CYTOGENETIC AND MOLECULAR EVALUATIONS OF GENETIC EFFECTS OF LEAF EXTRACT OF *Rhazya stricta* (Decne) ON *Allium cepa* ROOT TIP MERISTEMS

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Rhazya stricta (Decne) of the *Apocynaceae* family is a widely distributed plant in Saudi Arabia. Extract of its leaf is prescribed in folkloric medicine for the treatment of various disorders such as diabetes, sore throat, helminthiasis, inflammatory conditions and rheumatism (Ageel *et al*., 1987; Ali *et al*., 1995; Ali *et al*., 1998). This extract mainly contains alkaloids, glycosides, flavonoides, tannins and triterpenes (Ahmad *et al*., 1983; Al-Yahya *et al*., 1990). Several studies on rats and mice reported that the leaf extract causes sedation, analgesia, decreases motor activity and has anti-depressant, anti-oxidant activities, complex effects on brain endogenous monoamine oxidase activity and centrally-mediated hypotension (Ali *et al*., 1998; Tanira *et al*., 2000; Ali *et al*., 2000). Moreover, one of the studies ascribed anticancer activities to the indole alkaloids of *Rhazya stricta* (Mukhopadhyay *et al*., 1981). The genotoxicity of *Rhazya stricta* leaf extract was demonstrated by Baeshin *et al*. (2005) in *Saccharomyces cerevisiae* where auxotrophic mutants were recovered upon treatment with elevated concentrations of the extract. This necessitated a battery of tests to establish its genotoxic effect. The purpose of this study is to evaluate the genetic effects of the aqueous leaf extract of *Rhazya stricta* in root tip meristems of *Allium cepa* by cytogenetic and molecular assays.

MATERIALS AND METHODS

A. Preparation of the extract

Field identification of the plant was carried out by Prof. Baeshin following the work of (Batanouny and Baeshin, 1978). Leaf of *Rhazya stricta* were collected during the last week of December 2006 from naturally growing plants in their natural habitats located along the roadsides of Jeddah-Makkah highway. Collected leaf were kept in plastic bags in the field and later transferred in their bags to a fridge and kept overnight then subjected to extraction in the following day. The leaves were washed well with running water to dust and sands, hand-minced into small pieces, mixed with sterilized distilled water (1.5,

3 and 6 gm/L) and further blended in a blending machine. The mixture was then left for 24 hours at room temperature with mild hand-shaking at regular time intervals. The mixture was then filtered through a membrane filter and the filtrate (the extract) was either used directly in the experiment or kept in the fridge for no longer than 3 days for future use. Three concentrations of this stock extract were prepared (1.5, 3 and 6 gm/L) to be tested for genotoxic activities based on the dose prescribed in folkloric medicine (6 gm/L).

B. Allium cepa

The seeds and bulbs of *Allium cepa* were obtained from local nurseries.

C. Chemicals

All chemicals used in the present study obtained from Sigma Inc., except the followings: DNA polymerase (Perkin-Elmer Cetus), dNTPs (Boehringer Mannheim, Germany), DNA purification and extraction reagents and Agarose gel (Qiagen), oligonucleotides as random primers (Genetic laboratory, Cairo Univ.), DNA standard for Agarose gel electrophoresis (Gibco BRL), loading dye solution (Fermentas, Lithuania), protein assay reagents electrophoretic reagents, and protein standards (Bio-Rad).

D. Test of Genotoxicity

1- LD_{50} determination

Seeds of *Allium cepa* were germinated in elevated concentrations of aqueous extracts of the wild plant *Rhazya stricta* leaf (1, 1.5, 3, 6, 12, 24, 50, 100 and 200 gm/L) for seven days. The length of germinated seeds were measured followed by determining the concentration of leaf extraction which decreases the length of plant to 50% compared to control (LD_{50}).

2- Cytogenetic analysis

Roots of bulbs of *Allium cepa* were exposed to LD_{50}, 1/2 LD_{50} and 1/4 LD_{50} of aqueous extracts of the wild plant *Rhazya stricta* leaf and samples were taken at 6, 12, 18 and 24-hour intervals.

