African Journal of Biotechnology Vol. 10(43), pp. 8654-8661, 10 August, 2011
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB10.1704
ISSN 1684–5315 © 2011 Academic Journals

Full Length Research Paper

Free radical-scavenging and antimutagenic potential of acetone, chloroform and methanol extracts of fruits of *Argemone mexicana*

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Accepted 4 March, 2011

The antioxidant potency of acetone, chloroform and methanol extracts of *Argemone mexicana* was investigated by employing *in vitro* systems like nitroblue tetrazolium (NBT) and 1,1-Diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging assay whereas antimutagenic activity was determined by Maron and Ames assay using *Salmonella typhimurium* TA100 tester strain against sodium azide. In this study, dried fruits of *A. mexicana* were extracted with different solvents by maceration method in order of increasing polarity. It was observed that, acetone extracts were comparatively more effective than the other extracts in both the assays. The maximum inhibitory activities noticed were 79.0 and 71.0% in NBT and DPPH assay, respectively, at the maximum concentration tested. The inhibitory potential was compared with standard antioxidant (L-ascorbic acid). The IC_{50} value of the acetone extract of the fruit of *A. mexicana* was more than that of L-ascorbic acid showing the maximum inhibitory effect. Among the different extracts, the antimutagenic effect of methanol extract was found to be more followed by acetone and chloroform extracts. The methanol extract showed maximum inhibition of 80.5% at doses of 1.00×10^3 in co-incubation and 0.10×10^3 µg/ml in pre-incubation modes of the experiments, respectively. Chloroform extract showed maximum inhibition of 62.14 and 50.71% in co-incubation and pre-incubation mode of experiments at the highest concentration tested. Antimutagenicity of the acetone extract was more in co-incubation than pre-incubation mode of the experimentation. These results indicate that, *A. mexicana* fruit extracts have antioxidant as well as antimutagenic properties. The antioxidant and antimutagenic activities were significantly correlated.

Key words: *Argemone mexicana*, *a*ntioxidant, antimutagenic, sodium azide.

INTRODUCTION

Argemone mexicana belongs to the family Papaveraceae, commonly known as Bharband. It is an erect prickly annual herb with milky latex. It grows wild and is a troublesome weed, recently used in reclamation of Usar. Leaves are yellow with latex, exstipulate, alternate, sessile, simple and glaucous deeply dissected with spiny teeth. Seeds yield nauseous, bitter, non-edible oil, used in cutaneous troubles. Its cathartic presence of argemone oil in edible mustard oil is probably responsible for outbreaks and epidemic dropsy. Mixed with drying oils such as linseed oil, it may be used in the paint industry and also used for soap making.

Free radicals or ROS (reactive oxygen species) are highly reactive oxygen metabolites, which can attract electrons from surrounding molecule to induce accumulation of cellular damage and this result in skin aging (Ma et al., 2001: Cook and Samman, 1996), cardiovascular diseases, cancer, inflammatory diseases and a variety of other disorder (Finkel and Holbrook, 2000). As the free radicals play major role in causing the diseases, the supply of antioxidants, in the diet is of great importance for a healthy life (Scalbert and Williamson, 2000). Foods of plant origin and medicinal plants have been suggested as natural sources of antioxidants (Auddy et al., 2002; Choi et al., 2002; Mantle et al., 2000

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and Singh et al., 2007).

The protective effect of fruits and vegetables has generally been attributed to their antioxidant constituents, including vitamin C (ascorbic acid), vitamin E (α-tocopherol), carotenoids, glutathione, flavonoids and phenolic acids, as well as other unidentified compounds (Sies and Stahl, 1995). Total antioxidant capacity of many fruits and vegetables has been determined by the oxygen radical absorbance capacity (ORAC) assay, which measures the ability of plant extracts to scavenge peroxyl radicals (Cao et al., 1996 and Wang et al., 1996).

