GENOTOXIC AND MOLECULAR EVALUATION OF GENETIC EFFECTS OF *Rhazya stricta* (Decne) LEAF EXTRACT ON *Aspergillus terreus*

N. A. BAESHIN[1], J. S. M. SABIR[1], S. H. QARI[2] AND A. M. ALHEJIN[3]

1. *Department of Biological Sciences, Faculty of Science, King Abdulaziz University, P.O. Box 9028, Jeddah-21413, Saudi Arabia*

2. *Department of Biology, Teachers College, Umm Al-Qura University, P.O. Box 2203, Makkah, Saudi Arabia*

3. *Medical Biology Department, Faculty of Medicine, King Abdulaziz University, Jeddah, Saudi Arabia*

Rhazya stricta plant (Decne), a member of the Apocynaceae family, is widely distributed in Saudi Arabia. Extract of its leaves 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). The plant extract contains mainly 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 caused sedation, analgesia, decrease motor activity and have 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). Another study has described the anticancer effects of the indole alkaloids of *Rhazya stricta* (Mukhopadhyay *et al.*, 1981). Moreover, the genotoxicity of *Rhazya stricta* leaf aqueous extract was demonstrated for the first time by Baeshin *et al*. (2005), against *Saccharomyces cerevicae* auxotrophic mutant. However, more investigations are required to establish its genotoxic effects.

The aim of this study is to evaluate the genotoxic effects of the aqueous leaf extract of *Rhazya stricta* following the *Aspergillus terreus* auxotrophic mutant test and detect polymorphism across mutants and their wild type counterpart strain of *Aspergillus terreus* on the molecular (RAPD and SDS-PAGE) levels.

MATERIALS AND METHODS

A. Strains

1. Wild type strain

The haploid phase of the wild type strain of *Aspergillus terreus* was the target phase in the present study. Test organism was obtained from the Biology Department, Faculty of Sciences, King Abdulaziz University, Jeddah, where it has been maintained for several years.

2. Mutant strains

The induced mutant strains of *A.*

terreus were obtained by exposure to different concentrations of *Rhazya stricta* leaf extract, which were kept and maintained on Potato Dextrose Agar medium (PDA).

B. Media

1. Potato Dextrose Agar medium (PDA) was employed as the complete medium.

2. Difco Czapek Dox Agar medium (DOX) was used as the minimal medium either alone for the isolation of auxotrophic mutants or supplemented with the nutritional requirements for further characterization.

C. Preparation of the extract

Leaves of *Rhazya stricta* were collected from naturally growing plants located along the roadsides of Jeddah-Makkah highway. Collected leaves were kept in plastic bags in the field then transferred to refrigerator and kept overnight before being subjected to extraction in the following day. Leaves were washed well with running water to remove dust and sand, hand-minced into small pieces, mixed with sterilized distilled water and further blended in a blending machine. The blended mixtures were left for 24 hours at room temperature with mild hand-shaking at regular intervals. The mixture was then filtered and the filtrate (the extract) was either used directly in the experiment or kept in the refrigerator for no longer than 3 days for future use. Four concentrations of this stock extract were prepared (3, 6, 12, 24 g/l) to be tested for their genotoxic activities in light of the recommended dose prescribed in folkloric medicine (6 g/l).

D. Chemicals

Standard concentrations of hydrolysed casein (CAS), mixed vitamins solution (VITS), hydrolysed yeast ribonucleic acid (RNA), individual amino acids, individual vitamins and individual purines or pyrimidines were used as supplements to the minimal medium as indicated in Table (1). Chemicals used in the present study were purchased from Sigma-Aldrich, except DNA polymerase (purchased from Perkin-Elmer) and the dNTPs (purchased from Boehringer Mannheim). DNA purification and extraction reagents and agarose were purchased from Qiagen. Oligonucleotides (random primers) were purchased from Operon, UK. The DNA standard was purchased from Gibco BRL and the loading dye solution was purchased from Fermentas, Lithvuania. Protein assay reagents, electrophoretic reagents, and protein standard were purchased from Bio-Rad.

