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ASSESSMENT OF POSSIBLE MUTAGENICITY OF BETEL LEAF IN *SACCHAROMYCES CEREVISIAE*

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Abstract

Aqueous extract of betel leaf was tested for the induction of mitotic gene conversion, mitotic crossing over and reverse mutation in diploid strains of *Saccharomyces cerevisiae*. Tests performed with stationary and log phase cells showed that betel leaf extract was non-mutagenic in yeast without metabolic activation. It failed to increase the frequency of convertants, recombinants and revertants. Betel leaf extract neither caused cell killing nor inhibition of cell division. EMS, used as control, exhibited mutagenic and recombinogenic activity.

Introduction

Most, if not all, cancers may be due to environmental causes, and a positive correlation exists between carcinogenicity and mutagenicity. Thus there is need of identifying environmental carcinogens by employing short-term tests for mutagenicity. Although much attention has been focussed on the genotoxicity of industrial and agricultural chemicals, these alone cannot be wholly responsible for carcinogenic activity in man. There has been an increasing interest in naturally occurring compounds ingested as part of the normal diet and a relationship between diet and cancer has been established (Wynder & Gori, 1977). Many plant components have been shown to be potently mutagenic and some of them to be carcinogenic (Weisberger, 1979).

Chewing of betel quid is a widespread addiction in Pakistan, India and certain other parts of the world. A relationship between the incidence of oral and oesophageal cancers and habitual chewing of betel quid has been established (Bhide *et al.*, 1979; Ranadive *et al.*, 1979). Experiments with extract of betel quid yielded positive (Muier & Kirk, 1960), weak (Hamner, 1972) and negative (Dunham & Herbold, 1962) carcinogenic effects. Conflicting results have been obtained with mutagenicity testing. Betel quid failed to induce genetic damage in *Drosophilla* (Abraham *et al.*, 1979) but the saliva of its chewers had clastogenic effects in chinese hamster ovary (CHO) cells (Stich & Stich, 1982).

Betel leaf (*Piper betel*), an essential and one of the major components of betel quid, induced chromosomal damage in human leukocyte cultures (Sadasivan *et al.*, 1978), plant

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cells (Abraham & Cherian, 1976) and CHO cells (Stich & Stich, 1982). However, it failed to induce sex-linked recessive lethal mutations in *D. melanogaster* (Abraham *et al.*, 1979). The present study was undertaken to assess its possible mutagenic and recombinogenic potential in the diploid yeast, *Saccharomyces cerevisiae*.

Materials and Methods

1. *Test Agents:* Fresh betel leaves obtained from the Aabpara Market, Islamabad, Pakistan, were used for making aqueous extract by grinding 100g of leaves into a paste which was mixed in 200 ml distilled water, placed in a refrigerator for 48 h and then filtered (Sadasivan *et al.*, 1978). The filterate used for mutagenic treatment was membrane filtered prior to use. A 10% (v/v) stock solution of Ethyl methane sulfonate (EMS), a product of Koch-light Laboratories Ltd., prepared in distilled water was used as control.

2. *Biological Test Matrial:* Two strains of *S. cerevisiae* obtained from Dr. F.K. Zimmermann were used. D4 was used for the detection of induced mitotic gene conversion at two unlinked loci, *ade2* and *trp5* (Zimmermann, 1971). D7 was used for the simultaneous detection of mitotic crossing over at the *ade2*, mitotic gene conversion at the *trp5*, and reverse mutation at the *ilv1* locus (Zimmermann *et al.*, 1975).

3. *Treatment Conditions:* D4 and D7 were used as recommended by Zimmermann *et al.*, (1975). The tests were carried out with stationary as well as log phase cells. In stationary phase tests, cells were treated with betel leaf extract at a concentration of 0.1 and 0.2 ml per ml buffered at pH 5.91 and 8.05. Parallel positive control suspensions containing EMS were also prepared. Immediately on addition of the test solutions, about 10^6 cells from each suspension were spread onto the plates of appropriate selective synthetic minimal media lacking adenine, tryptophan or isoleucine for scoring convertants or revertants. For survival estimation and visual detection of mitotic recombinants in D7, serial dilutions were made to obtain 100-200 viable cells per YEPD plate. The remaining reaction mixtures were kept in the dark on a shaking reaction incubator at 30°C and after 4 h cells were harvested by centrifugation for dilution and plating.

