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EFFECT OF SEED EXTRACT OF *ZIZIPHUS MAURITIANA* ON THE MUTAGENIC POTENTIAL OF *SALMONELLA TYPHIMURIUM* (TA 98, TA100)

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ABSTRACT

Scientific data relevant to the use of large number of plant species in folk medicines in the form of various types of products and their consequences on human health has been a major area of interest in the past few years. *Ziziphus mauritiana* (family: Rhamnaceae) being an important medicinal plant, posses tremendously beneficial properties attributed by the presence of different secondary metabolites or phytoconstituents. The present study was carried out to verify the antimutagenic potential of aqueous-ethanolic seed extract of *Ziziphus mauritiana* 2MS through Ames Salmonella plate incorporation assay using two strains of *Salmonella typhimurium*, TA 98 and TA 100. Our results revealed that ZMS has shown its activity against both the strains in a concentration dependent manner. Although, the results were more pronounced with TA 98 as compared to TA 100. At a concentration of 1000µg/0.1ml ZMS has shown 60% inhibition of mutagenesis with TA 100 while the same concentration has shown 66% inhibition of mutagenesis in TA 98. The results of co-incubaion and pre-incubation studes have revealed that with of both the strains extract was more effective in co-incubation study rather than pre-incubation study. Previous studies have shown the presence of terpenoids and other phytoconstituents, which may be responsible for the bioactivity.

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INTRODUCTION

Mutations refer to the sudden inheritable and abnormal changes in the genetic makeup of an organism. There are many ways through which a gene mutation can affect the cell. Mutation is considered as the cause of innate metabolic defects in cellular system, triggering the morbidity and mortality in living organisms. An abundant amount of synthetic and natural substances, apart from various genotoxic, physical and biological agents, are known to act as mutagenic, co-carcinogenic or carcinogenic agents (Mitscher *et al*., 1986). The process of exposure or treatment of biological material to act as a mutagen, i.e., a physical or chemical agent that raises the frequency of mutation above the spontaneous rate, is described as mutagenesis (Rieger *et al.,* 1976) Antimutagens are the agents that interfere with the mutagenecity of physical and chemical mutagens most likely by reducing their deleterious effects (Mitscher *et al*., 1986). Evidences suggest that mutations in somatic cells causes cancer, genetic disorders and many other degenerative disorders including arthritis, hepatic disorders, neurodegenerative disorders, cardiovascular disorders, diabetes, chronic inflammation, ageing etc. Cancer is well associated with mutagens that cause oxidative stress in the cells via the production of excessive reactive oxygen species (ROS), (Halliwell and Gutteridge, 1984). Moreover, epidemiological studies indicate that many types of cancer are dependent on multiple mutational etiologies, as well as on inherited mutator phenotype (Hoeijmakers, 2001; Loeb, 1991; Marnett, 2000; Loeb *et al.,* 2003; Mills *et al.,* 2003; Bielas *et al.,* 2006; Hoffmann and Cazaux, 2010). High degree of correlation between carcinogenicity and mutagenicity has been shown through a study in which ninety percent of the carcinogens tested were found to be mutagenic in the Ames assay (McCann, 1975b). Plants and their products in general are rich in various types of phytochemicals compounds indeed they produce an extensive variety of antioxidant compounds that include flavonoids, terpenoids, terpenes and ascorbic acid (Hollman, 2001).