Reports have indicated that, plants in the Cactaceae family produce flavonol 3-O-glycosides (quercetin, kaempferol and isorhamnetin), dihydroflavonols, flavonones and flavanonols (Burret et al., 1982; Meyer and McLaughlin, 1982; Miller and Bohm, 1982, Richardson, 1978; Rosler et al., 1966). The presence of phenolic compounds in the fruit samples of cactus pears was observed by Kuti (2000) and Lee and Lim (2000) who reported a possible positive role of *Opuntia* cactus extract, as a natural antioxidant, in scavenging reactive oxidants.

Environmental mutagens are a threat to public health and cancer has become the number one cause of death in the world since the infectious diseases are now more or less under control (Tominaga et al., 1994). Chemical carcinogenesis was of great interest in scientific investigation but less attention had been paid to the substances in the environment/in diet that may protect against chemical mutagens or carcinogen acting as inhibitor in the carcinogenesis process. These chemicals are present in plants which may act as anticarcinogens or antimutagens by blocking ultimate carcinogen electrophiles in a nucleophilic chemical reaction to form innocuous products. A continuous input of these could serve as buffer against DNA damage. A wide array of phenolic substances particularly those present in dietary and medicinal plants have been reported to possess both antimutagenic and anticarcinogenic activities (Okuda et al., 1991; Kaur et al., 1997, 2002, 2007; Lopes et al., 1999).

Keeping in mind the medicinal value of *A. mexicana*, the present investigation was planned to study the antioxidant effect of acetone, choloroform and methanol extracts using nitroblue tetrazolium (NBT) and 1,1-Diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging assays, while the antimutagenic effect was done by Maron and Ames assay.

MATERIALS AND METHODS

Chemical

Ascorbic acid, nitroblue tetrazolium (NBT), nicotinamide adenine dinucleotide reduced (NADH), dimethyl sulfoxide (DMSO), histidine and phenazine methosulfate (PMS) were procured from Hi-media Laboratory Pvt. Ltd. Bombay.

Fruits

The fruits of *A. mexicana* were collected in the month of March from the plant growing in Mohindergarh district (North India). It was washed with tap water (twice), dried in oven at 40°C for 24 h and ground to fine powder.

Preparation of extract

1500 ml each of the solvent including chloroform, acetone and methanol were added serially to 500 g of the fruit powder (flow chart). After filtering through the several layers of mesclin cloth, filterate in different solvents was recovered. The respective solvent from the filterate was evaporated under vacuum at a temperature below 50°C. Extracts were re-dissolved in dimethyl sulfoxide (DMSO) and various concentrations were used for antioxidant and antimutagenic activities.

Culture

Salmonella typhimurium TA100 tester strain (base substitution tester) used in the anitmutagenic study was a kind gift from Dr. (Mrs.) Saroj Arora, of the Department of Botanical and Environment Sciences, Guru Nanak Dev University, Amritsar.

Antioxidant testing assays

*Nitroblue tetrazolium (*NBT) superoxide scavenging assay

The superoxide anion radical scavenging activity was assayed as described by Liu and Ng (1997). Superoxide anions were generated in a non-enzymatic phenazine methosulfate-nicotinamide adenine dinucleotide (PMS-NADH) system through the reaction of PMS, NADH and oxygen. It was assayed by the reduction of nitroblue tetrazolium (NBT). In this experiment, the superoxide anions radicals were generated in 3.0 ml of Tris-HCl buffer (16 µM, pH 8.0) containing 750 µl of nitroblue tetrazolium (NBT) (50 µM) solution, 750 µl of NADH (78 µM) solution and 300 µl of different concentrations (25 to 175 µg/ml) of the extracts. The reaction was initiated by adding 750 µl of PMS (10 µM) to the mixture.