E. Test of genotoxicity (auxotrophic mutation)

Induction of auxotrophic mutants and biochemical genetic activities of leaf extract were tested according to the method described by Baeshin (1976). A dense conidial suspension of *A. terreus*

was prepared and a number of conidia/ml was estimated using a hemocytometer. Five ml of this suspension was immediately added to 5 ml of the *R. stricta* leaf extract and 1 ml sample of this mixture was immediately diluted in 9 ml sterile distilled water to serve as untreated (positive) control. Subsequent samples were taken at regular intervals (every 15 minutes over one hour time of exposure) and were serially diluted in sterile distilled water to stop the mutagenic treatment. Samples of the final dilutions containing about 100 conidia were spread on PDA plates and incubated for 4 days at 28°C. This was repeated for each of the four different concentrations of the leaf extract (3, 6, 12, 24 g/l).

Mutants were isolated according to the method described by Fincham *et al*. (1979). At each time interval, a monoconidial inoculum was inoculated in each of 26 loci/plate containing PDA and served as a template. The template was, in turn, replicated on the minimal medium (DOX) to detect auxotrophic mutants. All replicates were incubated for 5 days at 28°C. Auxotrophic mutants were those which failed to grow on DOX after incubation for 5 days at 28°C. All mutant colonies were isolated on PDA templates and replicated on the following supplemented media for the determination of their nutritional requirements:

1. DOX
2. DOX + CAS + VITS - RNA
3. DOX + CAS - VITS + RNA
4. DOX - CAS + VITS + RNA
5. DOX + CAS + VITS + RNA

A colony which failed to grow on media number 2, 3 or 4 requires the chemical missing from that medium. The auxanographic technique of Pontecorvo (1949) was used to specify the particular nutritional requirement of each mutant. One ml of a dense conidial suspension of the mutant was mixed with cooled molten DOX (45°C) in dishes and left to solidify. A few crystals of the nutrients to be tested were placed at marked positions around the periphery of the agar plate. Each mutant grew after 5 days incubation in the immediate vicinity of the nutrient required.

F. Molecular analyses

The mutants were characterized at the molecular level using randomly amplified polymorphic DNA (RAPD) analysis following Williams *et al*. (1990) and their total cellular proteins were analyzed using SDS-polyacrylamide gel electrophoresis (SDS-PAGE) following Lammli (1971).

1- RAPD-DNA profile

Auxotrophic mutants were incubated for 5 days at 28°C in broth PDA media, then frozen in liquid nitrogen, ground with mortar and pestle and incubated in 1.5 ml of extraction buffer (100 mM Tris-HCl, pH 8.0, 100 mM NaCl, 50 mM EDTA, pH 8.0, 2% SDS and 0.1 mg/ml proteinase K) for 1.5 h at 37°C. DNA was extracted by using chloroform : isoamyl alcohol (24 : 1)

mixture twice, precipitated from the aqueous phase with 2 vol cold 90% ethanol (-20°C) during 48 h. DNA pellet was harvested by centrifugation, washed several times in 70% ethanol, air-dried and dissolved in deionized water. RNase treatment was performed according to Scott *et al*. (1991). DNA was analyzed using agarose gel electrophoresis.

RAPD-PCR was carried out in 25 pul containing 30 ng DNA, 3 mM $MgCl_2$, 20 pmol of the primer (primer sequences are shown in Table (2), 1 U AmpliTaq DNA polymerase, 250 puM of each of dCTP, dGTP, dATP, and dTTP in 10 mM Tris-HCl (pH 8.3), 50 mM KCl, covered with a 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 a final extension cycle at 72°C for 7 min. PCR amplification products were analyzed in 2.5 % (w/v) agarose gel in 1x TBE buffer and detected by staining with ethidium bromide (0.5 mg/ml). 100-bp DNA ladder was used as a standard. Electrophoresis was carried out at 80 V for 3.5 h, then the amplicons were visualized under UV light and photographed.

2- Protein profile

Total extracted proteins were separated on 12% (w/v) SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Protein samples were denatured in loading buffer (1% SDS, 1% mercaptoethanol, 20% glycerol, 0.02% bromophenol blue, 0.01 M Tris-HCl, pH 8.0) at 100°C for 5 min just before electrophoresis run. Electrophoresis was performed at room temperature for approximately 2 h, and the system was programmed to a two-step mode with applying constant current 10 mA in stacking gel (8%, w/v) and 20 mA in the separation gel. Gels were stained with silver nitrite.

