



# Mutagenic Potential of Ethanolic Extract of *Moringa oleifera* leaves by AMES *Salmonella* / Microsome Mutagenicity Assay

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## ABSTRACT

**Introduction:** *Moringa oleifera* is a miracle tree with a wide range of phytochemicals leading to its potential therapeutic effectiveness. Its use in allopathic medicine is increasing because of rising resistance against traditional drugs. Different phytochemicals can alter genetic material leading to increased chances of cancer and *Moringa oleifera* needs investigation in this regard. For testing mutagenic potential, AMES salmonella assay is used as a primary step. **Aims & Objectives:** To evaluate the mutagenic potential of ethanolic extract of *Moringa oleifera* leaves (EEMOL) by AMES *Salmonella* assay. **Place and duration of study:** This study was performed at Shaikh Zayed Postgraduate Medical Institute (SZPGMI), Lahore and Quality Operational Lab (QOL), University of Veterinary and Animal Sciences (UVAS), Lahore for a period of 3 months.

**Material & Methods:** Ethanolic extract of local *Moringa oleifera* leaves (EEMOL) was prepared. Fifteen different dilutions of this extract were used for standard plate incorporation and preincubation assay. These dilutions were tested to revert histidine dependent strains of *Salmonella* (TA-98 & TA-100) to histidine independent strains by mutations in the gene encoding for histidine synthesis. The mutagenic potential of these dilutions was assessed by calculating the mutagenic index from the number of reverted colonies at respective dilutions. Any dilution with mutagenic index equal to or above 2 was considered mutagenic by AMES salmonella/ mutagenicity assay. **Results:** In plate incorporation assay mean mutagenic indices were  $0.35 \pm 0.12$  (TA-100) and  $0.39 \pm 0.17$  (TA-98) while those in preincubation assay were  $0.33 \pm 0.13$  (TA-100) and  $0.39 \pm 0.17$  (TA-98). Maximum mutagenic index values in plate incorporation assay were 0.552 (TA-100) and 0.674 at 100ug/ml. In preincubation assay these maximum values came out to be 0.557(TA-100) and 0.674 (TA-98) at 100ug/ml. **Conclusion:** All the mutagenic index values for tested dilutions of ethanolic extract of *Moringa oleifera* leaves were below 2 which is indicative that these dilutions lack mutagenic potential by AMES *Salmonella*/mutagenicity assay.

**Key words:** EEMOL, Preincubation assay, Plate incorporation assay, Mutagenic index.

## INTRODUCTION

*Moringa oleifera* is a plant with extensive nutritive and medicinal value. It belongs to family *Moringaceae* and is abundantly present in Pakistan and India with the local name of Sohanjna. It is believed to be a miracle tree due to its significant hepatoprotective, anti-inflammatory, anti-

hyperlipidemic<sup>1</sup>, antioxidant<sup>2</sup>, anticonvulsant<sup>3</sup>, antidiabetic<sup>4</sup>, antibacterial<sup>5</sup> and antiviral properties<sup>6</sup>. Genotoxic phytochemicals result in changing DNA character which is termed as mutation. These mutations in the genetic makeup can lead to cancer and infertility<sup>7</sup>. Gene mutation is the one which can be easily detected in bacteria and other cells by changing the growth requirements of the cells. AMES *Salmonella* assay utilizes this principle in the screening of mutagenicity of different chemicals<sup>8</sup>. In

this assay two histidine dependent mutant strains of *Salmonella* TA-98 and TA-100 are used to check mutations of chemicals which make these bacteria histidine independent by mutation. These two strains are preferred because of their low rates of colony formation which makes it easier to count colonies. These strains are sufficient for mutagenic screening because they are sensitive to the frame shift (TA-98) and base pair (TA-100) mutations<sup>9</sup>.

This research was intended to find out the mutagenic potential of ethanolic extract of *Moringa oleifera* leaves using AMES *Salmonella* assay. By this we can find out the safety of the extract so that its chronic use as a therapeutic agent can be done.

## MATERIAL AND METHODS

This study was conducted at SZPGMI, Lahore and QOL, UVAS, Lahore, Pakistan.

### Ethanolic extraction of *Moringa oleifera* leaves:

Fresh leaves of *Moringa oleifera* were collected from district Lahore (Pakistan) and were identified from herbarium of The University of Punjab Lahore, Pakistan. These leaves were washed, air dried and ground to obtain powder. 100 grams of this powder was then extracted with 500ml of 95% ethanol using Soxhlet apparatus (CG-1368) followed by its filtration with autoclaved Whatmann's filter paper. This extract was dried to a semisolid paste in rotary evaporator (Stuart RE-300) and then percentage yield was calculated.