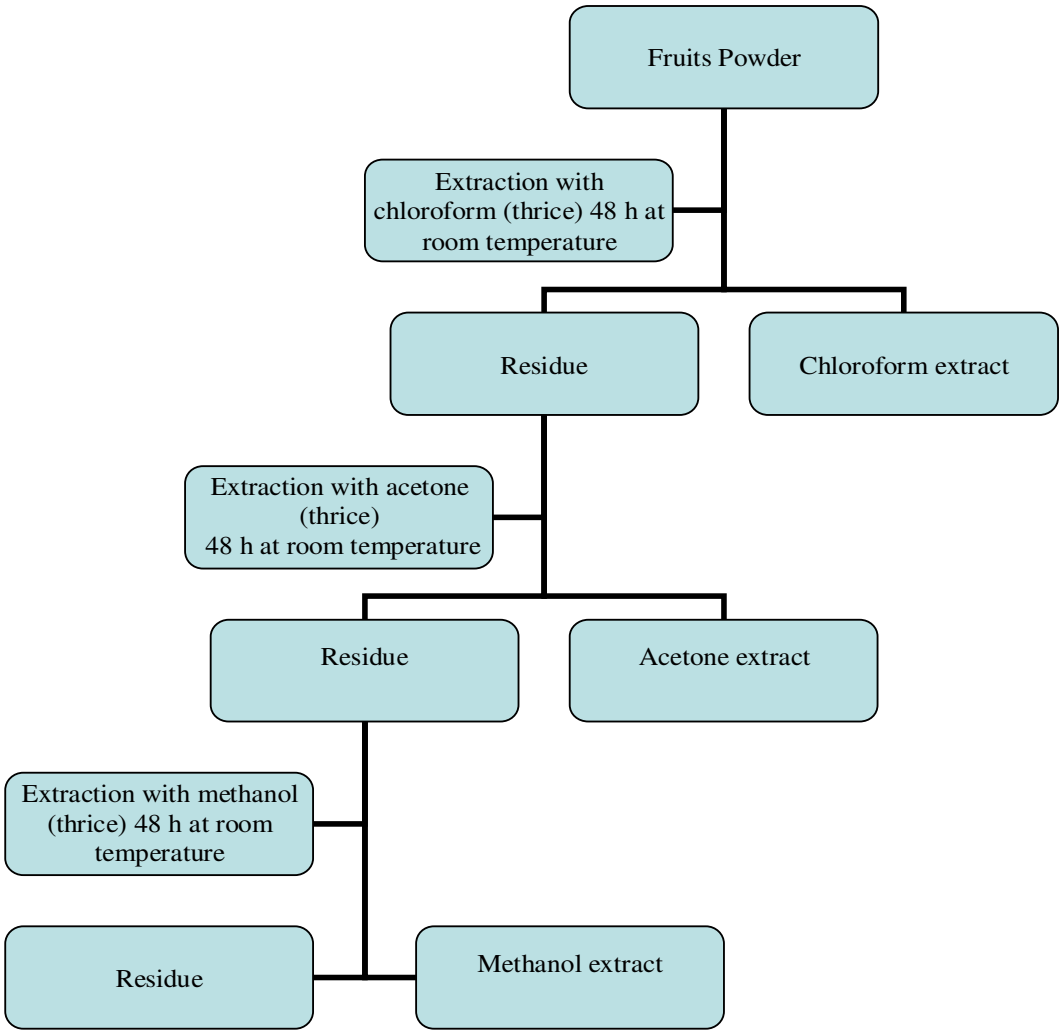
After 5 min of incubation at room temperature, the absorbance at 560 nm was measured in a spectrophotometer against the blank. Decreased absorbance of the reaction mixture indicates increased superoxide anion scavenging activity. L-ascorbic acid was used as a positive control. The antioxidant activity of the test samples was evaluated by calculating the percent inhibition of superoxide anion radicals by applying the following formula:

$$\% \text{ Inhibition} = \frac{A_0 - A_1}{A_0} \times 100$$

Where A_0, is the absorbance of the control (blank, without extract) and A_1, is the absorbance of the extract.