Treatments in log phase were given by growing cells at 30°C for 24 h, adding betel leaf extract (0.1 and 0.2 ml/ml) and allowing the cells to grow for anothr 24 h. Cultures with EMS served as positive control. The cells harvested by centrifugation, counted by haemocytometer were diluted and plated. All plates were incubated at 30°C, and the colonies counted after 3 and then after 5 days of incubation. The untreated samples, and the treated ones plated at 0 h in stationary phase tests, served as negative controls.

Results

1. *Cytotoxicity:* Percentage survival of stationary phase cells of D4 and D7 treated with betel leaf extract did not differ significantly from those of negative control at two pH levels (Table 1 & 2). Treatment with EMS also did not significantly affect the cell viability. Betel leaf extract was ineffective in inhibiting cell division in log phase tests as determined by cell counts.

2. *Genotoxicity:* Genotoxicity of a compound was assayed by its ability to induce mitotic gene conversion, mototic crossing over and reverse mutation in diploid yeast.

Gene conversion: Betel leaf extract failed to induce a significantly higher frequency of gene conversion in stationary phase cells at two pH levels, and also in log phase cells (Table 1). The positive control, EMS, induced a significantly higher frequency of convertants in stationary and log phase cells (Table 1).

Mitotic Recombinants and other aberrant colony formation: Various types of aberrant colonies resulted from the mutagenic treatment of D7. The red/pink twin-sectored colonies were due to mitotic crossing over at the *ade2* locus while other could result from either mitotic recombination, chromosomal aberrations or reverse mutations. Betel leaf extract did not induce higher frequencies of mitotic recombinants and other aberrant colonies than the negative control in both stationary and log phase tests (Table 2). EMS induced a significantly higher frequency of recombinants and other aberrant colonies.

Reverse Mutation: Betel leaf extract did not significantly increase the frequency of revertants in both stationary and log phase tests, while the positive control, EMS, significantly enhanced the rate of reversion over the negative control (Table 1).

Discussion

The results indicate the lack of mutagenic activity of betel leaf extract in diploid yeast without metabolic activation. This might suggest that no mutagenic substance is present in betel leaf or formed when it is metabolised by yeast. However, since the clastogenic activity of betel leaf has been detected in plant cells (Abraham & Cherian, 1976), it is possible that some mutagenic component is present in concentrations not high enough to be detected in yeast. In one study (Butool, 1983), high concentrations of the extract prepared as described by Abraham & Cherian (1976) failed to induce detectable genetic damage in growing cells of *S. cerevisiae* strain D7.

Only clastogenic activity of betel leaf exracts has been demonstrated (Abraham & Cherian, 1976; Sadasivan *et al.*, 1978; Stich & Stich, 1982) in direct assays. It is thus

Table 1. Frequency of convertants (per 10^6 survivors) at the *Ade2* and *Trp5* loci and revertsnts at *Ilv1* locus of *Saccharomyces cerevisiae* in stationary-phase and log-phase tests.

Test Compound	pH	% Survival	Cell count[a] (cells/ml x 10^8)	Convertants (D4) Ade+ 0 hr	Ade+ 4 hr	Trp+ 0 hr	Trp+ 4 hr	Revertants (D7) Ilv+	Ilv+ 4 hr
Water (negative	5.91	100 (100)		14.40	13.00	25.20 (22.90)	24.00 (20.43)	5.00	4.50
control)	8.05	99 (98)		15.66	13.20	26.00 (23.63)	24.33 (21.66)	5.80	5.00
		100 (100)	1.68 (3.50)	6.56		6.50 (35.00)		3.75	
Betel Leaf	5.91	99 (99)		15.00	13.80	28.00 (20.40)	27.40 (23.20)	6.00	5.75
extract 0.1 ml/ml	8.05	100 (97)		14.68	14.50	26.60 (22.00)	26.50 (21.90)	5.33	5.00
		98 (99)	1.65 (3.40)	7.50		6.70 (33.75)		4.10	
0.2 ml/ml	5.91	94 (100)		12.00	12.26	24.20 (21.45)	23.33 (22.66)	4.90	6.00
	8.05	97 (98)		13.33	14.20	27.30 (19.33)	25.66 (24.00)	5.25	6.50
		100 (97)	1.70 (3.20)	7.00		7.50 (38.37)		4.50	
EMS									
(Positive control)	5.91	96 (95)		15.62	79.00*	18.00 (24.37)	530.00* (234.37)*	5.75	158.00*
0.01 ml/ml	8.05	92 (90)		14.72	94.12*	16.70 (22.20)	529.20* (242.42)*	4.96	157.50*
		95 (95)		46.82*		163.30* (354.50)*		90.00*	

