver homogenate.

2.4. Chromosomal assay

The complete media was prepared using 7 ml of RPMI 1640, 1 ml of fetal bovine serum, 100 µl of 200 mM L-glutamine, 30 µl and 15 µl streptomycin (100mg/ml) and penicillin (100mg/ml) respectively, 100 µl of phytohemagglutinin (M form) and 0.5 ml of freshly collected human blood were mixed in a 15 ml graduated tubes. Different concentrations of (2.5 to 25 mg/ml) plant extracts added to the blood culture directly and after metabolic activation and incubated at 37^0C. Culture tubes were incubated in an incubator at 37^0C for 72 hours. The culture was mixed every day by inverting the tubes slowly in the morning and evening (Rooney, 2001) [13].

After 71 hours of incubation, 50 µL colchicines (2 mg/10 ml) was added under sterile condition, tubes were closed and mixed followed by addition of 75 µl ethidium bromide (10 mg/ml), mixing and incubation for 1 hour at 37^0C. After completion of incubation, culture tubes were centrifuged at 3500 rpm for 8 minutes. Resultant supernatant was removed and preheated hypotonic solution (60.75 M KCL) was added up to 8 ml in each culture tubes. The culture tubes were immediately incubated at 37^0C in water bath for 17 minutes. Fixative was added up to 10 ml in each culture tubes, mixed it slowly with the help of pasture pipettes until the color of culture turned to reddish brown. Culture tubes were centrifuged at 3500 rpm for 8 minutes and supernatant was discarded. Once again 8 ml of chilled fixative was added in same tubes and centrifuged same as before the supernatant was removed. Above washing steps were repeated with decreasing the volume of fixative (6 ml, 5ml, 4ml, 3ml) till clear and transparent white color pellet observed. The slide was prepared from the cell culture suspension and it was heat fixed at 40 ^{0}C. The glass slide then stained with Giemsa's stain for 8 to 10 minutes.

3. Results and Discussion

The yield of plant extracts was varied between 0.18 to 4.35 mg (Table 1). It was higher in distilled water extracts than chloroform and methanol. This variability may be due to differential solubility of compounds and also due to the geographical variation in the content of the compounds present in the plant materials.

Table 1: Variation in plant extracts yield per 15 gram of dried powder plant materials

Region	Sample name	Extracted material (g)	Sample name	Extracted material (g)
New V.V.Nagar *Calotropis procera*	AP-d	0.93	P3-Rd	0.19
	AP-c	0.45	P3-Rc	0.18
	AP-m	0.67	P3-Rm	0.25
New V.V.Nagar *Calotropis gigantean*	Ag-d	1.17	G3-Rd	0.69
	Ag-c	1.16	G3-Rc	1.02
	Ag-m	1.81	G3-Rm	1.5
Bardoli *Calotropis procera*	P3-d	2.51	P3-Rd	0.68
	P3-c	1.53	P3-Rc	1.24
	P3-m	1.90	P3-Rm	1.52
Bardoli *Calotropis gigantean*	G3-d	3.76	G3-Rd	2.04
	G3-c	3.29	G3-Rc	2.16
	G3-m	4.35	G3-Rm	2.31
Vadtal *Calotropis procera*	VP-d	2.62	Vp-Rd	0.62
	VP-c	2.38	Vp-Rc	0.83
	VP-m	3.64	Vp-Rm	1.27
Vadtal	Vg-d	3.98	Vg-Rd	1.33

Calotropis gigantea	Vg-c	2.65	Vg-Rc	1.95
	Vg-m	4.25	Vg-Rm	3.14

(g=gram)

Metabolic activation analysis in the presence of bovine liver tissue homogenate revealed detoxification of the compound present in 2.5 mg/ml of methanolic, chloroform and distilled water extracts of root and leaves of *Calotropis gigantea* by liver enzyme (Figure 1 & 2). However, there was significant alternation in the morphological appearance of chromosomes as compared with control at 2.5 mg/ml of extracts (Figure 3 & 4).

