



# Journal of Medical Sciences

ISSN 1682-4474

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**JMS (ISSN 1682-4474) is an International, peer-reviewed scientific journal that publishes original article in experimental & clinical medicine and related disciplines such as molecular biology, biochemistry, genetics, biophysics, bio-and medical technology. JMS is issued eight times per year on paper and in electronic format.**

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## Assessing Mutagenicity of Methanolic Extract of *Abrus precatorius* Seeds using Ames Bioassay

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*Abrus precatorius* seeds contain toxin abrin which inhibits protein synthesis and leading to cell death. This plant has Cytotoxic and pro-apoptotic effects. So, in this study, mutagenicity of methanolic seed extract of this plant was investigated using Ames test. The seeds were powdered and then maceration extracted with methanol for 48 h. Extract was analyzed for aflatoxin B1 and histidine contamination with TLC and MIC determined by broth dilution method. The test was performed using strain TA<sub>100</sub>. The genotypes was confirmed by presence of hisG46 Mutation, R factor plasmid and crystal violet sensitivity test. TA<sub>100</sub> carries mutations in genes involved in histidine synthesis, so that it requires histidine for growth. Tests were performed with 4 concentrations 0.2, 0.3, 0.4 and 0.5 mg mL<sup>-1</sup> (concentrations less than 0.1 MIC), negative control (methanol), positive control (NaN<sub>3</sub>), in the absence and presence of a rat liver metabolizing system. No increase in bacteria growth in the histidine-free medium was observed. In conclusion, *Abrus precatorius* was not genotoxic in Ames test. However, it is likely that the *Abrus precatorius* maybe inducing gonotoxicity at high concentrations. Thus, it is necessary to pay close attention while utilizing its products.

**Key words:** *Abrus precatorius*, mutagenicity, Ames test, *Salmonella typhimurium* (TA<sub>100</sub>)

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## INTRODUCTION

Medicinal Herbs and herbal products have been used as medicines for treatment of various diseases since ancient. The consumption of herbal medicines is increasing rapidly across the world and many people in both developed and developing countries are using these products. Although consumers believe herbal products are natural and therefore safe, they can cause adverse effects and toxicity (Gardiner *et al.*, 2007; Ernst, 2004a, b). The mutagenic effects of widely used medicinal plants are not investigated well. Recent study has indicated that some plant compounds such as Safrol, found in *Sassafras albidum*, Cycasin that is found in Cycadaceae family and Pyrrolizidine Alkaloids (PAs) that are found frequently in the Boraginaceae, Asteraceae and Orchidaceae families are toxic and carcinogenic (Ernst, 2004b; Rietjens *et al.*, 2005; Mei *et al.*, 2007; Fu *et al.*, 2004; Montbriand, 2005; Woo *et al.*, 1988; Birdsey, 1972; Louw and Oelofsen, 1975). Although there are few investigations on the toxicological properties of plants especially, their mutagenicity and carcinogenicity, recent findings raise questions about the safety and continued extensive use of all medicinal plants.

*Abrus precatorius* belongs to the family Fabaceae. The plant grows widely in tropical and subtropical areas of the world and known commonly as Jequirity bean, Rosary Pea, Crab's Eye and Indian Licorice. The roots, stems and leaves of this plant contain glycyrrhizin and are used in medicinal purposes (Ross, 2001; Rajaram and Janardhanam, 1992; Windholz, 1983). The seeds contain alkaloids, tannins, flavonoids and anti-hepatitis, purgative, diuretic, anti-microbial and anti-fertility activity. The seeds have protective effect against alcohol-induced renal damage and also used to treat chronic nephritis and diabetes mellitus (Jaja *et al.*, 2009; Watt and Breyer-Brandwijk, 1962).

