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Evaluation of Antimutagenic Activity of Neem Leaves Extract Against Sodium Arsenate Mutagenicity in Fresh Water Fish *Clarias lazera*

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ABSTRACT

Arsenic is a toxic metalloid commonly occurring in the natural ecosystem. Arsenic genotoxicity both *in vivo* and *in vitro* has been investigated. So, the identification of compounds possessing antimutagenic and anticarcinogenic properties against arsenic genotoxicity is of great importance. Neem *(Azadirachta indica)* is one of the most versatile multipurpose plant species. It has protective effects against the genotoxicity induced by numerous chemicals. The present study focused on the possible antimutagenic potential of ethanolic extract of Neem leaves against the clastogenicity induced by sodium arsenate in freshwater fish *Clarias lazera*. Chromosomal aberrations (CAs), micronucleus (MN) frequencies and DNA fragmentation were genotoxic end points that were used in this investigation. In the present work, fish were exposed to sub lethal conc. of sodium arsenate (1 mg/L), Neem extract (3 ppm) and sodium arsenate plus Neem extract along with negative control at duration 96 hours. Results showed that sodium arsenate when used alone induced a significant increase in the frequency of CAs, MN and DNA fragmentation percentage. Neem extract did not show genotoxic potential. In combined treatment, Neem extract significantly reduced the frequency of CAs, MN and DNA fragmentation induced by sodium arsenate with reduction percent of 71.5 %, 86.29 % and 91.66 %, respectively. Overall, we can conclude that sodium arsenate is a potent genotoxic agent for fish and the Neem extract effectively reduced the genotoxicity induced by sodium arsenate.

Key Words: *Clarias lazera*, fish, sodium arsenate, neem extract, genotoxicity, chromosome aberration, micronucleated erythrocytes, DNA fragmentation.

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INTRODUCTION

The assessment of biological effects on aquatic vertebrate species is frequently employed to monitor water pollution because it provides meaning for information on bioavaibility and effective concentration levels. Numerous toxic and genotoxic contaminants such as trace metals, pesticides, dyes and pharmaceuticals can be identified as the components of complex aquatic environmental mixtures *(Ohe et al., 2004)*. So, fish living in different water bodies carry the risk of direct or indirect exposure to various toxicants, industrial and agricultural chemicals. Arsenic is a toxic metalloid commonly occurring in the natural ecosystem. It is found in biological systems as inorganic oxy forms (oxides, arsenites and arsenates) and as organic arsenic compounds. Inorganic arsenic is used in wood preservative treatments and prior to this decade was widely used as a pesticide. As a result of agricultural and industrial activities large quantities of arsenicals are realized into environment that may be hazard to fish and wild life *(Eisler 1988)*. Arsenic is carcinogen to which numerous human groups around the world are exposed *(Del Razo et al. 1990)*. The most common route of exposure for the general public is food and drinking water. It is absorbed by the lung, gastrointestinal system and skin; it crosses to the placental barrier and concentrates to a significant extent in the fetus *(Galvao and Corey 1987)*. Moreover, arsenic exposure causes chromosomal abnormalities, with a preponderance of end-to-end fusions.

These chromosomal end fusions suggested that telomerase activity may be inhibited by arsenic *(Chou, et al. 2001)*. The putative genotoxic effects of arsenic both *in vivo* and *in vitro* have been investigated. Higher incidences of micronuclei, chromosomal aberrations, sister chromatid exchanges and aneuploidy have been reported from the human populations exposed to arsenic through drinking water in various countries such as Mexico *(Ostrosky-Wegman et al. 1991 and Gonsebatt et al. 1997)*, Finland *(Maki-Paakkanen et al. 1998)*, Argentina *(Dulout et al. 1996 and Lerda 1994)* and Taiwan *(Liou et al. 1999)*. Also, Arsenic may function as a tumor promoter in carcinogenesis in humans *(Mo et al. 2009)*.