The concentrations of *Rhazya stricta* were freshly prepared in distilled water. Root meristems were examined at various intervals by thoroughly washing root tips and fixing in Carrnoy`s fixative (1:3 - ethanol: glacial acetic acid) for 24-hour followed by washing in 70% ethanol. Then, root tips were stained in acetocarmine and preserved in 70% ethanol. The root tips were squashed in 45% acetic acid after being macerated in 1 N HCl for 5-10 minutes at a maintained temperature of about 60°C (Dyer, 1979). Cells were screened under a light microscope for mitotic index, mitotic phases and chromosomal aberrations. Genotoxic and cytotoxic effects were estimated as numbers of dividing cells/ 3000 counted cells (approximately).

3- RAPD-DNA profile

Samples of roots of bulbs of *Allium cepa* were frozen in liquid nitrogen, ground with mortar and pestle

and incubated in 1.5 ml of extraction buffer (100 mM Tris-HCl pH 8, 100 mM NaCl, 50 mM EDTA pH 8, 2% SDS and 0.1 mg/ml proteinase K) for 1.5 h at 37°C. DNA was extracted twice with chloroform: isoamyl alcohol (24:1) mixture, precipitated from the aqueous phase with 2 vol. of cold ethanol at -20°C during 48 h. DNA pellet was harvested by centrifugation, washed several times in ethanol, air-dried and dissolved in deionized water. RNase treatment was performed accordingly to Scott (1991). DNA was analyzed using agarose gel electrophoresis.

PCR of RAPD was carried out in 25 ul containing 30 ng of treated isolated DNA, 3 mM MgCl2, 20 mol of the 10-mer primer (primer: OPB-20, OPA-09, OPA-16), 1 U of AmpliTaq DNA polymerase, 250 uM each of dCTP, dGTP, dATP, and dTTP in 10 mM Tris HCl (pH 8.3), 50 mM KCl, overlaid with drop of mineral oil. A Perkin-Elmer TC480 thermal cycler was used for 40 cycles of amplification (94°C for 1 min, 36°C for 1 min, 72°C for 2 min) and then 72°C for 7 min.

Amplicons were analyzed in 2.5% (w/v) agarose gel in 1xTBE buffer. PCR products (25 µl per sample) were mixed with 3-5 µl Gel Loading Dye Solution and loaded onto the agarose gel, containing ethidium bromide (0.5 mg/ml). DNA ladder 100 bp was used for agarose gel. Electrophoresis was carried out at 80 V for 3.5 h, then the results were visualized under UV light and documented using Poloride camera.

4- Protein profile

Total extracted proteins were separated by polyacrylamide gel electrophoresis (SDS-PAGE), the total monomer concentration of the stacking gel was 8% (w/v) and the separation gel was 12% (w/v). Dimensions of the gel immersed in running buffer (0.1% SDS, 0.05 M Tris/0.384 M glycine buffer, pH 8.3) were 8 × 8 × 0.1 cm. Samples in loading buffer (1% SDS, 1% mercaptoethanol, 20% glycerol, 0.02% bromophenol blue, 0.01 M Tris-HCl, pH 8.0) were incubated at 100°C for 5 min before electrophoresis. Electrophoresis was performed at room temperature for approximately 2 h, and the system was programmed to a two-step mode with applying constant current of 10 mA in stacking gel and 20 mA in the separation gel. Gels were stained with silver nitrite.

E. Statistical analysis

Data were statistically analyzed using student's t-test, Duncan test of significance of differences and linear regression calculations for the detection of linear relationship between concentration of the extract or time of exposure to each concentration and mitotic index or mutational frequency using Stat-graphics V 5.0 and Microsoft Excel 2003 for Ms-Windows.

RESULTS

The mitotic index, frequency of mitotic phases and percentage of chromosomal aberrations in root meristems of *Allium cepa* treated with aqueous extracts

of the wild plant *Rhazya stricta* leaf are given in Tables (1-3) and Figs (1-4).

The data in Tables (1-3) and Figs (1-4) show that all treatments led to a significant decrease in the mitotic index and frequency of mitotic phases, meanwhile the interphase was highly increased, and so did the mutational frequency and chromosomal aberrations in all different treatments compared with control. The decrease of mitotic index was highly significant ($p>0.05$) at all treatments.

Most of the recorded chromosomal aberrations were stickiness, disturbance of chromosomes, C-metaphase and binucleate telophase, meanwhile insignificant number of micronuclei and chromosomal breakage were scored. The microscopic investigation recorded an extensive cell death (pyknosis), which is called necrosis in animal tissues. All these cytogenetic results suggest a cytotoxic, genotoxic and anti-carcinogenic effects.