The short term genotoxicity test such as Ames *Salmonella*/microsome mutagenicity assay (*Salmonella* test; Ames test) is an appropriate and relatively inexpensive test for estimating the carcinogenic potentials of the chemicals and natural products. The main goal of this mutagenicity test is to identify genotoxic and carcinogenic ability of the test compound. This *Salmonella* mutation assay is performed under both non-metabolic and metabolic conditions. The reverted bacteria in Ames test are strain that are able to grow in condition containing trace of histidine. The colonies are made only by histidine independence (*hisC*) bacteria. The number of such colonies is increased in a dose-dependent manner by a mutagen (McCann *et al.*, 1975). TA 100 strain was used in all experiments.

*Abrus precatorius* is known as a poisonous plant. Signs of poisoning include vomiting, dehydration, abdominal pain, drowsiness and convulsions (Lucas, 2006). Its triterpenoid showed moderate cytotoxicity against different cell lines (Xiao *et al.*, 2011). The seeds contain potent phytotoxin abrin. Abrin is classified as type 2 Ribosome-inactivating Proteins (RIPs) which inhibit protein synthesis and leading to cell death (Stirpe and Battelli, 2006; Olsnes *et al.*, 1975). This plant also has cytotoxic and pro-apoptotic effects (Sofi *et al.*, 2013). The presence of toxic lectins in seeds may act as a potential limitation for its pharmacologic utilities. *Abrus spinosus* does not exhibit cytotoxic effect such as sperm toxic effects like effects produced by *A. precatorius* (Gigani *et al.*, 2012). The aim of this study was to confirm the possibility of developing health problems such as mutagenicity arising from the use of *Abrus precatorius*. The genotoxicity of this plant has not yet been studied. So, in this study, the risk of carcinogenesis induced by *Abrus precatorius* was investigated using Ames bioassay.

## MATERIALS AND METHODS

*Abrus precatorius* seeds were obtained from reliable sources and authenticated by Department of Pharmacognosy, Faculty of Pharmacy, Jundishapur University of Medical Sciences. *Salmonella typhimurium* strain TA<sub>100</sub> was purchased from Persian Type Culture Collection (PTCC) organization.

**MIC determination of abrus precatorius extract:** Dilution method for the determination of MIC (minimum inhibitory concentration of bacteria) was used. The medium used was LB broth supplemented with histidine and biotin. Concentrations 20, 15, 10, 5 and 2.5 of extract were prepared. As a negative control 2 tubes and 2 tubes lacking the plant extract were used as positive control. Then, 0.1 mL of overnight bacterial culture was added to each tube. The tubes for 24 h at 37°C and the lowest concentration of the tube without turbidity were considered as the MIC (Maron and Ames, 1983).

**Aflatoxin detection in the plant extract:** Thin Layer Chromatography (TLC) was used for the detection of aflatoxin. Mobile phase consisting of chloroform, 5.5, acetone, 12.5, water 2.5 and 1 µg mL<sup>-1</sup> Aflatoxin B1 solution was used as the standard solution. Spots were observed with UV (366 nm) (Mahmoud *et al.*, 1992).

**Assessing the presence of histidine in the plant extract with thin layer chromatography (TLC):** The presence of

histamine in samples of methanolic extract can raise apparent spontaneous revertant mutation rates, so, it was indicated that no histidine is present in samples with a TLC according to the methods given by Freid and Sherma (1982).

**Preparation of test compounds for mutagenicity assay bacterial strain growth:** Overnight cultures of *Salmonella* TA<sub>100</sub> was grown in LB broth supplemented with histidine and biotin for 48 h at 37°C to reach the concentration of  $1.5 \times 10^8$  bacteria per mL.

**Genotype confirming:** The tester strain was confirmed prior to use for different requirements and characteristics according to the methods given by Maron and Ames (1983).

**Histidine dependence (his):** A loop full of the culture was streaked across a LB agar plate supplemented with an excess of biotin. Because all the *Salmonella* strains are histidine dependent, there should be no growth on the plates.

**Biotin dependence (bio):** A loop full of the culture was streaked across a LB agar plate supplemented with an excess of histidine. There should be no growth on the plate.