DPPH radical scavenging assay

1 mg extract powder was dissolved in 1 ml of 50% ethanol solution to obtain 1000 µg/ml sample solution. This solution was serially diluted into 100, 250, 400, 550, 700, 850 µg/ml with 50% ethanol. In each reaction, the solutions were mixed with 1 ml of 0.1 mM 1,1-Diphenyl-2-picrylhydrazyl (DPPH), 0.45 ml of 50 mM Tris-HCl buffer (pH 7.4) and 0.05 ml samples at room temperature for 30 min. 50%



Flow chart. Extraction by maceration of fruit powder of *A. mexicana* by increasing polarity.

ethanol solution was used as the negative control. The reduction of the DPPH free radical was measured by reading the absorbance at 517 nm. DPPH is a purple-colored stable free radical; when reduced it becomes the yellow-colored diphenylpicrylhydrazine. L-ascorbic acid was used as the positive control. The antioxidant activity of the test samples was evaluated by calculating the percent inhibition of superoxide anion radical by applying the following formula:

$$\% \text{ inhibition} = ((A_0 - A_1)/A_0) \times 100$$

Where A_0, is the absorbance of the control (blank, without extract) and A_1, is the absorbance of the extract.

The IC_{50} values (the concentration required to inhibit superoxide radical formation by 50%) of the plant extracts were determined and compared with L-ascorbic acid, used as the positive control.

Antimutagenicity test

The plate incorporation assay, as suggested by Maron and Ames (1983) was used in the investigation to check the inhibitory activity of the acetone, chloroform and methanol extracts. To check the antimutagenicity of the extracts in TA100 tester strain of *S. typhimurium*, two modes of experimentation were followed together with co-incubation and pre-incubation. During co-incubation, to 2 ml of top agar containing 0.5 ml histidine, 0.1 ml of fresh overnight grown *Salmonella* culture of tester strain (TA100), 0.1 ml of sodium azide and 0.1 ml of extract were added. A pre-incubation procedure was also followed where equal volume of the positive mutagen and extract were mixed in sterile capped tube and allowed to stand for 30 min at 37°C; 0.2 ml of this was added to 2 ml of the soft agar with 0.1 ml of bacterial culture.

After pouring the soft agar on minimal agar plates, the plates were titled, rotated and placed on a leveled surface to harden for half an hour and then, incubated at 37°C for 48 h; similar experiments were also designed for other concentrations and extracts. A set of positive control (where only mutagen was added), negative control (only extract) and spontaneous reversion (only bacterial inoculum) was added in each experiments. After 48 h of incubation, the numbers of revertant colonies were counted against a background lawn. The concentrations of the various extracts used for antimutagenicity assay were 1.00×10^3, 0.5×10^3 and 0.10×10^3 µg/0.1 ml/plate. The test samples were tested against direct acting mutagen that is, sodium azide (2.5 µg/0.1ml/plate) in TA100 tester strain of *S. typhimurium*. All the test samples were dissolved in

