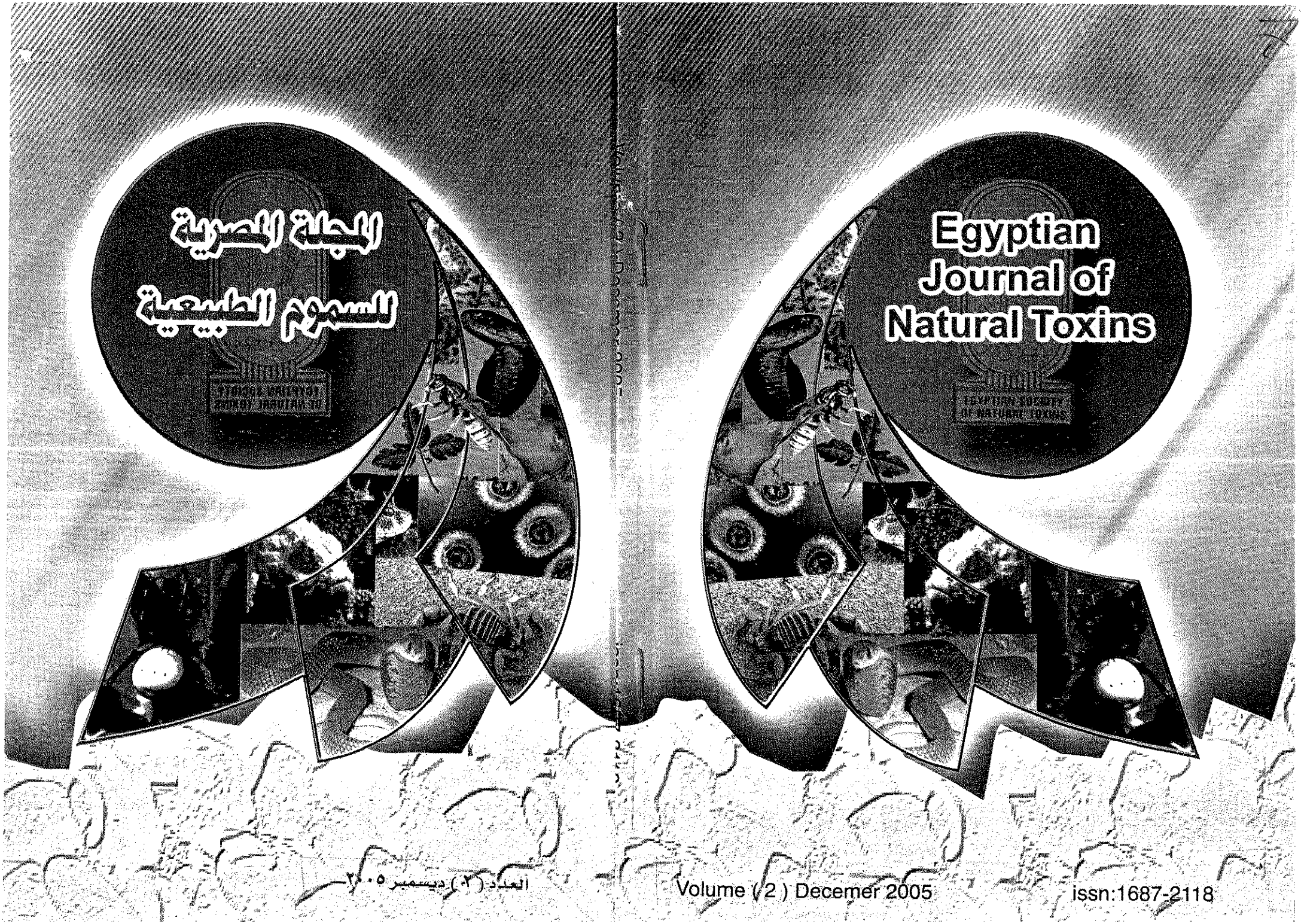
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EVALUATING THE GENOTOXICITY OF *RHAZYA STRICTA* LEAVES EXTRACT BY THE *SACCHAROMYCES CEREVISIAE* AUXOTROPHIC MUTANTS TEST

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ABSTRACT

Dense cell suspensions of *Saccharomyces cerevisiae* were treated with elevated concentrations of aqueous extracts of the wild plant *Rhazya stricta* leaves. Samples were taken at regular intervals in each treatment and assayed for survival percentage and auxotrophic mutants.