**Biotin and histidine dependence (bio; his):** A loop full of the culture was streaked across a LB agar plate supplemented with an excess of biotin and histidine. Growth should be observed with all strains.

**RFA marker:** A loop full of the culture was streaked across a LB agar plate supplemented with an excess of biotin and histidine. A sterile filter paper disk was placed in the center of the streak and 10 mL of a sterile 0.1% crystal violet solution was applied. The *Salmonella* strain showed a zone of growth inhibition surrounding the disk.

**R factor:** 0.1 mL of overnight bacterial culture was added to ampicillin plates and was incubated for 12 h at 37°C. Bacterial growth in the ampicillin plates confirms presence of R factor in the bacteria (Maron and Ames, 1983).

**Preparation of rat-liver S9 fraction and mix:** For checking the metabolic activation of the test compounds, incubation with 400 µL of S9 fractions were carried out. Male Wistar rats (body weight~250 g) were injected with 100 mg kg<sup>-1</sup> phenobarbitone sodium daily for 5 days. Sixth Day, rats were sacrificed by cervical dislocation and

livers were collected, homogenized in 0.15 M KCl. The homogenate was centrifuged at 9,000 g for 10 min. The supernatant was aliquoted (2 mL portions) and stored at -18°C until used (Garner *et al.*, 1972). The S9 mix was prepared according to the recipe recommended by Maron and Ames (Maron and Ames, 1983) and Mortelmans and Zeiger (Mortelmans and Zeiger, 2000). 0.4 mL per plate of the high S9 mix was used in the experiment.

**S9 mix:** The S9 mix composing of 8 mM MgCl, 33 mM KCl, 5 mM glucose-6-phosphate, 4 mM NADPH, 4 mM NADH, 100 mM sodium phosphate (pH 7.4) and 2 mL of S9 was used.

**Ames/Salmonella typhimurium mutagenicity test:** Four concentrations of plant extract (0.2, 0.3, 0.4 and 0.5 mg mL<sup>-1</sup> in methanol) were tested. Ames test was carried out as standard plate incorporation test (Ames *et al.*, 1975) with *Salmonella typhimurium* strain TA<sub>100</sub> with and without *in vitro* microsomal activation (by S9 rat liver homogenate). The assay was performed as follows: molten LB agar containing 0.08 mg per mL histidine and 0.12 mg mL<sup>-1</sup> biotin was poured into plates (with or without 0.4 mL per plate metabolic activation (S9) mix). In following, 0.5 mL of plant extracts/or 0.5 mg mL<sup>-1</sup> NaN<sub>3</sub> in distilled water as positive control/or methanol as negative control was used and spread. The mixtures were then preincubated 0.2 mL of the overnight culture of *Salmonella*TA100 and distributed with a sterile loop on top of the LB agar. The plates were incubated in an inverted position for up to 4 days at 37°C. All the experiments were repeated twice in triplicate. All experimental data were expressed as Mean±SD.

## RESULTS AND DISCUSSION

**MIC determination of abrusprecatorius extract:** The minimum inhibitory concentration of plant extracts on microbial growth was determined 5 mg mL<sup>-1</sup>. This determination is routine mainly to confirm resistance, as a research tool by diagnostic laboratories and researchers. This determination was carried out to diminish possible antibacterial activity effects of extract with respect to bacterial species used by Ames test.

**Aflatoxin detection in plant extract:** No Aflatoxin was detected in the plant extract with TLC method. The non-microsomal aflatoxin mutagenicity was showed previously in *Salmonella typhimurium* TA100 and TA98 (Yourtee *et al.*, 1987). This detection is performed in order to fully exclude this mutagenicity.

**Assessing the presence of histidine in the plant extract with thin layer chromatography (TLC):** No histidine was detected with TLC method. Therefore, the presence of histidine in plant extract cannot contribute to the spontaneous revertant rate.