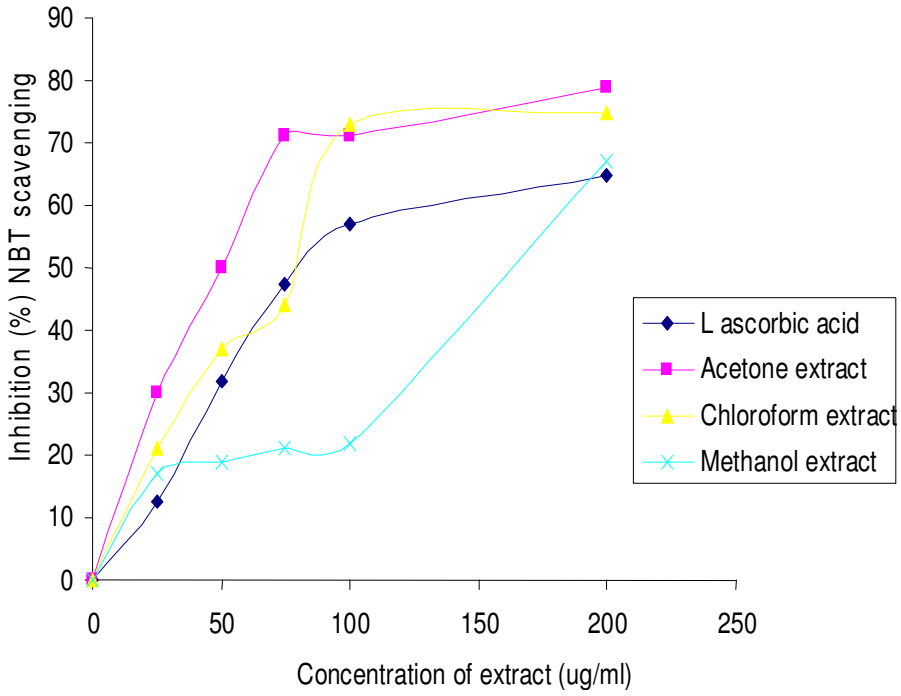


Figure 1. Free radicals scavenging activity of *A. maxicana* fruit by NBT assay.

dimethyl sulfoxide (DMSO). Sodium azide was dissolved in water. In each case, there was no over toxicity observed and the numbers of spontaneous revertants were identical to the DMSO vehicle control. Non toxic concentrations were determined to be those without statistically significant difference in the number of the spontaneous revertant colonies, size of colonies and intensity of the background lawn when compared with the control where no extract was added. Each concentration was tested in triplicate and the entire experiment was repeated thrice.

The inhibitory activity was expressed as percentage decrease of reverse mutation:

$$\text{Percent inhibition} = \frac{(X-Y)}{(X-Z)} \times 100$$

Percent mutagenic activity was calculated as:

$$\text{Percent of control} = \frac{Y}{X} \times 100$$

Where X, is the number of histidine revertants induced by the mutagen alone (sodium azide); Y, is the number of histidine revertants induced by the mutagen in the presence of the extract and Z is the number of revertants induced in the negative control.

Statistical analysis

All the experiments were repeated at least three times. Results were reported as mean ± SE (not shown in graphs). Correlation coefficient (r) between the two type activities is also presented. T-test was applied for significance of the results. IC_{50} values were also calculated.

RESULTS AND DISCUSSION

Antioxidant activity

The acetone, chloroform and methanol extracts of the fruits of *A. mexicana* quenched NBT and DPPH free radicals in a dose dependant manner because as the concentration of the extracts increased, the NBT and DPPH quenching activity was also increased. The order of effectiveness of the extracts in NBT assay (Figure1) was: acetone extract (79.0%) > chloroform extract (75.0%) > methanol extract (67.0%), while in the DPPH assay (Figure 2) was: acetone extract (71.0%) > methanol extract (66.0%) > chloroform extract (63.0%). Table 1 shows that, the IC_{50} value for the acetone extract was found to be 52.0 and 69 µg/ml in NBT and DPPH assay, respectively, which were more than that of L-ascorbic acid (81.0 µg/ml). So, the acetone extract of the fruits of *A. mexicana* had greater potential in inhibiting the free radicals' formation than that of L-ascorbic acid. The antioxidant capacity of cactus fruits may be attributed to their flavonoid, ascorbic acid and carotenoid contents (Kuti, 2004).

The observed antioxidant activity of the extract may be due to the neutralization of free radical character of NBT and DPPH, either by transferring of an electron or hydrogen atom (Naik et al., 2003). The ability of the extract to scavenge the NBT or DPPH radical has also been related to the inhibition of lipid peroxidation (Rekka and Kourounakis, 1991). The free radical chain reaction