In addition, the data in Table (4) and Fig. (4) show that the molecular analysis for all treatments revealed a significant decrease in the DNA quantity ($P>0.05$) for all treatments compared with control. The results of total protein profile and SDS-PAGE techniques revealed significant decrease in the total protein of all treatments compared with the control, as well as polymorphism of protein bands, thus correlated with polymorphic numbers of genetic bands, which were electrophoretic PCR products of all treatments compared with the control in the RAPD technique. All these results support the cytogenetic data hat the leaf extract of *R. stricta* is clastogenic, mutagenic and anti-carcinogenic agent.

DISCUSSION

Mitotic index was shown to be decreased as duration of treatment or concentration increased. Depression of mitotic index could be explained as a result of preventing cells from proceeding into prophase or from depressing the mitotic phases following prophase which is indicative of the induction of molecular change in the genetic material, suggesting either DNA lesion, or interference with cell cycle (Sabir *et al*., 1998). All treatments were found to significantly decrease all mitotic phases in root meristems of *Allium cepa*; these results are similar in many respects to the data reported earlier (Baeshin *et al*., 1997; Wusheng *et al*., 1994; Sarbhoy *et al*., 1991). The change of mitotic phases may be resulted by linked or intercalated the one of component of *Rhazya stricta* with proteins (histones) or nucleotides of DNA in interphase (Jacobson and turner, 1980). Also, all treatments of *A. cepa* cells by leave extract of *Rhazya stricta* led to significant increase of chromosomal aberrations as concentrations increased or exposure time of treatments. These chromosomal aberrations include binuclear cells which were induced with significant frequency, and are indicative of the ability of one substance of *R. stricta* extract to interfere with spindle and cell wall formation. Similar cytogenetic results were obtained by Sarbhoy *et al*. (1991)

and Baeshin *et al*. (1997). Other aberrations as disturbance, stickiness and C-metaphase were induced with significant frequencies. Similar result were obtained by different authors (Baeshin and Al-Ahmadi, 2004) when some natural products were tested. All these cytogenetic results quite consistent with that obtained by *A. cepa* test, which are together strongly, support the role of *R. stricta* as anti- carcinogenic agent.

The molecular study of DNA and protein of the treatments and the control showed that DNA and protein quantities revealed a significant decrease in all treatments compared with the control, suggesting a molecular changes as a deletion in one or more loci which affect gene expression and interruption in the nucleotide chain of DNA and protein synthesis consequently as alkaloids in *Rhazya stricta* leaf extract often do. These results are consistent with the results obtained earlier (Mukhopadhyay *et al*, 1981; David *et al.*, 1997; Adam *et al.,* 2000; Morita *et al*., 2005).

Polymorphic DNA bands were obtained in treatments comparing to control with all RAPD primers used. This observation gives good evidence to the ability of *Rhazya stricta* extract to induce point mutations as a result of deletion compromising at least one nucleotide as revealed by the disappearance of many genetic bands as compared with wild type. Some of the components of *R. stricta* may act as intercalation agent or generates free radicals which are interacted with DNA to account for the observed deletions, as suggested by similar results obtained by Ansah *et al*. (2005) in their study with *Cryptolepis sangvinolehta*. The obtained results of SDS-PAGE protein profile confirm the ability of *R. stricta* to cause frame shift mutations in genes of root meristems of *A. cepa*.

SUMMARY

Root tip meristems of *Allium cepa* were treated with elevated concentrations of leaf aqueous extract of the wild plant *Rhazya stricta*. Samples were taken at regular time intervals in each treatment and subjected to cytogenetic and molecular genetic assays (RAPD-DNA and SDS-PAGE protein profiles). It was found that the extract has a potent lethal, clastogenic and mutagenic activities. Mitotic index decreased as concentration or time of exposure increased. Frequency of chromosomal aberrations increased with increase in concentration or exposure time. Most chromosomal aberrations were stickiness, disturbance of chromosomes, C- metaphase and binucleate telophase. Pycnotic cells were extensively frequent. The RAPD results demonstrated a polymorphic numbers of genetic bands, which were the electrophoretic products of PCR for all treatments compared with the control. The results indicated a decrease in the total protein contents of all treatments compared to the control, and a polymorphism of protein bands via SDS-PAGE. Results strongly suggest that the leaf extract of *R. stricta* is a clastogenic,

mutagenic and anti-carcinogenic agent along with differential expression of genes encoding bulb proteins.