**Fig-1:** Ethanolic extract of *Moringa oleifera* leaves

### Preparation of stock solution and dilutions:

Stock solution of ethanolic extract of *Moringa oleifera* was made by dissolving 8 mg of the extract

in 10ml of distilled water to obtain strength of 800µg per ml. It was then followed by serial two fold dilutions to get solutions having concentrations of 400 µg/ml, 200 µg/ml, 100 µg/ml, 50 µg/ml, 25 µg/ml, 12.5 µg/ml, 6.25 µg/ml, 3.12 µg/ml, 1.56 µg/ml, 0.78 µg/ml, 0.39 µg/ml, 0.19 µg/ml, 0.09 µg/ml, 0.04 µg/ml and 0.02 µg/ml.

### AMES *Salmonella*/ microsome mutagenicity assay:

Standard plate incorporation and preincubation assay were used for assessment of mutagenic potential of ethanolic extract of *Moringa oleifera* as described by Kristien Mortelmans<sup>9</sup>. All the preformed dilutions of the extract were tested against two mutant strains of *Salmonella typhimurium* (TA-98 & TA-100) procured from EBPI Canada in lyophilized form.

Minimal glucose agar was used as bottom agar in the Petri dishes. It was prepared by adding agar, distilled water, *Vogel Bonner* salt solution and glucose solution (10% v/v)

### Standard plate incorporation assay:

Top agar for standard plate incorporation and preincubation assays was prepared by mixing 2ml of molten agar with 0.50ml of rat metabolic (S9) activation mix (EBPI, Canada), 0.05ml of test extract dilution and 0.05-0.10ml overnight culture of *Salmonella* strain (about 1–2 X 10<sup>8</sup> bacteria per tube).

Petri dishes with bottom agar (Glucose minimal agar) were labeled followed by their layering with top agar. These petri dishes were placed in an incubator at 37°C for 48 hours followed by counting of number of histidine revertant colonies. Mutagenic index was calculated by comparison of these revertant colonies with that of negative control.

In positive control group 0.05 ml of Sodium azide was used whereas in negative control group 0.05ml of distilled water was used.

### Preincubation assay:

In preincubation assay top agar was incubated at 37°C for 20 minutes before layering on the bottom agar to allow formation of active metabolites of the extract. After incubation this top agar was layered over the bottom agar and incubated at 37°C for 48 hours. Number of revertant colonies were counted and compared to negative control for calculation of mutagenic index.

### Statistical analysis:

The data was analyzed by statistical package for social sciences (SPSS IBM statistics 20). For comparing mutagenic potential in terms of mutagenic index, Kruskall Walis ANOVA was used and Mann Whitney U test was applied for post hoc analysis where required. P value  $\leq 0.05$  was considered statistically significant and value between 0.05 and 0.10 was considered indicative.

## RESULTS

### Percentage yield of the extract:

12.4g of extract was obtained from 100g of *Moringa oleifera* leaves powder which shows that the percentage yield was 12.4%.

### Mutagenic index calculation:

Mutagenic index was calculated by using the following formula

Mutagenic index (M.I.) = Number of histidine revertant colonies with test extract / Number of natural histidine revertant colonies with negative control

Ethanolic extract of <i>Moringa oleifera</i>					
Sr. No	Conc. ug per plate	Mutagenic Index Values			
		TA- 100+S9	TA- 100-S9	TA- 98+S9	TA- 98-S9
1	0.02	0.152	0.194	0.176	0.116
2	0.04	0.177	0.254	0.137	0.209
3	0.09	0.240	0.164	0.255	0.186
4	0.19	0.127	0.254	0.314	0.256
5	0.39	0.266	0.238	0.235	0.349
6	0.78	0.329	0.433	0.392	0.279
7	1.56	0.278	0.298	0.451	0.419
8	3.125	0.354	0.343	0.412	0.349
9	6.25	0.380	0.403	0.569	0.488
10	12.5	0.418	0.448	0.608	0.581
11	25	0.468	0.463	0.647	0.651
12	50	0.494	0.492	0.588	0.512
13	100	0.557	0.552	0.686	0.674
14	200	0.392	0.433	0.510	0.395
15	400	0.342	0.328	0.372	0.326
+Control	5	13.089	12.582	8.784	4.698

**Table-1:** Mutagenic index of Ethanolic extract of *Moringa oleifera* with TA-100 and TA-98 by plate incorporation and preincubation assay

### Plate incorporation method:

**TA-100:** Mean mutagenic index measured was  $0.35 \pm 0.12$ . Minimum mutagenic index was 0.16 at

0.09ug/ml while maximum mutagenic index was 0.55 at 100ug/ml.