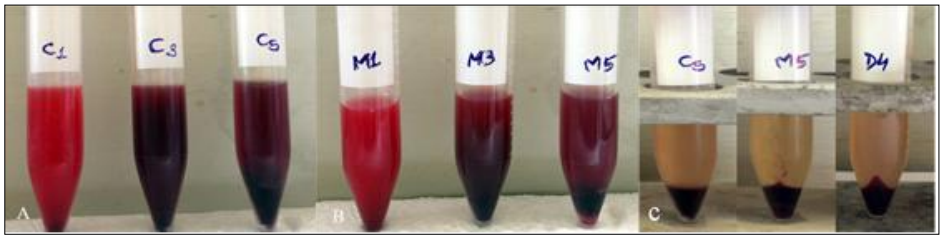

Fig 1: Genotoxic effect of chloroform (A) and methanolic (B) extract of *C. gigantea* leaves on human chromosomes at 2.5 mg/ml concentration following metabolic activation, (C) Chromosomes from control (without metabolic activation).

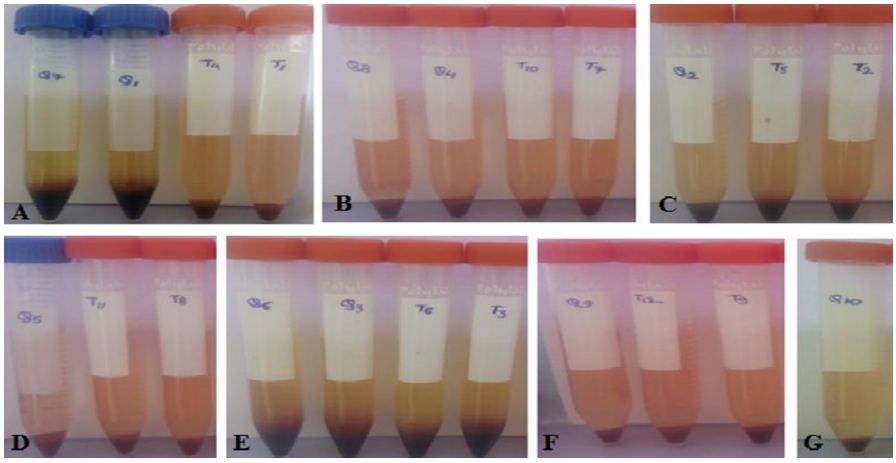

Fig 2: Genotoxic effect of chloroform (A & B), methanolic (C & D) and distilled water (E & F) extract of *C. gigantea* roots and leaves on peripheral blood lymphocytes culture at 2.5 mg/ml concentration with and without metabolic activation. (A): Q7, Q1 are root chloroform extract without metabolic activation and T4, T1 are root chloroform extracts with metabolic activation; (B): Q8, Q4 are leaves chloroform extract without metabolic activation and T10, T7 are leaves chloroform extracts with metabolic activation; (C): Q2 is root methanol extracts without metabolic activation and T5, T2 are root methanol extract with metabolic activation; (D): Q5 is leaves methanol extract without metabolic activation and T4, T1 are leaves methanol extract with metabolic activation; (E): Q6, Q3 are root distilled water extract without metabolic activation and T6, T3 are root distilled water extracts with metabolic activation; (F): Q9 is leaves distilled water extract without metabolic activation and T12, T9 are leaves distilled water extract with metabolic activation; (G) is control.