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Table (1): Mitotic index in root meristems of *Allium cepa* treated in different duration times with *Rhazya stricta* leaf extract.

Concentration of treatment (gm/L)	Duration of treatment (h.)	Total observed cells	Total dividing cells	Mitotic index
Control	Control	3003	282	9.391
1.5	6	3000	237	7.900
	12	3000	155	5.167*
	18	3001	138	4.598*
	24	3000	115	3.833*
3.0	6	2999	147	4.902*
	12	3003	133	4.429*
	18	3003	85	2.831*
	24	3001	63	2.099*
6.0	6	3002	126	4.197*
	12	3001	103	3.432*
	18	3003	76	2.531*
	24	3004	41	1.365*

(*) Significant from control at the 5% level.

Table (2): Frequency of mitotic phases in root meristems of *Allium cepa* treated in different duration times with *Rhazya stricta* leaf extract.

Concentration of Treatment (Gm/L)	Duration of Treatment (H.)	Number of Interphase (Rest Phase)	Mitosis Phases				% Mitosis Phases
			% Prophase	% Metaphase	% Anaphase	% Telophase	
Control	Control	90.310	3.896	2.098	1.789	1.598	9.391
1.5	6	90.400	3.200	1.900	1.100	1.700	5.632
	12	92.933	3.100	0.800	0.567	0.700	
	18	93.369	2.999	0.700	0.467	0.433	
	24	93.900	2.400	0.667	0.433	1.365	
3.0	6	92.698	3.668	0.467	0.300	0.467	3.565
	12	93.373	2.997	0.500	0.333	0.599	
	18	94.438	2.331	0.233	0.067	0.200	
	24	96.501	1.699	0.100	0.100	0.200	
6.0	6	93.671	1.899	1.299	0.600	0.400	2.881
	12	94.668	1.799	0.566	0.500	0.566	
	18	96.104	1.399	0.300	0.333	0.500	
	24	97.570	1.189	0.100	0.000	0.067	

(*) Significant at $P< 0.05$ compared to control.

Table (3): Percentage of clastogenesis abnormalities and mutational frequency in root meristems of *Allium cepa* treated in different duration times with *Rhazya stricta* leaf extract.

Concentration (gm/L)	Duration (h)	Total defected mitotic phases	Clastogenesis								Total % of Abnormalities	Mutational frequency
			Micronuclei	Brake and Fragments	Bridges	Stickiness	C-Metaphase	Disturbance	Binucleate	Pyknosis Cells		
0	0	9	0.000	0.000	0.000	0.200	0.000	0.100	0.000	0.000	0.300	0.032
1.5	6	51	0.000	0.000	0.200	0.700	0.300	0.500	0.000	0.000	1.700*	0.215**
	12	57	0.000	0.000	0.100	0.667	0.600	0.600	0.800	0.167	2.933**	0.368**
	18	61	0.000	0.200	0.500	0.666	0.900	0.700	0.200	0.233	3.399**	0.442**
	24	68	0.000	0.200	0.300	1.167	0.900	0.800	0.100	0.500	3.967**	0.591**
3.0	6	72	0.000	0.000	0.000	2.001	0.333	0.333	0.167	0.200	3.034**	0.490**
	12	66	0.100	0.000	0.100	1.565	0.200	0.200	0.200	0.366	2.731*	0.496**
	18	52	0.000	0.000	0.000	1.499	0.033	0.033	0.133	0.999	2.697*	0.612**
	24	42	0.000	0.000	0.000	1.200	0.100	0.033	0.067	1.666	3.066**	0.667**
6.0	6	64	0.067	0.000	0.100	0.799	0.400	0.500	0,200	0.466	2.532*	0.508**
	12	57	0.000	0.000	0.000	0.933	0.333	0.233	0.100	0.666	2.266*	0.553**
	18	41	0.000	0.000	0.000	0.999	0.133	0.100	0.067	2.897	4.196**	0.539**
	24	32	0.000	0.000	0.000	1.032	0.000	0.000	0.333	4.382	5.692**	0.780**

(**) Significant from control at the 1% level (*) Significant from control at 5% level.

Table (4): Quantification of DNA and Total Protein Were isolated from control and root meristems of *Allium cepa*. After exposure to elevated concentration of *Rhazya stricta* in deferent duration times.