F. Statistical analysis

Data of survival percentage and frequency of auxotrophic mutants were subjected to linear regression analysis for the detection of linear relationship between concentration of the extract or time of exposure and survival percentage or auxotrophic mutants frequency using Microsoft Excel 2003 for MS-Windows.

RESULTS AND DISCUSSION

The survival percentages and recovery of auxotrophic mutants among survivors of *Aspergillus terreus* cultured cells, as a function of treatment with different concentrations of leaf extract of *Rhazya stricta*, are shown in Figs (1-3). It is clearly shown that the increase in concentration and exposure time led to a decrease in survival percentage and an increase in auxotrophic mutation percentage as confirmed by linear regression calculation. The highest possible percentage of mutation (1.7%) is achieved with the dose of 6 g/l at exposure time of 45 min, which is the optimal dose for the induction of auxotrophic mutants with leaf extract of *Rhazya stricta*. This

dose is similar to the prescribed dose in folkloric medicine. Table (3) shows a list of auxotrophic mutants recovered from *Rhazya stricta* leaf extract-treated conidia of *Aspergillus terreus.* All 14 mutants are amino acid-requiring. Some of the mutants restore growth with any one of different alternative nutritional requirements (e.g. arginine or proline). These are probably leaky auxotrophic mutants, i.e., they fail to completely prevent the action of the target gene and permit some residual functions (incomplete knockout).

Results of the molecular analysis for all auxotrophic mutants are shown in Table (4) and Figs (4 & 5). The RAPD results show a number of polymorphic bands, which were the electrophoretic products of PCR for all mutants compared with the wild type. Table (4) illustrates that the highest number of polymorphic bands among mutants was generated in reactions with the primers OPA-16 (80 bands) and the lowest number of polymorphic bands was obtained with primer OPB-03 (48 bands), whereas the number of polymorphic bands of primers OPA-02 and OPA-04 were 65 and 50, respectively. The percentages of polymorphic bands are 32.92, 19.75, 20.58 and 26.75% for primers OPA-16, OPB-03, OPA-04 and OPA-02, respectively. The results of SDS-PAGE also express polymorphism of several protein bands. All these results strongly point out the mutagenic potency of the leaf extract of *R. stricta*.

The present study indicated that dose and exposure time to *Rhazya stricta* leaves extract are inversely proportionate to survival percentage, which means that an increase in the dose and exposure time is met by a decrease in survival percentage, whereas mutation percentage increased as dose and exposure time increased. These results are in general agreement with those mentioned by Fincham *et al*. (1979), who stated a constant relation between the dose and mutation percentage which increases to a certain limit with the increase in dose when using chemical mutagens. This provides additional evidence for the mutagenicity of this extract demonstrated before, for the first time, by Baeshin *et al*. (2005) in *S. cerevicae*.

The present study confirmed that *Rhazya stricta* leaf extract exhibit strong mutagenic activities as compared with the potent chemical mutagenic agent NTG. It was found by Baeshin and Sabir (1987) that treatment with NTG resulted in a percentage of 3.8% auxotrophs in *A. terreus* with the optimal dose of 0.0075 g/10 ml at 70 min of exposure, whereas *Rhazya stricta* leaf extract, in the present study, resulted in a percentage of 1.7% auxotrophic mutants with the dose of 6 g/l at 45 min of exposure.

Most of the auxotrophic mutants obtained from the treatments of the wild type strain of *A. terreus* by *Rhazya stricta* leaf extract were amino acids-requiring, specifically arginine, proline or cystine-methionine, suggesting the selected target

locus of this extract on the molecular level. Similar results were obtained by Tayl (1975), Baeshin (1976), Baeshin and Sabir (1987) and Sabir (2005). This observation led to the necessity to make molecular study of DNA and proteins of the auxotrophic mutants as compared to the wild type (control).

Polymorphic DNA bands were obtained in mutants comparing to wild type with all RAPD used primers. This observation gives evidence to the ability of *Rhazya stricta* leaf 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 the wild type. Some of the components of *R. stricta* may act as intercalation agent or generates free radicals, which interact with DNA, to account for the observed deletions, as suggested by similar results obtained by Ansah *et al.* (2005) in their study on *Cryptolepis sangvinolehta*. The obtained results of SDS-PAGE protein profile also confirm the ability of *R. stricta* to produce frame shift mutation in *A. terreus*.