Ziziphus mauritiana, family Rhamnaceae, is a spiny, evergreen shrub or small fruit bearing tree, native to the tropical and subtropical regions. The fruits of *Ziziphus mauritiana* tree, commonly known as ber has been reported to be a rich source of vitamin C and considered for nutritional and phytochemical

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studies in the past few years (Azam *et al.,* 2006 and Obeed *et al.,* 2008). Evidences from the studies conducted on almost every part of this plant suggest their role as a potent antioxidant and anticancer agent. Our previous studies had shown that the aqueous-ethanolic seed extract of *Ziziphus mauritiana* possessed antioxidant potential (Bhatia and Mishra, 2010). In addition to this the extract has shown potential effect against various cell lines and benzo (a) pyrene induced cancer (Mishra *et al*., 2012). As literature revealed that there is strong relationship between cancer and mutation. Thus, keeping in mind, the present study was aimed to investigate the antimutagenic potential of aqueous-ethanolic seed extract of *Ziziphus mauritiana* through Ames Salmonella plate incorporation assay using two strains of *Salmonella typhimurium*, TA 98 and TA 100.

MATERIALS AND METHODS

Plant Source, Extract Preparation, and Standardization (ZMS)

Fruits of *Ziziphus mauritiana* (Lamk.), variety Umran were collected from Botanical Gardens of Punjabi University, Patiala, Punjab, India and authenticated by Professor R.C. Gupta, Botany Department, Punjabi University, Patiala, Punjab, India. Plant sample has been kept in Voucher specimen DOB (305) PUP at Punjabi University, Patiala. Aqueous-ethanolic seed extract of *Ziziphus mauritiana* (ZMS) was prepared and standardized as described earlier (Mishra and Bhatia, 2010). The test sample was kept at 4˚ C until further use. For the present investigation the test sample with varying concentration were dissolved DMSO (0.1ml).

Bacterial Strains

The antimutagenic potential of extract was evaluated in the present investigation by using the Ames Salmonella plate incorporation assay (Maron and Ames, 1983). The two strains of *Salmonella typhimurium* were used viz. TA 98 and TA 100. The genotype of both the strains was checked regularly for their histidine requirement, deep rough character (rfa), UV sensitivity, uvrB mutation and presence of R factor plasmid. The spontaneous reversion frequency of tester strain TA100 was in the range of 115 to 240 revertants per plate whereas that of TA98 was in the range of 25 to 60 revertants per plate. *Salmonella typhimurium* TA 98 is a frame shift mutation that contains the his3052 mutation and Salmonella *typhimurium* TA 100 contains base pair substitution mutation hisG46.

Diagnostic mutagens (Positive controls)

Sodium azide, one of the recommended direct-acting diagnostic mutagens, was used for TA 100 strain. For TA 98 strain, 4-nitro-o-phenylenediamine (NPD) was used. In the case of S9-dependent mutagen, 2-aminofluorene was used for the tester strains.

Negative control

Different concentrations of extracts added to the test tubes containing nutrient broth culture and soft agar supplemented with histidine and biotin. The mixture was poured onto minimal agar plates which were then incubated at 37°C for 48 hours and the number of revertant colonies was determined. Non-toxic concentrations were determined to be those, where there was no statistically significant difference in the number of spontaneous revertant colonies, size of the colonies and intensity of the background lawn, as compared to the control where no extract/fraction was added.

Antimutagenicity screening

Antimutagenic potential of ZMS was tested in two sets of experiments as described by Maron and Ames, 1983. For the co-incubation bacterial culture (0.1 ml; 2-5 X 10^8 cells/ml), 0.1 ml NPD or sodium azide and 0.1 ml of non-toxic concentration of extracts were added in the above order into sterile test tubes containing 2 ml of soft agar and poured onto minimal agar plates. In the case of indirect-acting mutagen, 2-aminofluorene (2AF), 0.1 ml of bacteria, 0.1 ml of 2AF, 0.5 ml of S9 mix and 0.1 ml of extract/fraction were added into 2 ml of soft agar, mixed and poured onto minimal glucose agar plates. For the Pre-incubation, Positive mutagen (0.1 ml of NPD or sodium azide) and 0.1 ml of non-toxic concentration of extracts/fractions were added into sterile empty test tubes. The mixture was placed in gyrorotary incubator set at 37^{o}C for 30 minutes. After incubation, 2 ml of molten agar was added into the test tubes along with 0.1 ml of culture. The contents of the tube were well mixed and poured onto minimal agar plates. In the case of 2AF, 0.1 ml of 2AF, 0.5 ml of S9 mix and 0.1 ml of ZMS were incubated. Once the soft agar was poured onto minimal agar plates, it was evenly distributed by rotating the dish and then placed on a leveled surface. After solidification, the plates were placed in incubator at 37^{o}C in an inverted position.