No.	Treatments		DNA Quantity ug/mL	Total Protein Quantity ug/mL
	Con. (gm/L)	Time (minutes)		
1	Control	Control	895	2302
2	1.5	6	590	2014
3		12	450	1950
4		18	425	2006
5		24	655	2008
6	3.0	6	170	1950
7		12	840	1950
8		18	95	2001
9		24	70	1800
10	6.0	6	125	1874
11		12	45	1805
12		18	65	1817
13		24	20	1793

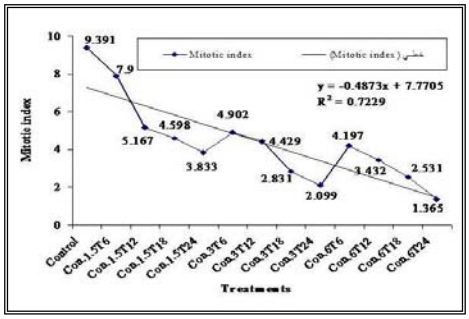


Fig. (1): Mitotic index of root tip cells of *Allium cepa* that which treated by leaf aqueous extract of *Rhazya stricta* in different duration times.

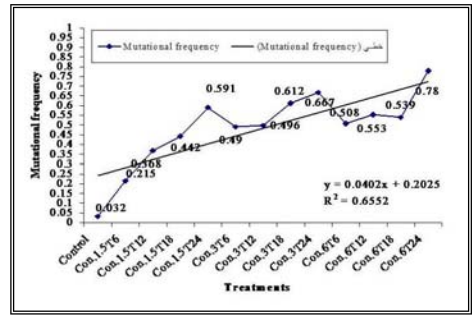
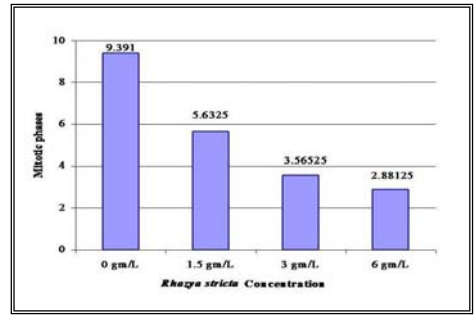


Fig. (2): Mutational frequency in root tip cells of *Allium cepa* that which treated by leaf aqueous extract of *Rhazya stricta* in different duration times.

Fig. (3): Relationship between concentration of the leaf aqueous extract of *Rhazya stricta* and average of percentage of mitotic phases of root tip cells of *Allium cepa*.



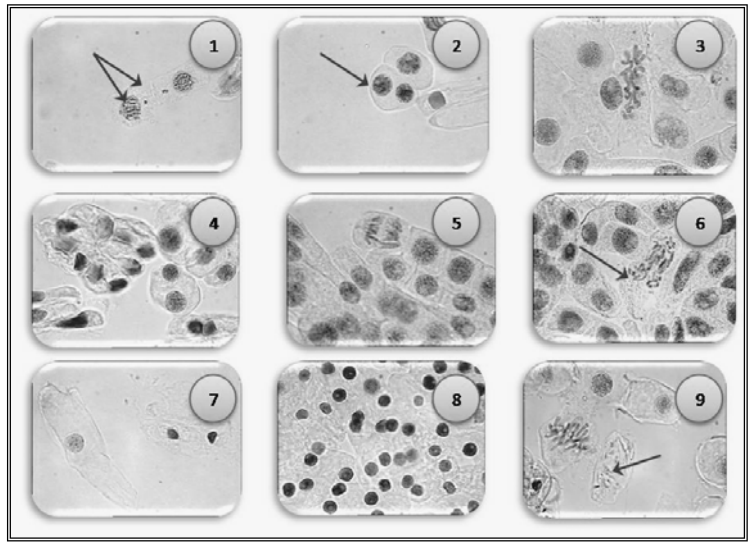


Fig (4): Chromosomal aberrations and abnormalities induced by *Rhazya stricta* leaf extract in root meristems of *Allium cepa*. (1), micronucleus (6 gm/L). (2), binucleate (3 gm/L). (3), C-metaphase (3 gm/L). (4), stickiness (6 gm/L). (5), anaphase bridge (3 gm/L). (6), disturbance cells (1.5 gm/L). (7), compare between normal cell (left) and pyknosis cell (right). (8), pyknosis cells (6 gm/L). (9), degrada nuclei (6 gm/L).