SUMMARY

Dense conidial suspensions of *Aspergillus terreus* were treated with elevated concentrations of aqueous extract of the wild *Rhazya stricta* plant leaves. Samples were taken at regular intervals for each treatment and assayed for survival percentage, auxotrophic mutants, and RAPD and SDS-PAGE analyses. It was found that the extract has potent lethal and mutagenic activities. Survival percentage decreased as the concentration or time of exposure increased. Frequency of auxotrophic mutants increased with the increase in concentration or exposure time. Most auxotrophic mutants were amino acid-requiring. The RAPD results demonstrated a number of polymorphic bands across mutants as compared to the wild type strain. SDS-PAGE results also expressed polymorphism of protein bands. Generally, the results strongly point out to the mutagenic potency of the leaf extract of *R. stricta* on conidial suspensions of *Aspergillus terreus*.

REFERENCES

Adam, S. E. A., H. Al-Farhan and M. A. Al-Yahya (2000). Effects of combined *Citrullus colocynthis* and Rhazga stricta use in Najdi sheep. Am. J. Chin. Med., 28: 385-390.

Ageel, A. M., J. S. Mossa, M. A. Al-Yahya, M. Tariq and M.S. Al-Said (1987). Plants used in Saudi folk medicine. King Saud University Press, Riyadh, Saudi Arabia.

Ahmad, Y., K. Fatima and P. W. Le-Quesne (1983). Further alkaloidal constituents of the leaves of *Rhazya stricta.* Phytochem., 27: 1017-1019.

Ali, B. H., H. Al-Qarawi, H. M. Mousa and A. K. Bashir (2000). Concentration of amino acids in brains of mice treated with the traditional medicinal plant *Rhazya stricta. Dence.* Indian J.

Pharmacol., 32: 253-254.

Ali, B. H., A. K. Bashir and M. O. Tanira (1998). The effect of *Rhazya stricta*, a traditional medicinal plant, on the forced swimming test in rats. Pharmacol. Biochem. Behav., 59: 547-550.

Ali, B. H., A. K. Bashir, M. O. Tanira and S. Banna (1995). Some nervous system actions of *Rhazya stricta* in mice. Clin. Exp. Pharmacol. Physiol., 20: 496-502.

Al-Yahya, M. A., I. A. Al-Meshal, J. S. Mossa, A. A. Al-Badr and M. Tariq (1990). Saudi Plants: A Phytochemical and Biochemical Approach. King Saud University Press, Riyadh, Saudi Arabia.

Ansah, C., A. Khan and N. J. Gooderham (2005). *In vitro* genotoxicity of the West African anti-malarial herbal *Cryptolepis sanguinolenta* and its major alkaloid cryptolepine. Toxicology, 208: 141-147.

Baeshin, N. A. (1976). The Genetics of Nectria cosmariospora Ces and Der Not. Ph.D. Thesis: The University of Dundee, Dundee, Scotland, UK.

Baeshin, N. A., N. R. Abdel-Rahman and N. H. Twaty (1987). Induction of mutation in *Aspergillus terreus* by Nitrosoguanidine. Researches Sci., 222: 141-148.

Baeshin, N. A. and J. M. Sabir (1987). Some genetic studies on *Aspergillus terreus* Thom. Researches Sci., 222: 215-224.

Baeshin, N. A., N. Twaty and A. Al-Hebshi (2005). Evaluation the genotoxicity of *Rhazya stricta* leaves extract by the *Saccharomyces cerveisiae* auxotrophic mutats test. Egyp. J. Natu. Toxi., 2: 87-100.

Batanouny, K. H. and N. A. Baeshin (1978). Studies on the flora of Arabia: I. The Jeddah-Mecca Road, Saudia Arabia. Taeckholmia, 9: 67-81.

David, B., T. Sevenet, O. Thoison, K. Awang, M. Pais, M. Wright and D. Guenard (1997). Hemisynthesis of *rhazinilam* analogues: structure-activity relationships on tubulin-microtubule system. Bioorg. Med. Chem. lett., 7: 2155-2158.