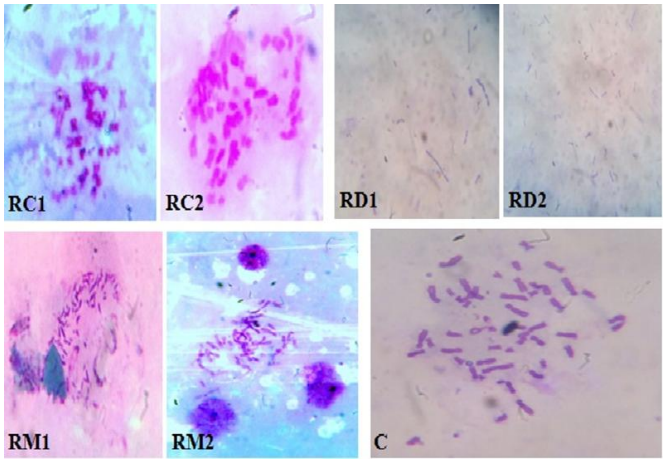

Fig 3: Effect of chloroform (RC1, RC2), methanolic (RM1, RM2) and distilled water (RD1, RD2) extract of *C. gigantea* roots on human chromosomes. RC1, RD1 and RM1 are the cultures treated with chloroform, distilled water and methanol extracts of *C. gigantea* root respectively with metabolic activation by bovine lever homogenate. RC2, RD2 and RM2 are the cultures treated with chloroform, distilled water and methanol *C. gigantea* root respectively without metabolic activation by bovine liver homogenate. C is control chromosomes.

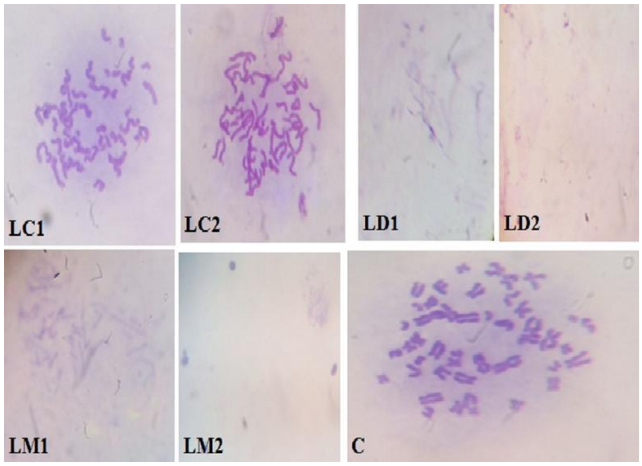

Fig 4: Effect of chloroform (LC1, LC2), methanolic (LM1, LM2) and distilled water (LD1, LD2) extracts of *C. gigantea* leaves on chromosome. LC1, LD1 and LM1 are the cultures treated with chloroform, distilled water and methanol extracts of *C. gigantea* leaves respectively with metabolic activation by bovine liver homogenate. LC2, LD2 and LM2 are the cultures treated with chloroform, distilled water and methanol *C. gigantea* leaves respectively without metabolic activation by bovine liver homogenate. C is control chromosomes.

Cytogenetic analysis of treated whole blood cells revealed visible change in the appearance of blood culture after 24 hours and 48 hours of incubation in the presence of higher concentration of plants extracts. Methanolic extract exhibited least reddish appearance as compared with chloroform which was red in color (Figure 1). There is a significant alteration in the cell pellet appearance and volume between chloroform, methanol and distilled water extracts treated blood culture tubes of *Calotropis procera* and *Calotropis gigantea* plants as compared with control (Figure 2).

Initially, there were two different random concentrations (20 and 25 mg/ml) of *C. procera* and *C. gigantea* methanolic, chloroform and distilled water extracts were used for the evaluation of genotoxicity. However, there were no chromosomes in chloroform and methanolic extract treated blood culture in comparison with control (without metabolic activation) following Giemsa's staining and microscopic observation. In the second phase, systematic genotoxicity analysis using reduced concentration of plant extracts (25 mg/ml, 10 mg/ml, 5 mg/ml and 2.5 mg/ml) without metabolic activation. Results revealed that there were absence of chromosomes in *Calotropis gigantea* and *Calotropis procera* root extract treated blood culture than control which showed chromosomes (Table 2). The absences of chromosomes in distilled water, methanol and chloroform extracts treated blood culture are due to their strong genotoxic effect. Further searching literature, we found that the concentration used in our study is 12.5 to 25 times higher than that was prescribed (0.5mg-1mg) for the preparation of Ayurvedic medicines from Calotropis. Chromosomal aberration study using lower concentration of *Calotropis procera* and *Calotropis gigantea* aqueous, methanolic and chloroform extracts were repeated. Microscopic observation revealed absence of metaphase in slide prepared for tested concentration. Control in both, the methanolic and chloroform extracts of *Calotropis procera* and *Calotropis gigantea*. The slides showing high contamination of bacteria in case of aqueous extract of *Calotropis procera* and *Calotropis gigantea* leeds to culture failure.