It was found that the extract has potent lethal and mutagenic activities. Survival percentage decreased as concentration or time of exposure increased. Frequency of auxotrophic mutants increased with increase in concentration or exposure time. Most auxotrophic mutants were amino acid requiring mutants, less abundant were the nitrogen bases requiring ones and come last the vitamins requiring mutants.

Key words: Genotoxicity - *Rhazya stricta* - *Saccharomyces cerevisiae*

INTRODUCTION

Rhazya stricta Decne of the Apocynaceae family is a wild plant widely distributed in the sandy plains of Saudi Arabia and comparable habitats throughout the world. Extract of its leaves is prescribed in folkloric medicine for the treatment of various disorders such as *diabetes mellitus,* sore throat, syphilis, helminthiasis and inflammatory conditions and rheumatism (Ageel *et al.*, 1987; Ali *et al.,* 1995 and Ali *et al.,* 1998). The leaves of the plant contain mainly alkaloids, glycosides, triterpenes, tannins and volatile bases (Ahmad *et al.,* 1983; Al-Yahya *et al.,* 1990).

Several works on rats and mice reported that leaves extract induced sedation, analgesia, decreased motor activity, had antidepressant-like and antioxidant activities, had complex effects on brain endogenous monoamine oxidase activity and centrally-mediated hypotension (Ali *et al.*, 1998; Tanira *et al.*, 2000 and Ali *et al*, 2000). Moreover, Mukhopadhyay *et al.* (1981)

ascribed to the indole alkaloids of *Rhazya stricta* anticancer effects. Many studies revealed that some plants extracts can modify the genotoxity of some other known potent mutagens by acting as inhibitors of mutagenesis and/or clastogenesis or even they can be themself genotoxic agents (Sarkar *et al*, 1996). *Rhazya stricta* has not yet been tested for genotoxic activities. The purpose of this study is to evaluate its genotoxicity by treating *Saccharomyces cerevisiae* cells with leaves extract of this plant and assaying for survival percentage and auxotrophic mutants.

MATERIALS AND METHODS

A. Preparation of the extract:

Field identification of the plant was carried out by Dr. Baeshin following the work of Batanouny and Baeshin (1978). Leaves of *Rhazya stricta* were collected during the last week of December of 2003 from naturally growing plants in their natural habitats located along the roadsides of Jeddah – Makkah highway. Collected leaves were kept in plastic bags in the field and later transferred in their bags to a frigidaire and kept most of the time overnight then subjected to extraction in the following day. The leaves were washed well with running water to get red of dust and sands, hand-minced into small pieces, mixed with sterilized distilled water (15g/3L) and further blended in a blending machine. The mixture was then left for 24hrs at room temperature with mild hand-shaking at regular 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 frigidaire for no longer than 3 days for future use. Three concentrations of this stock extract were prepared (13.4, 15, 20.1 gm/10ml) to be tested for genotoxic activities based on the dose prescribed in folkloric medicine (15g/10ml.)

B. Strains:

1. Wild Type Strains:

The haploid phase of the wild type strain of *Saccharomyces cerevisiae* was the test organism in the present study. It was kindly provided by Prof. A.A. Abuzaid of the Biochemistry Department, Faculty of Sciences, King Abdulaziz University who obtained the original culture (Labeled NRRL Y 12632) from the Northern Regional Research Laboratory, U.S.A. The culture was grown and maintained on Yeast Malt Agar medium (YMA).