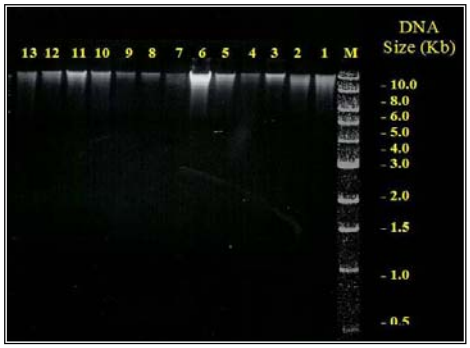


Fig. (5): DNA isolated from root tip cells of *Allium cepa* which treated by leaf aqueous extract of *Rhazya stricta.* In deferent duration times.

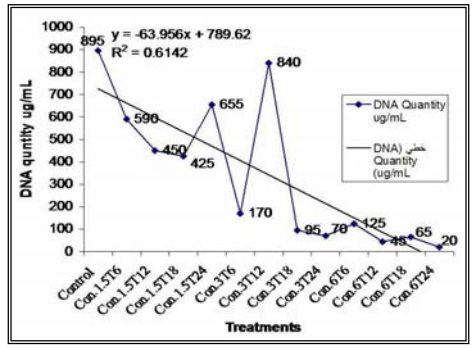


Fig. (6): Relationship between treatments of leaf aqueous extract of *Rhazya stricta* and DNA quantity of root tip cells of *Allium cepa*.

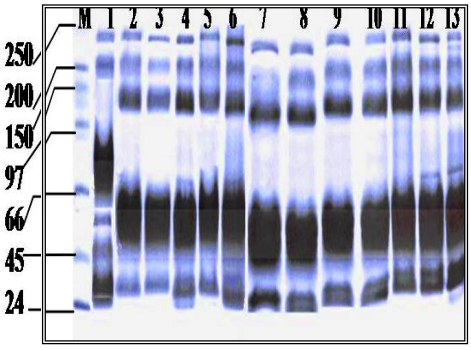


Fig. (7): Protein bands of root tip cells of *Allium cepa* that which treated by leaf aqueous extract of *Rhazya stricta.*

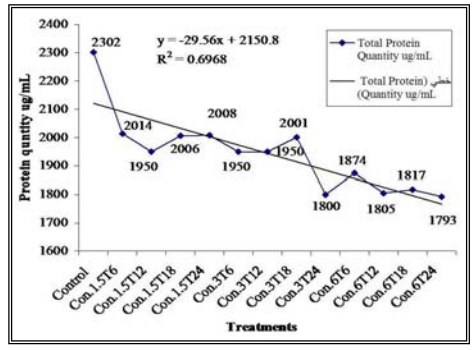


Fig. (8): Protein quantity of root tip cells of *Allium cepa* that which treated by leaf aqueous extract of *Rhazya stricta* compared to control.

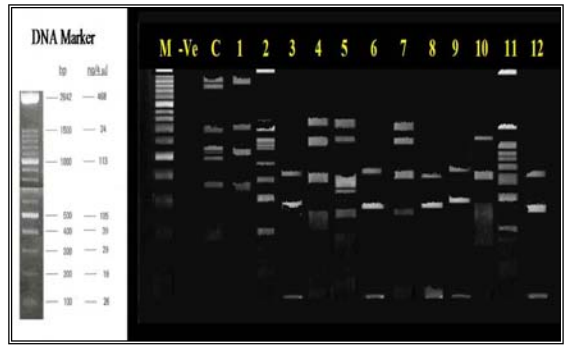


Fig. (9): RAPD-DNA genetic bands of *Allium cepa* that which caused by leaf aqueous extract of *Rhazya stricta.*

M: Standard -ve: Negative Control
+ve: Positive Control
1: Concentration 1.5 for 6 hrs
2: Concentration 3.0 for 6 hrs
3: Concentration 6.0 for 6 hrs
4: Concentration 1.5 for 12 hrs
5: Concentration 3.0 for 12 hrs
6: Concentration 6.0 for 12 hrs
7: Concentration 1.5 for 18 hrs
8: Concentration 3.0 for 18 hrs
9: Concentration 6.0 for 18 hrs
10: Concentration 1.5 for 24 hrs
11: Concentration 3.0 for 24 hrs
12: Concentration 6.0 for 24 hrs