a Values in parentheses are for the strain D7.

b Values in every 3rd row are for the long-phase cells.

* Differ significantly from the negative control values ($P > 0.01$).

Table 2. Frequency of morphologically aberrant colonies in *Saccharomyces cerevisiae* strain *D7* in stationary and log phase test.

Test	Test compound	% Survival	Number of colonies examined	Total number of aberrant colonies	Aberrant colonies per 10^3 survivors	Number of twin-sectored colonies	Twin-sectored colonies per 10^3 survivors
Stationary-phase test	Water (negative control)	100	15525	25	1.61	2	0.12
	Betel leaf extract						
	0.1 ml/ml	99	14455	18	1.24	2	0.13
	0.2 ml/ml	100	12435	16	1.28	2	0.16
	EMS (Positive control)						
	0.01 ml/ml	95	9547	90	9.42*	10*	1.04*
Log-phase test	Water (negative control)	100	14285	21	1.47	2	0.14
	Betel leaf extract						
	0.1 ml/ml	99	15325	22	1.43	2	0.13
	0.2 ml/ml	97	10575	14	1.31	1	0.09
	EMS (positive control)						
	0.01 ml/ml	95	10870	105	9.65*	14*	1.28*

*Differ significantly from the negative control values ($P > 0.01$).

possible that only specific damage is induced by extracts. However, since mitotic gene conversion can detect any type of genetic damage inflicted on DNA (Murthy, 1979; Zimmermann *et al.*, 1975) the negative response in the present study could not be due to failure of detecting the specific damage induced, and hence the chances of false negative results are very meagre.

Most of the plant products, such as condiments and spices, that induced chromosome damage in plant systems, and to a certain extent, in cultured mammalian cells, were nonmutagenic when tested in mice or rats, and *Drosophilla* (S.K. Abraham, personal communication) and in diploid yeast, *S. cerevisiae* (S.R. Chughtai, Masooma Qazalbash & Sarah Butool, unpublished observations).

Eugenol, one of the major constituents of betel leaf (Atal *et al.*, 1975), was nonmutagenic in direct assays with *Salmonella typhimurium* but some of its metabolites were mutagenic in the absence of metabolic activation (Sekizawa & Shibamoto, 1982; Yoshimura *et al.*, 1981). Eugenol was negative in reversion assays with *E. coli* (Sekizawa & Shibamoto, 1982) and has been reported both as negative (Yoshimura *et al.*, 1981) and positive (Sekizawa & Shibamoto, 1982) in rec-assay with *B. subtilis* in the absence of metabolic activation. It was clastogenic and also had potentiating effect on the clastogenic activity of other ingredients of betel quid (Stich *et al.*, 1981). It, therefore, appears that eugenol is negative in direct assays and its activity is observed only in the absence of metabolic activation.

The discrepancies may be due to the differences in the metabolic potential of different test systems, and the genetic end-point utilized. The information about the mutagenicity of eugenol in microbial tests (Sekizawa & Shibamoto, 1982; Yoshimura *et al.*, 1981), the clastogenicity of betel leaf in plant and mammalian cells (Abraham & Cherian 1976; Sadasivan *et al.*, 1978; Stich & Stich, 1982), and the observations that the clastogenic activity of betel leaf in CHO cells was reduced by the addition of rat liver enzymes (Stich and Stich, 1982), suggest that a mutagenic component, in the form of eugenol, is present in betel leaves which is inactivated by the metabolizing system of yeast. Eugenol is present in various plant products, sometimes in large quantities as in the widely used condiments, cloves and fennel. It would be interesting to test such products and eugenol itself in diploid yeast.

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