2. Mutant strains:

Induced mutant strains by *Rhazya stricta* leaves extract in this experiment was isolated and maintained on YMA and used later in this study for determining their nutritional requirements.

C. Chemicals:

Standard concentrations of hydrolysed casein (CAS), mixed vitamins solution (VITS), hydrolysed yeast ribonucleic acid (RNA), individual amino acids, individual vitamins and individual purines and pyrimidines were used as supplements to the minimal media as indicated in table (1).

Table 1: Concentrations of Medium Supplements.

Supplement	Conc. of stock solution (mg/ml)	Conc. used in media (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 mixed, and 0.2 ml of this mixture added to 200 ml media (0.2 ml 1/10 dilution/plate).	

D. Media:

1. Complete medium: Yeast Malt Agar medium (YMA) was employed as the complete medium.

2. Minimal Medium: 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 work.

E. Test of Genotoxicity:

Leaves extract was tested for genotoxic activities following the method described by Baeshin (1976) for the induction of auxotrophic mutants. A dense cell suspension was made and number of cells/ml was estimated using a hemocytometer. 5ml of this suspension was immediately added to 5ml of the extract and one ml sample of this mixture was immediately diluted in 9ml 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 serially diluted in sterile distilled water to halt the mutagenic treatment. Samples of the final dilutions containing about 100 cells were spread on YMA plates and incubated for 5 days at 28°C. This was repeated for each of the three different concentrations of the leaves extract (13.4, 15 and 20.1g/10ml).

Total isolation method described by Fincham *et al.* (1979) was followed for detecting and isolating mutants. At each interval a monocellular inoculum was incubated in each of 26 loci/plate containing YMA 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 are those which fail to grow on DOX after incubation (5 days at 28°C). All colonies behaving in this manner were isolated on YMA templates and replicated on the following supplemented media for the determination of the nutritional requirements of the mutants:

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 any media of 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 cell 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. Statistical Analysis:

Data of survival percentage and frequency of auxotrophic mutants were subjected to Linear Regression calculations for the detection of linear relationship between concentration of the extract or time of exposure to each concentration and survival percentage or auxotrophic mutants frequency using Microsoft Excel 2002 for Ms-Windows.

RESULTS

The survival percentage and recovery of auxotrophic mutants among survivors of *Saccharomyces cerevisiae* cultured cells, as a function of treatment with different concentrations of leaves extract of *Rhazya stricta*, are shown in the figures (1-8).

It is clearly shown that the increase in concentration and exposure time lead 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 (3%) is achieved with the dose of 20.1 g/10 ml at exposure time of 60 minutes which is the optimal dose for the induction of auxotrophic mutants with leaves extract of *Rhazya stricta*. This dose is higher than the prescribed dose in folklore medicine.

Table (2) represents list of auxotrophic mutants recovered from *Rhazya stricta* leaves extract treated cells of *Saccharomyces cerevisiae*. Thirteen of these mutants are amino acid -requiring ones, five are vitamins-requiring ones whereas eight of them are nitrogen bases-requiring ones. 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, ie., they fail to completely prevent the action of a gene and permit some residual functions. It is noticed from Table 1 that all amino acid-requiring mutants are arginine-requiring ones and most nitrogen bases-requiring mutants are adenine-requiring ones.

DISCUSSION

The present study showed for the first time that *Rhazya stricta* leaves extract is mutagenic in *Saccharomyces cerevisiae* cell culture.

Duration of exposure to the extract or the dose (concentration of the extract) was found to be inversely proportionate to survival percentage: an increase in the dose or exposure time is met by a decrease in survival percentage. It was also found that percentage of auxotrophic mutants

increased as the dose or duration of exposure to the extract increased. All these results are in agreement with Fincham *et al.* (1979) who stated that by using chemical mutagens there was a constant relation between the dose and mutation percentage which increased to a certain limit with the increase in the dose. This was found true by many authors in may different fungal species (Kolmark and kilbey, 1968; Ingle, 1972; Baehsin, 1976; Baeshin *et al.*, 1987). The highest possible percentage of auxotrophic mutations induced by the leaves extract of *Rhazya stricta* in the present study was 3% with the dose of 20.1 g/10 ml at exposure time of 60 minutes. This is similar to that of the potent chemical mutagen nitrosoguanidine (N.T.G.) which induced 3% auxotrophic mutations in *Saccharomyces cerevisiae* with the optimal dose (Baeshin *et al.*, 1978). This might indicate that extract of *Rhazya stricta* leaves is a potent chemical mutagen for the induction of point mutation.