**Genotype confirming:** Microbial growth on plates containing histidine and biotin was observed after 24 h. While on plates containing histidine or biotin only no microbial growth was observed.

**Rfactor:** The Rfactor was confirmed by resistance to ampicillin. This test should be done in order to show full resistance of TA<sub>100</sub> strain.

**Ames/Salmonella typhimurium mutagenicity test:** The results of the bacterial reversion assay with four concentrations of plant extracts are presented in Table 1 and 2. In Table 1, the results of extract concentrations in absence of liver-metabolizing enzymes were presented. In Table 2, the results of extract concentrations in presence of liver-metabolizing enzymes were presented. According to the EPA and GenPharmTox guidelines, a mutagenic potential of a test item, tested with Ames test, is confirmed if the mutant frequency (expressed as induction factor) is 2.0 or higher (USEA, 1996; OECD, 1997). The results suggested that the dose of 0.2-0.5 mg mL<sup>-1</sup> (concentrations less than 0.1 MIC) of *Abrus precatorius* was not mutagenic to the TA<sub>100</sub> strain of *S. typhimurium*, either with or without metabolic activation. The analysis

of induction factor of administered 0.2-0.5 mg mL<sup>-1</sup> of *Abrus precatorius* extract showed that the numbers were not affected significantly by any of the doses of the extract tested compared with control (Table 1, 2). The highest numbers of induced mutation were estimated 1.73 and 1.72, in absence and presence of liver-metabolizing enzymes, respectively. Therefore, it is likely that ingredients in *Abrus precatorius* such as toxic lectins don't affect the key enzymes by means of activation or inhibition.

The experiments were analyzed in triplicate and were repeated again to confirm the result. The interpretation was carried out similar to those described in regulatory guidelines. The number of induced mutation should be at least twice the activity observed in negative control and there must be a reproducible dose response curve. The influence of metabolic activation was carried out by using 400 µL of S<sub>9</sub> mixture.

A dose effect relationship could underlay this conclusion. A possible mutagenic potential is assumed if the IF quotient ranges 1.7 to 1.9 in combination with dose effect relationship. No mutagenic potential is assumed if all IF quotients range 1.0 (and lower) to 1.6. A nonexistent dose effect relationship could underline this conclusion (USEA, 1996; OECD, 1997). In our study none of the results of the Ames test (+S<sub>9</sub> and -S<sub>9</sub>) exceeded the critical value 2.0 in some concentrations IF quotients was higher than 1.7, but there was not a dose effect relationship. Therefore, no mutagenic activity was observed in any of extract samples tested on

Table 1: Results of *Abrus precatorius* extract concentrations in absence of liver-metabolizing enzymes

						Controls	
Concentration (mg mL <sup>-1</sup> )		C <sub>1</sub> (0.2)	C <sub>2</sub> (0.3)	C <sub>3</sub> (0.4)	C <sub>4</sub> (0.5)	Control+ (NaN <sub>3</sub> 0.01)	Control- (methanol)
Repeat 1	Plate 1	147	155	155	154	385	92
	Plate 2	159	160	159	161	400	90
Repeat 2	Plate 1	151	154	163	157	410	95
	Plate 2	150	155	158	165	390	89
Repeat 3	Plate 1	153	148	168	165	395	97
	Plate 2	158	157	162	166	415	96
Mean±SD		153±4.49	154.83±4.47	160±4.5	161±4.9	399±11.68	93±3.13
Induction factor (IF)±SD		1.64±0.073	1.66±0.091	1.73±0.038	1.73±0.076	4.29±0.136	

No. of colonies and Induction factor (IF) were showed as Mean±SD

Table 2: Results of *Abrus precatorius* extract concentrations in presence of liver-metabolizing enzymes