**TA-98:** Mean mutagenic index measured was  $0.39 \pm 0.17$ . Minimum mutagenic index was 0.12 at 0.02ug/ml while maximum mutagenic index was 0.67 at 100ug/ml.

### Preincubation assay:

**TA-100:** Mean mutagenic index measured was  $0.33 \pm 0.13$ . Minimum mutagenic index was 0.13 at 0.19ug/ml while maximum mutagenic index was 0.56 at 100ug/ml.

**TA-98:** Mean mutagenic index measured was  $0.39 \pm 0.17$ . Minimum mutagenic index was 0.14 at 0.04ug/ml while maximum mutagenic index was 0.69 at 100ug/ml.

## DISCUSSION

Botanicals have extensively been used in traditional medicine because of their therapeutic applications. Long term nutritional or therapeutic use of these plants was considered safe in the past but according to recent scientific data their possible carcinogenicity, mutagenicity and toxicity have been established. Tannins are among one of the chemical constituents of these plants which are the reason for their antioxidant activity and they are now considered to modify the expression of certain genes and signaling pathways of different cells<sup>10</sup>.

*Moringa oleifera* locally known as Sohanjana is among the native herbal plants of Pakistan with extensive therapeutic uses. Its seeds and leaves have traditionally been utilized for the treatment of skin disorders, convulsions, Diabetes Mellitus, cardiovascular pathologies and influenza<sup>11</sup>.

AMES *Salmonella* assay is one of the preliminary tests used for identification of frame shift and base pair mutations induced by different extracts and chemicals<sup>8</sup>. If any of the concentrations of the tested chemicals or extracts gives positive result in AMES test either in the presence or absence of S9 metabolic extract, it is enough to label that chemical or extract as mutagenic. This assay utilizes two histidine dependent mutant strains of *Salmonella* TA-98 and TA-100 which are reverted from histidine dependence to histidine independence after frame shift or base pair mutations respectively<sup>9</sup>. Results of the assay are given in the form of mutagenic index values and any chemical or extract with these values equal to or greater than 2 are considered mutagenic<sup>12</sup>.

Mutagenic potential of different plant extracts has already been established based on their AMES assay results. In a study frame shift mutation was observed by metabolites of aqueous extracts of *Mimosa bimucromata*, *Sambucus australis* and *Bauhnea forficata* while base pair mutation was also observed with aqueous extract and metabolites of *M. bimucromata*<sup>13</sup>. In another study aqueous extracts of root, leaf and stem of *L. globuliferum* were tested for mutagenicity by AMES test and all the concentrations of leaf and stem extracts came out to be mutagenic while only two root concentrations showed positive results<sup>14</sup>. Similarly aqueous extracts of *T. turcica* flowers, root and stem were tested for mutagenicity by AMES test and it was observed that all these extracts showed frame shift mutation in the presence of S9 metabolic activation complex. In addition base pair mutation was also observed with leaf extract of *T. turcica* in the presence and absence of S9 metabolic activation complex<sup>15</sup>.

In present study it was observed that none of the concentrations shown mutagenic index above 2 for either TA-98 or TA100 which is suggestive that ethanolic extract of *Moringa oleifera* leaves lacks mutagenic potential due to absence of any ingredient leading to frame shift or base pair mutation. Metabolites of the extract also lack any mutagenic potential as the mutagenic index value for all tested concentrations in the presence of S9 metabolic activation complex also came below 2.

These results of the study are in accordance with a research in which aqueous extract of *Moringa oleifera* seeds was reported non mutagenic by AMES test<sup>16</sup>. As this extract has no mutagenic potential it can be used safely for different therapeutic indications.

Although non mutagenic potential and safety of the extract is established by AMES test but this assay tests only certain type of mutations so there is need for further evaluation of the extract using COMET assay and other novel techniques.

## CONCLUSION

Ethanolic extract of *Moringa oleifera* leaves is safe for use as it does not induce any point or frame shift mutations in histidine dependent *Salmonella Typhimurium* when used in AMES assay.

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