The result of this study showed that some auxotrophic mutations restored growth with more than one of different alternative nutritional requirements (e.g., Arginine or Proline). Such observation encountered by Baeshin and Sabir (1987) in inducing auxotrophic mutations in *Aspergillus terreus* by N.T.G. Such mutations are most likely to be leaky mutations that they fail to completely prevent the action of a gene and permit some residual functions. All auxotrophic mutations requiring amino acids were arginine-requiring ones whereas most of the nitrogen bases-requiring mutants were adenine-requiring ones. These are mutations in mutator genes, genes that are more susceptible to mutation than others and/or probably they are selected by the extract for mutagenic action.

The fact that survival percentage is inversely proportionate to duration of exposure to the extract or its dose is indicative of cytotoxic activities of the extract. Many previous studies ascribed cytotoxic activities to leaves extract of *Rhazya stricta* (Mukhopadhyay *et al*, 1981; Adam, 1998, 1999; El-Hag *et al*, 1999). Particulary of interest is the study of Mukhopadhyay *et al.* (1981) who found that cytotoxic effect is associated with antitumor activity of the extract. These observations deserve further research on genotoxicity and anticarcenogicity of the leaves extract of this plant on the cytological and the molecular levels and this is what is being currently carried out in our laboratory.

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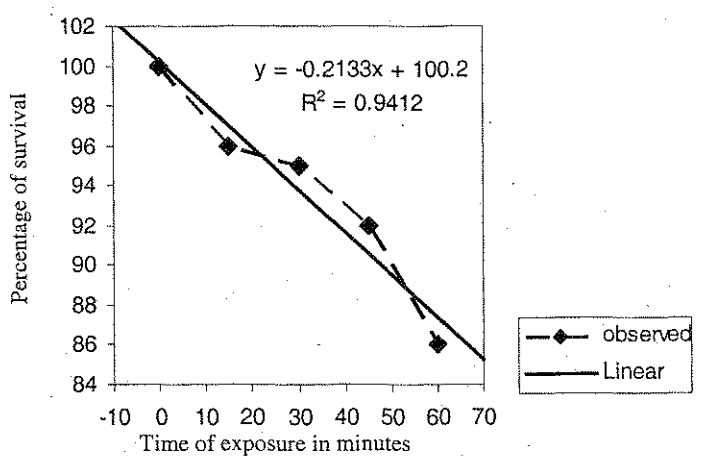

Figure (1): Effect of time exposure to 13.4g/10ml of the leaves aqueous extract of *Rhazya stricta* on percentage of survival of *saccharomyces cerevisiae* cultured cells.

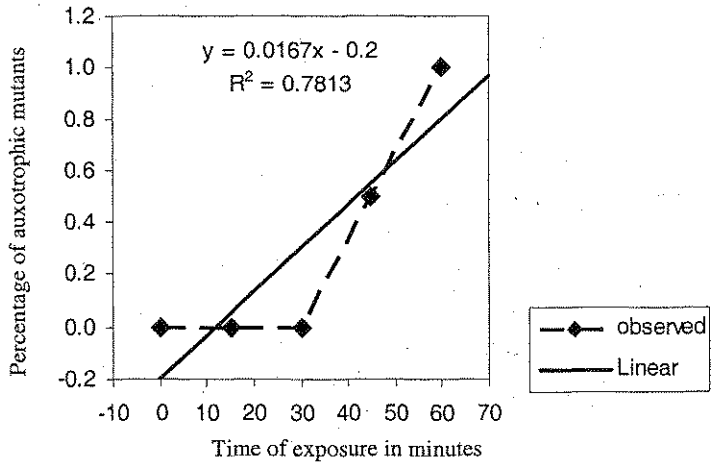

Figure (2): Effect of time exposure to 13.4g/10ml of the leaves aqueous extract of *Rhazya stricta* on percentage of auxotrophic mutants in *saccharomyces cerevisiae*