El-Sharkawi, H. Malibari, A., El-Shaieb, M. and K. Tawfik (1981). Some edapho-ecological factors controlling the distribution of soil fungi in the western region of Saudi Arabia. Bull. Fac. Sci., KAAU, Jeddah, 5:103-115.

Fincham, J. R. S., P. R. Day and E. Radford (1979). Fungal Genetics. University of California Press., Berkeley and Loss Angeles.

Morita, H., K. Awang, A. H. Hadi, K. Takeya, H. Itokawa and J.

Kobayashi (2005). Conformational analysis of *rhazinilam* and three-dimensional quantitative structure-activity relationships of *rhazinilam* analogues. Bioorg. Med. Chem. Lett., 15: 1045-1050.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, 227: 680-685.

Mukhopadhyay, S., G. A. Handy, S. Funayama and G.A. Cordell (1981). Anticancer indole alkaloids of *Rhazya stricta.* J. Nat. Prod., 44: 696-700.

Pontecorvo, G. (1949). Auxanographic technique in biochemical genetics. J. Gen. Microbiol., 3: 122-127.

Sabir, J. S. M. (2005). Assaying the single and combined mutagenicity of *Calotropis procera* Ait latex and Chlorcyrin in *Aspergillus terreus*. J. Agri. Res., Tanta Univ., 31: 820-839.

Scott, D., S. M. Galloway, R. R. Marshall, M. Jr. Ishidate, D. Brusick, J. Ashby and B. C. Myhr (1991). Genotoxicity under extreme gultue conditions . A report from ICPEMC Task Group 9. Mutat. Res., 257: 147-204.

Tanira, M. O., B. H. Ali, A. K. Bashir, S. Dhanasekaran, E. Tibirica and L. Alves (2000). Mechanism of the hypotensive action of *Rhazya stricta* in rats. Pharmacol. Res., 41: 369-387.

Tayl, A. A. (1975). Genetical studies with *Nectria comariospora Ces & De Not*., Ph.D. Thesis: the University of Dundee, Dundee, Scotland, UK.

Williams, J. G. K., A. R. Kubelik, K. J. Livak, J. A. Rafalski and S. V. Tingey (1990). DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Res., 18: 6531-6535.

Table (1): Concentrations of medium supplements to the minimal medium.

Supplement	Stock solution conc. (mg/ml)	Conc. used in medium (mg/ml)
Individual amino acids	10	10^{-1}
Individual purines and pyrimidines	4	2×10^{-2}
Vitamins		
Aneurin	5	5×10^{-4}
Biotin	0.02	2×10^{-6}
Choline chloride	20	2×10^{-3}
Inositole	40	4×10^{-3}
Nicotinic acid	10	10^{-3}
Pyridoxine	5	5×10^{-4}
P-aminobenzoic acid	1	10^{-4}
Pantothenic acid	20	2×10^{-3}
Riboflavine	10	10^{-3}
Others		
Hydrolysed casein	60	3
Hydrolysed yeast ribonucleic acid	10	5×10^{-1}
Mixed vitamins	1 ml of each of the individual vitamins solutions were mixed, and 0.2 ml of this mixture was added to 200 ml medium (0.2 ml 1/10 dilution/plate)	

Table (2): Primer sequences.

Primer	Sequence	% G+C
OPA-16	5` TGC CTT GCA G `3	60
OPB-03	5` CAT CCC CCT G `3	70
OPA-04	5` GTC GAA CGA G `3	60
OPA-02	5` AGC CTT CGC T `3	60

Table (3): List of auxotrophic mutants recovered from *Rhazya stricta* leaf extract-treated conidia of *Aspergillus terreus.*

Code no.	Extract conc. (g/l)	Treatment (min)	Requirement
AMR3T45	3 g/l	45 min	Cystine
AMR3T60	3 g/l	60 min	Lysine or histidine
AMR6T30	6 g/l	30 min	Lysine or histidine
AMR6T45A	6 g/l	45 min	Tyrosine
AMR6T45B	6 g/l	45 min	Arginine
AMR6T60A	6 g/l	60 min	Cystine
AMR6T60B	6 g/l	60 min	Glutamine
AMR12T45	12 g/l	45 min	Alanine
AMR12T60A	12 g/l	60 min	Glutamine
AMR12T60B	12 g/l	60 min	Tyrosine
AMR24T30	24 g/l	30 min	Glutamine
AMR24T45	24 g/l	45 min	Proline or arginine
AMR24T60A	24 g/l	60 min	Proline or arginine
AMR24T60B	24 g/l	60 min	Methionine or proline