Concentrations of extracts/fractions

The extract/fraction solutions were prepared in DMSO. The concentrations that were used in the case of direct-acting mutagens (NPD and sodium azide) were 10, 50, 100, 500, 1000 µg/0.1 ml/plate. The antimutagenic activity of each fraction was expressed as percent decrease of reverse mutations as follows:

Inhibitory activity (%) = a-b/a-c × 100

a = Number of histidine revertants induced by mutagen (sodium azide and NPD)
b = Number of histidine revertants induced by mutagen in the presence of extract
c = Number of histidine revertants induced in the presence of extract alone and solvent (negative control)
The Data expressed as mean value ± S.D. (n=3 plates)

RESULTS AND DISCUSSION

The antimutagenic potential of *Ziziphus mauritiana* plant extract was checked against two strains of *Salmonella typhimurium i.e.* TA 98 and TA 100. The extract was co-incubated and pre-incubated with the strains separately. The decrease in number of colonies was taken as the measure of inhibition of mutagenesis. It was evident from results (Table 1 and 2) that the extract exhibited the inhibition of mutagenesis in a concentration dependent manner and the effect was more pronounced with co-incubation as compared to pre-incubation. It was observed that extract exerted more of its antimutagenic potential with TA 98 strain instead of TA 100. The extract exhibited inhibition of mutagenesis in a concentration dependent manner. In the pre-incubation, extract at minimum

(10 µg/ml) and maximum (1000 µg/ml) concentrations showed the rise in activity by 1.8 folds and 1.4 folds respectively when compared with that of the activity expressed during the pre-incubation with TA 100 strain. Similarly, the rise noticed in co-incubation with TA 98 at minimum and maximum concentration was 1 and 2 folds higher as compared to co-incubation with TA 100 strain.

Table 1 Effect of ZMS on TA 100 strain and inhibition of mutagenesis

Treatment	Concentration (µg/0.1 ml)	TA 100 (Sodium azide) His^+ revertants/plate	% Inhiition of mutagenesis
Spontaneous (Only Bacterial culture)		20.01 ± 2.03	
Positive Control (Mutagen+ Bacterial culture)	20	1490 ± 2.42	
Negative Control Bacterial culture + plant extract	10	21.12 ± 2.56	
	50	19.10 ± 1.13	
	100	20.04 ± 2.42	
	500	20.01 ± 4.33	
	1000	19.13 ± 2.23	
Co-Incubation	10	1356.11 ± 2.32	9.11
	50	1148.12 ± 1.85	23.26
	100	912.02 ± 2. 78	39.31
	500	766.32 ± 3.71	49.25
	1000	**602.31 ± 2.45**	**60.40**
Pre-Incubation	10	1455.23 ± 2. 32	2.38
	50	1308.12 ± 3.21	12.37
	100	1171.24 ± 3.12	21.70
	500	1001.15 ± 2.76	33.26
	1000	**795.23 ± 4.65**	**47.27**

Data are mean value ± S.D. (n=3 plates) and representative of one of the three experiments