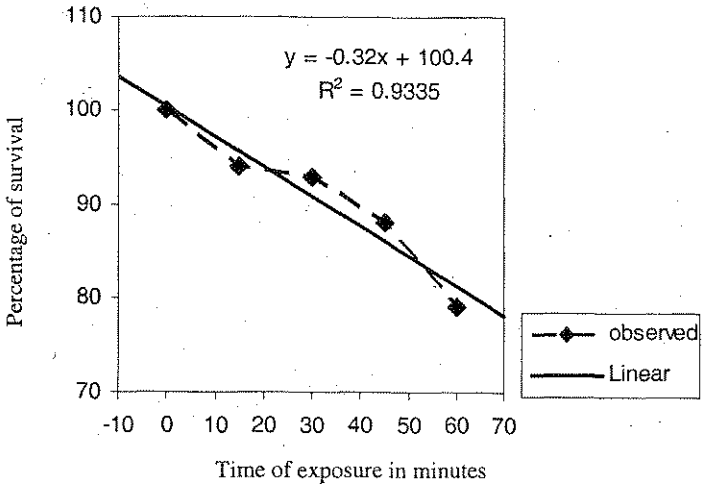

Figure (3): Effect of time exposure to 15g/10ml of the leaves aqueous extract of *Rhazya stricta* on percentage of survival in *saccharomyces cerevisiae*

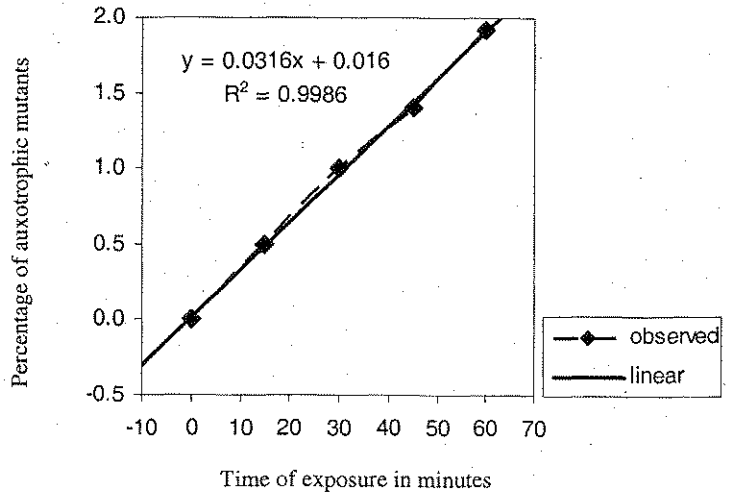

Figure (4): Effect of time exposure to 15g /10ml of the leaves aqueous extract of *Rhazya stricta* on percentage of auxotrophic mutants in *saccharomyces cerevisiae*