Table 4: Polymorphic bands of each genetic primers and percentage of polymorphism in auxotrophic mutants of *Aspergillus terreus.*

Primer	Total no. bands	No. monomorphic bands	No. polymorphic bands	% polymorphic bands from total bands
OPA-16	129	49	80	19.42
OPB-03	91	43	48	11.65
OPA-04	98	48	50	12.14
OPA-02	94	29	65	15.78
Total	412	169	243	----

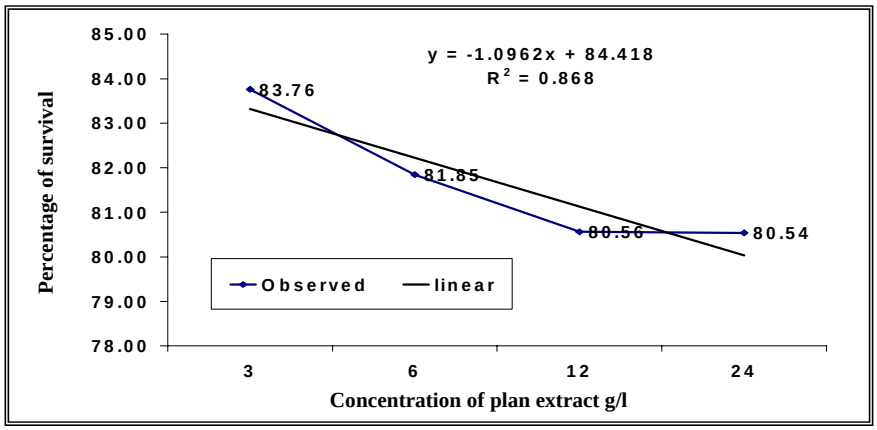

Fig. (1): Relationship between concentration of the leaf aqueous extract of *Rhazya stricta* and average percentage of survival in *Aspergillus terreus.*

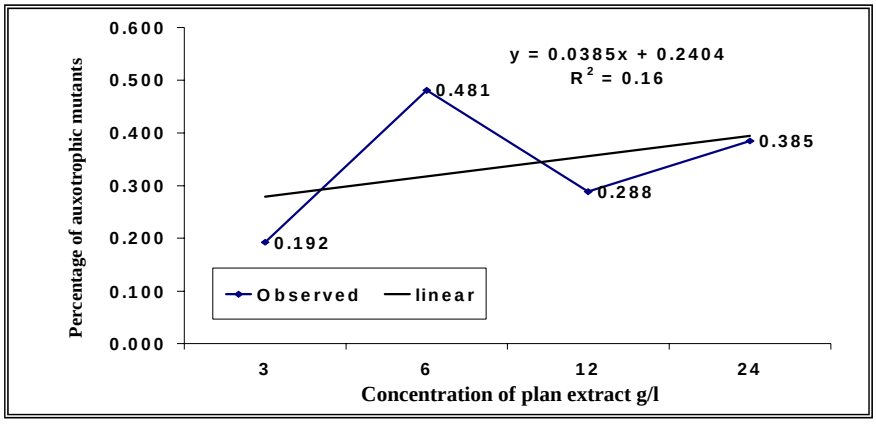

Fig. (2): Relationship between concentration of the leaf aqueous extract of *Rhazya stricta* and average percentage of auxotrophic mutants in *Aspergillus terreus*

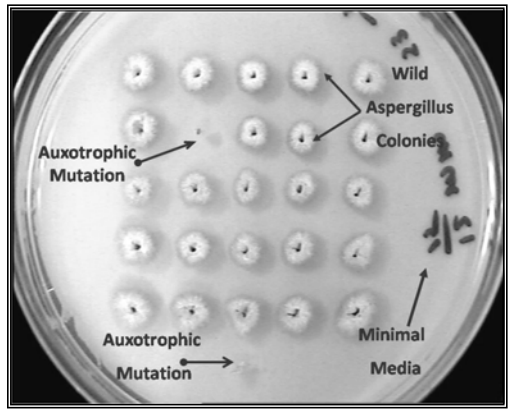

Fig. (3): Auxotrophic mutants in *Aspergillus terreus* caused by leaf aqueous extract of *Rhazya stricta.*