						Controls	
Concentration(mg mL <sup>-1</sup> )		C <sub>1</sub> (0.2)	C <sub>2</sub> (0.3)	C <sub>3</sub> (0.4)	C <sub>4</sub> (0.5)	Control+ (NaN <sub>3</sub> 0.01)	Control- (methanol)
Repeat 1	Plate 1	150	155	149	155	380	89
	Plate 2	157	148	155	159	395	91
Repeat 2	Plate 1	153	159	161	154	420	96
	Plate 2	147	150	156	162	415	87
Repeat 3	Plate 1	158	155	165	158	385	95
	Plate 2	151	161	155	166	400	90
Mean±SD		152.67±4.23	155±5	156.8±5.53	159±4.47	399.17±15.94	91±3.55
Induction factor (IF)±SD		1.68 ±0.044	1.69±0.066	1.72±0.044	1.72±0.1	4.38±0.235	

No. of colonies and Induction factor (IF) were showed as Mean±SD

*Salmonella typhimurium* strain TA<sub>100</sub>. Also, the statistical significance of genotoxic potentials in any of the samples according to the negative control was not proven ( $p > 0.05$ ). The results of short-term genotoxicity/mutagenicity tests on *Salmonella typhimurium* did not show the presence of genotoxic compounds in samples of plant extract. The bacterial mutagenicity assays can be carried out in 48 h and considered as rapid prescreens for distinguishing between carcinogenic and non-carcinogenic chemicals.

The recent literatures review show that constitutes such as PA (pyrrolizidine alkaloids), Safrol, cycasin are toxic and carcinogens (Ernst, 2004b; Rietjens *et al.*, 2005; Mei *et al.*, 2007; Fu *et al.*, 2004; Montbriand, 2005; Woo *et al.*, 1988; Birdsey, 1972; Louw and Oelofsen, 1975). These ingredients can penetrate the cell wall of bacteria, suggest the Ames test is suitable for evaluating the genotoxicity of extract. It is well worth to note that extracts showed genotoxic effects in bacterial tests also showed genotoxicity in at least one of the genotoxicity tests in human blood such as the micronucleus test (for detection of chromosome breakage and aneuploidy) and the alkaline comet assay (for DNA damage) (Taylor *et al.*, 2003). This test allowing many thousands of compounds in our environment, not previously tested, to be screened for potential hazard. A good correlation has been observed by several groups, for a number of carcinogenic drugs in their ability to induce mutation in the above strain and the ability to induce a response in animals. Thus Ames test can easily and quickly assess mutagenic potential of these chemicals. For this initial screening, the tester strain TA<sub>100</sub> was used due to its sensitivity to a broad range of mutagens and carcinogens. However, many substances are inactive in the TA<sub>100</sub> assay and active against other tester strains, e.g., TA<sub>98</sub>. Thus, the use of strains in addition to TA<sub>100</sub> should be considered in more comprehensive screening programs.

However, medicinal plants are complex ingredients make it difficult to guess on the compounds responsible for the toxicity and also mutagenic effects. This plant with diverse pharmacological activity has different active metabolites in the seed such as abrin, abrus agglutinin, glycyrrhizin, gallic acid, trigonelline, precatorine and lipolytic enzymes. Glucine, coumestrans, resin asparagines and sterols are other ingredients (Ross, 2001; Jaja *et al.*, 2009). Our results contribute toward validation that these ingredients are not potential or effective genotoxic.

The evaluation of the significance of these findings through clinical follow-up will be continued. Clarification

of these mechanistic elements in mutation induction and determining the differences in specificity between bacterial and mammalian systems remains an interesting goal for further investigation.

## CONCLUSION

In conclusion, *abrus precatorius* was not genotoxic in Ames test. However, it is likely that the *abrus precatorius* maybe inducing gonotoxicity at high concentrations. Thus, it is necessary to pay close attention while utilizing its products. Therefore, screening for potential genotoxic effects of wildy used medicinal plants in traditional medicine is recommended.

## ACKNOWLEDGMENTS

This study is a part of a Pharm. D. thesis. We would like to gratefully and sincerely thank department of microbiology of Faculty of Medicine. This work is granted by Ahvaz Jundishapur University of Medical Sciences.

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