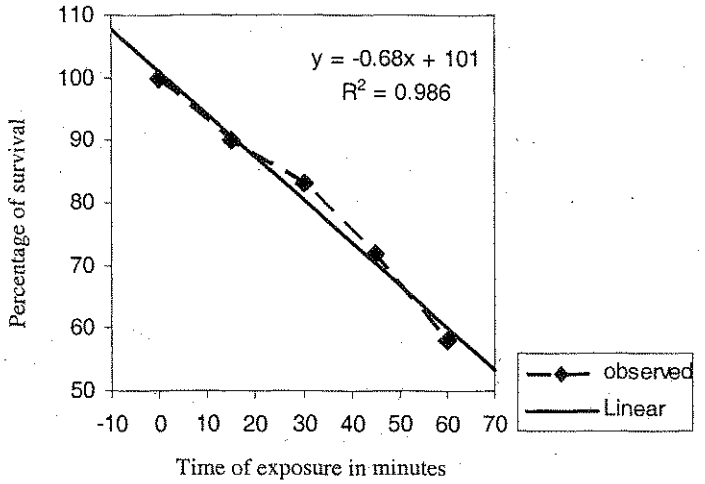

Figure (5): Effect of time exposure to 20.1g/10ml of the leaves aqueous extract of *Rhazya stricta* on percentage of survival in *saccharomyces cerevisiae*

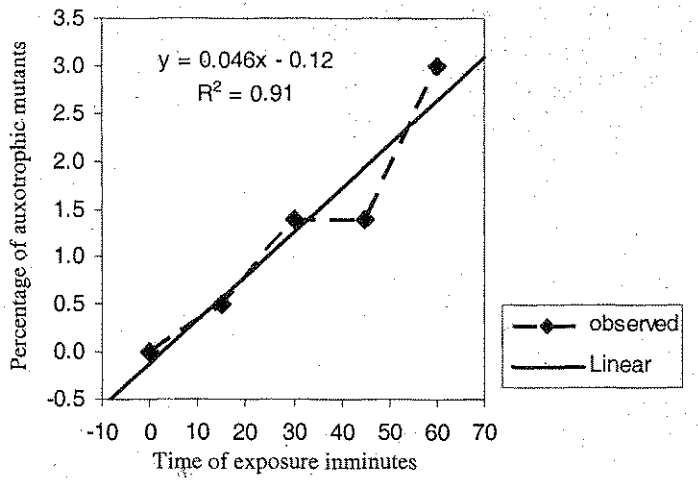

Figure (6): Effect of time exposure to 20.1g/10ml of the leaves aqueous extract of *Rhazya stricta* on percentage of auxotrophic mutants in *saccharomyces cerevisiae*

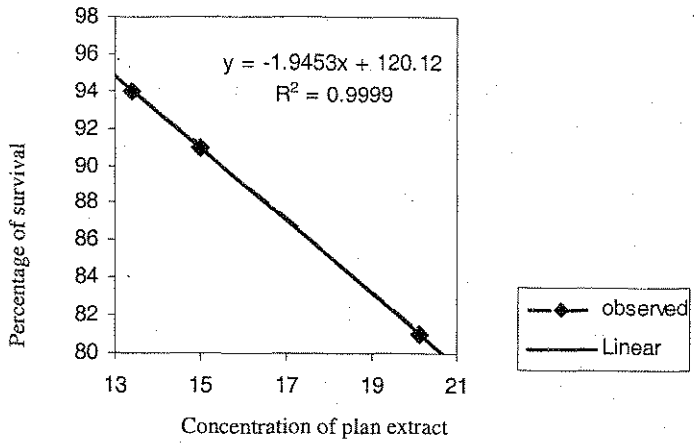

Figure (7): Relationship between concentration of the leaves aqueous extract of *Rhazya stricta* and average of percentage of survival in *saccharomyces cerevisiae*

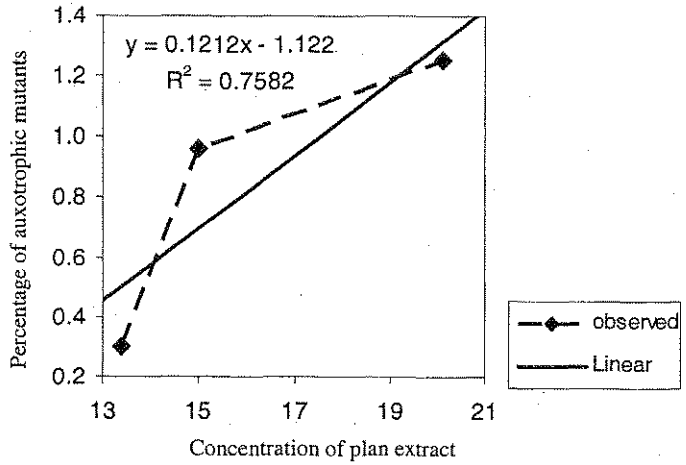

Figure (8): Relationship between concentration of the leaves aqueous extract of *Rhazya stricta* and average of percentage of auxotrophic mutants in *saccharomyces cerevisiae*