Table 2 Effect of ZMS on TA 98 strain and inhibition of mutagenesis

Treatment	Concentration (µg/0.1 ml)	TA 98 (NPD) His^+ revertants/plate	% Inhiition of mutagenesis
Spontaneous (Only Bacterial culture)		20.01 ± 2.03	
Positive Control (Mutagen+ Bacterial culture)	20	1521 ± 1.21	
Negative Control Bacterial culture + plant extract	10	21.12 ± 2.56	
	50	19.10 ± 1.13	
	100	20.04 ± 2.42	
	500	20.01 ± 4.33	
	1000	19.13 ± 2.23	
Co-Incubation	10	1387.12 ± 3.01	11.23
	50	1149.01 ± 2.99	27.27
	100	972.07 ± 2.65	38.18
	500	704.11 ± 3.56	55.58
	1000	**530.21 ± 2.04**	**66.88**
Pre-Incubation	10	1497.15 ± 2. 09	4.09
	50	1342.02 ±2.97	14.15
	100	1158.10 ±3.01	26.10
	500	972.13 ±2.87	38.18
	1000	**746.07 ±4.01**	**52.85**

Data are mean value ± S.D. (n=3 plates) and representative of one of the three experiments.

It was observed that co-incubation of plant extract with TA100 strain resulted in 60% inhibition of mutagenesis at 1000µg/0.1 ml, while the pre-incubation resulted in only 47% inhibition of mutagenesis at the same concentration. The results were more pronounced with TA 98 strain as with TA98 strain co-incubation resulted in 66% inhibition of mutagenesis at the same concentration and 52% with pre-incubation. Mutations are the cause of metabolic defects in cellular system that may trigger the onset of some diseases including cancers and increases the morbidity and mortality in living organism. A plethora of synthetic and natural substances, apart from various genotoxic physical and biological agents are known to act as mutagens, co-carcinogens/carcinogens agents (Mitscher *et al*., 1986). Mutations are not only involved in carcinogenesis but also play an important role in pathogenesis of many chronic degenerative diseases which share common mechanism such as DNA damage, oxidative stress and inflammation.

These factors not only lead to cancer but can also result in atherosclerosis and other heart diseases (De Flora *et al*., 1996). Since, the mutagens are involved in the initiation or promotion of many diseases and the phytocompounds counteract the promutagenic and carcinogenic effects of mutagens and hence, act as antimutagens. Antimutagenic and anticarcinogenic properties of a wide variety of dietry constituents and plants secondary metabolites are very well reported (Stavric, 1994; Sangwan *et al*., 1998; Shon *et al*., 2004). Mutagens that cause base pair substitution mutations primarily at one of the GC pairs can revert the mutated *S. typhimurium* TA 100 strain to wild type. In contrast, TA 98 is a frame shift mutagen sensitive strain (Mortelmans and Zeiger, 2000). The results of our study demonstrated that ZMS was effective against both the mutagens: NPD and NaN_3.

This antimutagenic activity of seed extract of *Ziziphus mauritiana* against directly acting mutagens may probably be due to protection of bacterial genome from the binding of mutagens either by eliminating the mutagen from the bacteria or by inhibiting the entry of mutagen to the bacterial wall. The free radicals may be involved in the NPD/NaN_3 induced mutation in the *Salmonella* tester strains. Hence, the antioxidant activity of seed extract of *Ziziphus mauritiana* (Bhatia and Mishra, 2009) could also be responsible for antimutagenic effect. The antimutagenic effect of γ-tocopherol by trapping the mutagenic electrophiles is very well reported (Christen *et al*., 1997). Moreover, antioxidant was reported to be effective against NPD and NaN_3 induced mutation in *Salmonella typhimurium* strain (Ajith *et al*., 2005). The exhibited activity is not due to the toxic effect of extract on bacterial strain. This was evident from the plate treated with only bacterial strain and extract as the background lawn was clear and number of revertants of bacterial colonies was similar to the plate treated with only the bacterial culture. Our results are supported by the findings of Negi *et al*. (2003) where they studied the antimutagenic and antioxidant effect of pomegranate peel extracts and found it to be a good antioxidant as well as antimutagenic agent. Wide array of data is available mentioning the Antimutagenic activity of various plant species is due to their phytoconstituents.

In conclusion this study suggests that plant extract exhibited more pronounced antimutagenic potential against TA 98. However further investigations are required to elucidate the component responsible for its biological activity.

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