Table 2: Extract and concentration wise metaphase chromosome analysis

Extracts	Concentration in mg/ml	Total No. of metaphase	
		Calotropis procera	***Calotropis gigantean***
Distilled Water (Aqueous)	DY1(Control)	11	25
	2.5 (MA)	00	00
	2.5	00	00
	5.0	00	00
	10	00	00
	15	00	00
	25	00	00
Methanol	MY1(Control)	10	24
	2.5 (MA)	3	5
	2.5	00	00
	5.0	00	00
	10	00	00
	15	00	00
	25	00	00
Chloroform	CY1(Control)	18	29
	2.5 (MA)	00	00
	5.0	00	00
	10	00	00
	15	00	00
	25	00	00

There are many reports related to genotoxicity analysis of chemicals using peripheral blood lymphocytes (Siddique, et al. 2006; 2009). Medicinal plant extracts toxicity also studied on *Vicia faba* root tip chromosomes and *Salmonella typhimurium* based bacterial system (Ames test) (Sobita and Bhagirath. 2005; Hong and Lyn. 2011). *In vitro* cytotoxic activity of *C. procera* latex and flowers extracts are evaluated against MCF-7 (Breast cancer) and HeLa (Cervical cancer) cell line and they found that ethanolic extract of dried latex and flowers showed cytotoxic properties against MCF-7 and HeLa cells in dose dependent manner (Pusapati et al. 2012).

Recently, the demand plant derived medicine are increased for the treatment of variety of diseases, therefore, it is extremely important to evaluate their genotoxicity. The genotoxicity test provides widely used for human risk assessment. The mutagenic hazard can be manifested as a heritable change resulting from gametic or somatic cells mutation (cancer) (Roncada et al. 2004). Chromosomal aberration is a routinely used for cytotoxicity analysis of various compound used in the treatment of human diseases. For this reason in the current study, evaluation of genotoxic effect of *Calotropis procera* and *Calotropis gigantea* methanolic, chloroform and aqueous extracts carried out on human lymphocytes followed by chromosomal analysis.

It is difficult to compare present findings, as there was not a single report in the literature related to genotoxicity of root and leaves of *Calotropis procera* and *Calotropis gigantea* to the best of our knowledge using chromosomal aberration assay.

4. Conclusion

As plants are great source of medicines for various ailments. Their effective concentration can be found out to avoid genotoxicity in human. *Calotropis procera* and *Calotropis gigantea* methanol, chloroform and distilled water extracts are toxic to human chromosomes at a concentration of 2.5 - 25 mg/ml. The results of present study suggest a genotoxic effect of *Calotropis* extracts in the presence of bovine liver homogenate at 2.5 mg/ml in human peripheral blood culture under *in vitro* condition with altered morphology of chromosomes as compared to control. However, further detail study needed to characterize this altered morphology with respect to various types of structural chromosomal aberrations. The toxicity evaluation by cytogenetic alteration is an initial step in the risk assessment procedure for genotoxic substances. It is therefore, advisable to use optimal acceptable lowest possible doses of the plant extracts as medicine and to minimize any risk otherwise lead to mutation.

5. Acknowledgements

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6. Conflict of interest

None

7. References

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