Table (2): List of auxotrophic mutants recovered from *Rhazya stricta* leaves extract treated cells of *Saccharomyces cerevisiae*.

Code No.	Extract concentration (gm/10ml)	Treatment (min)	Requirement
Amino acids			
Ar, Pr-RS2114	15	15	Arginine or Proline
Ar, Al-RS2417	15	60	Arginine or Alanine
Ar, Or-RS2419	15	60	Arginine or Ornithine
Ar, OR, Pr-RS2408	15	60	Arginine or Ornithine or Proline
Ar, Tr-RS3119	20.1	15	Arginine or Tryptophan
Ar, Gl-RS3410	20.1	60	Arginine or Glycine
Ar, Or-RS3219	20.1	30	Arginine or Ornithine
Ar, Gl-RS3225	20.1	30	Arginine or Glycine
Ar, Gl, Pr-RS3220	20.1	30	Arginine or Glycine or Proline
Ar, Or-RS3324	20.1	45	Arginine or Ornithine
Ar, Gl-RS3425	20.1	60	Arginine or Glycine
Ar, Or, Ly-RS3405	20.1	60	Arginine or Ornithine or Lysine
Ar, Pr, Ly-RS3418	20.1	60	Arginine or Proline or Lysine
Vitamins			
Ane-RS1419	13.4	60	Aneurine
Cho, Ino-RS2224	15	30	Cholinechloride or Inositol
Cho-RS3423	20.1	60	Cholinechloride
Ane-RS3319	20.1	45	Aneurine
Ribo-RS3407	20.1	60	Riboflavine
Nitrogen bases			
Ad-RS1321	13.4	45	Adenine
Ad-RS1413	13.4	60	Adenine
Hyp-RS2211	15	30	Hypoxanthine
Ad-RS2319	15	45	Adenine
Ad-RS2318	15	45	Adenine
Hyp-RS2311	15	45	Hypoxanthine
Ad-RS2412	15	60	Adenine
Ad-RS3318	20.1	45	Adenine

الملخص العربي

تقييم السمية الوراثية لمستخلص أوراق نبات الحرمل باستخدام اختبار طفرات العوز الغذائي في خميرة الخباز

نبيه عبدالرحمن باعشن - ندى حسن التواتي - علوية الحبشي
قسم الاحياء - كلية العلوم - جامعة الملك عبدالعزيز
ص.ب ٨٠٢٠٣ ، جدة ٢١٥٨٩ - المملكة العربية السعودية

تمت معاملة معلقات خلوية من خميرة الخباز بتراكيز متصاعدة من المستخلصات المائية لأوراق نبات الحرمل البري. وقد سحبت العينات على فترات منتظمة لكل معاملة لرصد النسبة المئوية للبقاء وطفرات العوز الغذائي. وأشارت النتائج أن للمستخلص تأثير طفوري وابادي قوي. فقد انخفضت النسبة المئوية للبقاء بزيادة التركيز او بزيادة مدة التعريض لكل تركيز. كما أن معدل حدوث طفرات العوز الغذائي زاد بازدياد التركيز أو بزيادة مدة التعريض.

وقد كانت معظم طفرات العوز الغذائي حدوثا هى تلك التي تحتاج لاحدى الأحماض الأمينية، ويأتي بعدها من حيث الكثرة تلك التي تحتاج احدى القواعد النيتروجينية، في حين أن أقل الطفرات حدوثاً هي التي تحتاج احدى الفيتامينات.