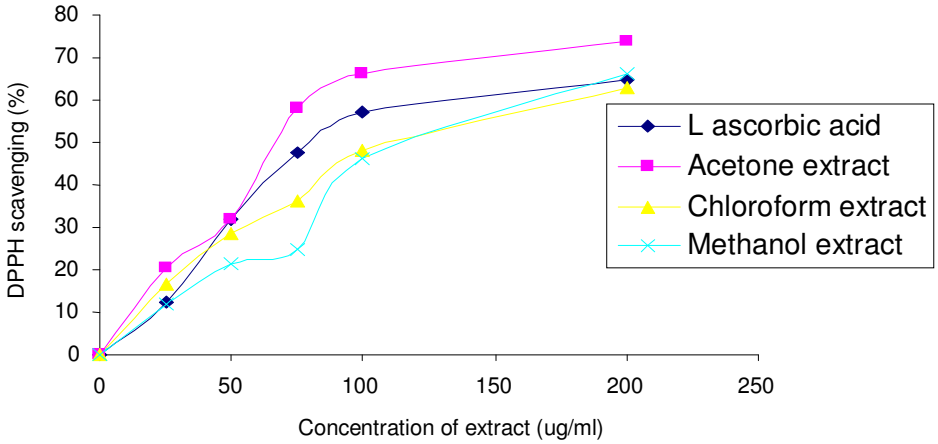


Figure 2. Scavenging of the DPPH radical by different extracts of fruit of *A. maxicana* by DPPH scavenging assay.

Table 1. IC_{50} values of acetone, chloroform and methanol extract of fruit of *A. mexicana* in antioxidant system.

S/N	Assay	Extract/standard	IC_{50} values (µg /ml)
1	NBT superoxide scavenging assay	L-ascorbic acid	81
		Acetone	52
		Chloroform	82
		Methanol	148
2	DPPH radical scavenging assay	L-ascorbic acid	81
		Acetone	69
		Chloroform	122
		Methanol	124

of auto-oxidation are also inhibited by antioxidants by donating the hydrogen of the phenolic hydroxyl group and thereby, giving rise to a stable end product which does not initiate the further oxidation of lipids (Sherwin, 1978). The data shows that, these extracts are free radical scavenger and may act as primary antioxidants, which may react with the free radical by donating hydrogen. Similar work was carried out by Singh et al. (2007) on *Acacia auriculiformis* A. cunn. Hence, *A. mexicana* fruit may be an important source of scavenger of free radicals.

Antimutagenic activity

The results of the antimutagenic activity of the different extracts are shown in the Figure 3 and Table 2. The inhibitory effect observed at different concentrations (0.10 × 10^3, 0.50 ×10^3 and 1.00 × 10^3 µg/0.1 ml) in co-incubation assay was found to be dose dependent. The order of effectiveness of the various extracts of the fruit of *A. mexicana,* in co-incubation mode of the experiment was: methanol extract (80.5 %) > acetone extract (70.7 %) > chloroform extract (62.14 %), respectively, at the highest concentration tested. But in pre-incubation mode of experiment, methanol extract showed maximum inhibition (80.5%) at the lowest concentration tested which was just reverse of the co-incubatuion mode. The maximum inhibitory effect was observed in the co-incubation and pre-incubation studies in the case of methanol extracts with the reduction in the frequency by 80.5 % of histidine revertants. Table 2 indicates that, this extract contained some antimutagenic substances which inhibited the mutagenicity of sodium azide. The antimutagen present in the extracts may interact with the specific enzyme systems, which are necessary for the activation of mutagens (Van der Hoeven et al., 1986)

Tea extract showed strong antimutagenic action against five indirect mutagens in TA98 and TA100 tester strains of *S. typhimurium* (Yen and Chen, 1995). Polyphenols which are the important constituents may be one of the factors for the antimutagenicity as they have been reported as antimutagens in number of systems (Shih et al., 2000; Kaur et al., 2005). The enhanced anti-mutagenic activity of methanol extract may also be due to its oligomeric nature. Gali et al. (1992) suggested that, oligomeric hydrolysable tannins have more antitumor promoting activities than monomeric hydrolysable tannins but the exact mechanism by which the antimutagen