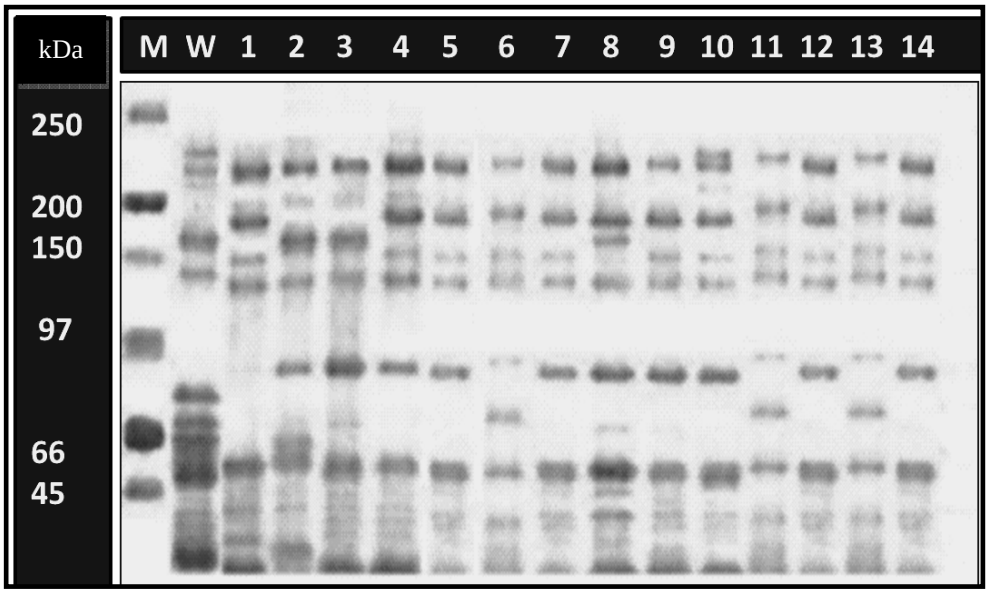

Fig. (4): SDS-PAGE protein bands of auxotrophic mutants in *Aspergillus terreus* treated with leaf aqueous extract of *Rhazya stricta.*

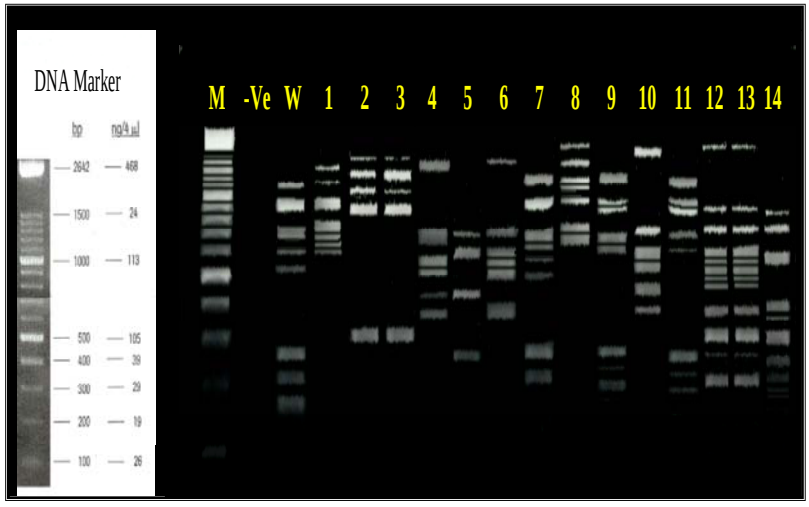

Fig. (5): RAPD-PCR products of auxotrophic mutants of *Aspergillus terreus* treated with leaf aqueous extract of *Rhazya stricta* for OPA-04 primer as a model.

M	-Ve	W	No	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Marker	Negative Control	Wild type	Mutation Code	AMR3T45	AMR3T60	AMR6T30	AMR6T45A	AMR6T45B	AMR6T60A	AMR6T60B	AMR12T45	AMR12T60A	AMR12T60B	AMR24T30	AMR24T45	AMR24T60A	AMR24T60B