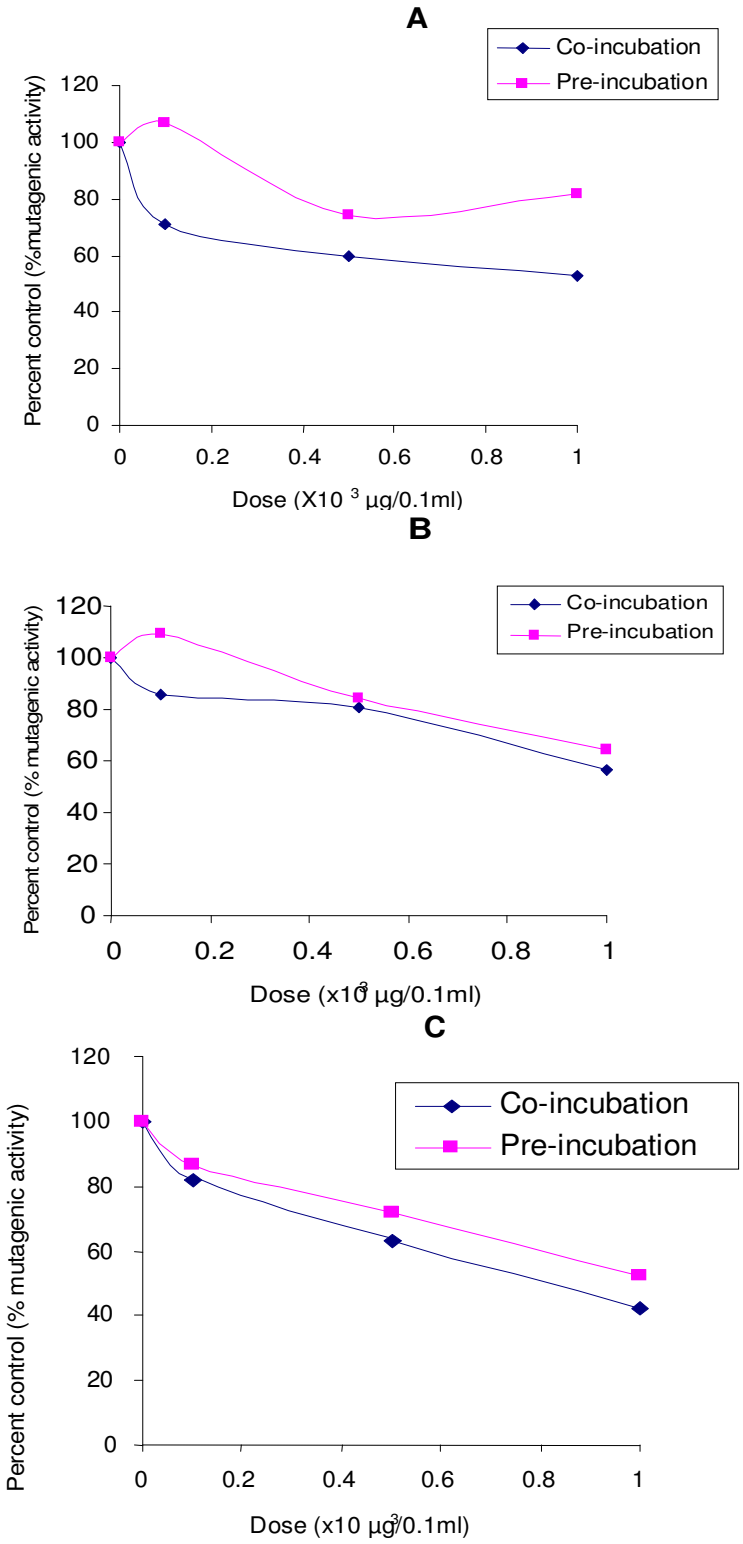


Figure 3. Effect of acetone (A), chloroform (B) and methanol (C) extracts of the fruits of *A. mexicana* on sodium azide induced mutagenicity in TA 100 tester strain of *S. typhimurium.*

$$\% \text{ of control} = \frac{\text{His+ revertant/plate with mutagen and antimutagen}}{\text{His+ revertant/plate with mutagen}} \times 100$$

Table 2. Effect of various extracts of fruit of *A. mexicana* on mutagenicity of sodium azide in TA100 tester strain of *S. typhimurium*.

Concentration of extract (µg/0.1 ml)	TA100 tester strain of *S. typhimurium*					
	Acetone extract (AE)		Chloroform extract (ChE)		Methanol extract (ME)	
	Sp = 1000 Sp + AE = 660 SA = 2000 (2.5 x 10^3 µg/0.1 ml)		Sp = 1000 Sp + ChE = 600 SA = 2000 (2.5 x 10^3 µg/0.1 ml)		Sp = 1000 Sp + ME = 560 SA = 2000 (2.5 x 10^3 µg/ 0.1 ml)	
	Mean ± SE	% inhibition	Mean ± SE	% inhibition	Mean ± SE	% inhibition
Co-incubation						
0.10 ×10^3	1420 ± 48.09	43.2	1720 ± 56.57	20.0	1640 ± 28.28	25.71
0.50 × 10^3	1192 ± 22.63	60.2	1614 ± 990	27.57	1256 ± 16.97	51.6
1.00 × 10^3	1052 ± 36.77	70.7	1130 ± 219.23	62.14	840 ± 28.28	80.5
Pre-incubation						
0.10 ×10^3	2138 ± 38.18	-40.5	2184 ± 50.95	13.14	840 ± 28.28	80.5
0.50 × 10^3	1488 ± 19.80	38.20	1680 ± 28.28	22.85	1430 ± 7.07	39.5
1.00×10^3	1632 + 133.10	27.46	1290 + 49.50	50.71	1040 + 84.86	66.6

Data shown are mean ± SE of two repeated experiment. Sp, Spontaneous reversion; SA, sodium azide.

Table 3. Co- relation coefficient (r) between antioxidant and antimutagenic activities of different extracts of fruit of *A. mexicana*.

S/N	Antioxidant assay	Antimutagenic activity					
		Co-incubation			Pre-incubation		
		AE	ChE	ME	AE	ChE	ME
1	NBT	0.94	0.95	0.97	0.88	0.96	0.48
2	DPPH	0.96	0.98	0.96	0.87	0.98	0.64

Values above 0.63 were significantly correlated at 5% level. AE, Acetone extract; ChE, chloroform extract; ME, Methanol extract

Table 4. T-test statistics of antioxidant and antimutagenic activities of fruit of *A. mexicana*.

S/N	Antioxidant activity	Antimutagenic activity					
		Co-incubation			Pre-incubation		
		AE	ChE	ME	AE	ChE	ME
1	NBT assay	6.0484*	2.0568*	6.8485*	0.6937	0.7336	4.6398*
2	DPPH assay	0.7256	1.7265**	2.2088*	0.5462	1.1984	1.5193

*Significant at 5% level of significance; ** significant at10% level of significance; AE, acetone extract, ChE, chloroform extract; ME, methanol extract.

present in the acetone and chloroform extracts inhibited the mutagenicity is not known.

Antioxidant and antimutagenic activity were more significantly correlated in all the three extracts of the fruit in co-incubation when compared with the pre-incubation mode of the experiment; both the activities were significantly correlated in acetone and chloroform extracts but not in the methanol extract of the fruit as shown in Tables 3 and 4.

The work further reveals that, *A. mexicana* could be an interesting source of antioxidants and antimutagenic agents and may be used in different fields, like pharmaceutical, food and cosmetics. Further studies are underway to confirm these results with other antioxidants and antimutagenic assays and to isolate and characterize the bioactive compounds responsible for the antioxidant and antimutagenic activities in these extracts.

ACKNOWLEDGEMENTS

We are grateful to Dr.(Mrs) Saroj Arora, Department of Botanical and Environment Sciences, Guru Nanak Dev University, Amritsar, for the kind help and providing the

TA100 strain of *S. typhimurium*, which she got from Professor B.N. Ames, of the Department of Biochemistry, University of California, Berkely, U.S.A.

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