The exact mechanism of arsenic-induced carcinogenicity still remains elusive; however, short-term assays indicate that arsenic does not induce point mutations but rather acts as a clastogen, inducing the formation of chromosomal aberrations and micronuclei in animal and human systems *(Basu et al. 2001). Ramirez and Garcia (2005)* demonstrated the significant genotoxic effect of arsenic bioaccumulation in zebra fish gill cells. Public health concerns have centered on carcinogenic, cardiovascular and nervous system effects seen in populations exposed to arsenic in drinking water *(SAB DART ID Committee Draft 1996).* Therefore, the discovery and exploration of compounds possessing antimutagenic and anticarcinogenic properties are of great importance. Many indigenous substances posses some inhibitory activity towards environmental and man made genotoxic agents *(Grover and Bala 1992 and Brokman et al. 1992).* Neem (*Azadirachta indica* A. Juss), is an indigenous plant commonly grown in India and its subcontinent. It is one of the most versatile multipurpose plant species well known for its insecticidal and various types of biomedical properties *(Govindachari 1992 and ICAR 1993).*

Almost every part of Neem tree has been known to posses a wide range of pharmacological properties *(Biswas et al. 2002).* It is traditionally used to treat large number of diseases including malignancies *(Van Der Nat et al. 1991).* The chemo preventive potential of dietary Neem flowers has been demonstrated in inhibiting Aflatoxin B1 and 9, 10 dimethyl-1, 2-benzanthracene (DMBA) induced liver and mammary gland carcinogenesis in rats *(Tepsuwan et al. 2002).* Also, previous studies confirmed the protective effects of ethanolic and aqueous Neem leaf extract against N-methyl-N-nitro-N-nitrosoguanidine (MNNG) induced oxidative stress, gastric carcinogenesis, clastogenicity and genotoxicity in rats and mice *(Subapriya et al. 2003 and 2004, Subapriya and Nagini 2003 and Arivazhagan et al. 2003).* Recently, the ameliorating effect of vitamin C, β-carotene and azadirachtin (principle compound of Neem) against genotoxicity of ethyl methanesulfonate (EMS) and cadmium chloride has been demonstrate in a fish, *Oreochromis mossambicus (Guha and Khuda - Bukhsh, 2002 and 2003 and Chandra and Khuda-Bukhsh,2004).*

More recently, the strong effect of ethanolic Neem leaf extract against pentachlorophenol (PCP) and 2,4-dichlorophenoxyacetic acid (2,4-D) in fresh water fish *Chana punctatus* has been reported *(Farah et al., 2006).* On the contrary, some reports about the genotoxicity of crude extract of Neem have been appeared in terms of chromosome breakages in bone marrow and germ cells of male mice *(Awasthy et al. 1999, Awasthy 2001 and Salchzadeh et al. 2003).* However, *Abou-Tarboush et al. (2005)* confirm the lack of genotoxic potential of azadirachtin of Neemix-45 in the bone marrow cells of male and female mice. The aim of the present study is to evaluate the possible antimutagenic potential of Neem leaves ethanolic extract against the genotoxicity of sodium arsenate in fresh water fish *Clarias lazera* by using the chromosome aberrations, micronucleus formation and DNA fragmentation as genotoxic end points.

MATERIALS AND METHODS

Specimen:

A total number of sixty four clinically normal mature catfish *Clarias lazera* with an average weight of 150 – 200 g were obtained from a local market. The fish were transferred from the market to the lab in tanks then kept in a well aerated full glass aquaria supplied with dechlorinated tap water and left for 10 days at a temperature 25±1°C to acclimate our lab conditions prior to be used. The fish were supplied with commercial pellets while there faeces and uneaten food were aspirated regularly. Two days pre and during the period of experiment the fish were fasted to prevent the undesirable effect of excreta on the chemical composition of water. The fish were divided into 4 groups of 16 fish each. Each group were put in well aerated glass aquaria measured 1×0.5×0.5 m and supplied with dechlorinated tap water at a temperature 25±2°C.

Chemicals:

Sodium arsenate (inorganic form of arsenic) was purchased from Sigma Company, USA.

Preparation of Ethanolic Extract of Neem Leaves:

Fresh Neem leaves were obtained from the Ministry of Agriculture, Egypt and were cleaned by washing in tap water and air dried. Cleaned leaves made semi powder by crushing and then the ethanolic extract of Neem leaves was prepared according to the procedure described by *Chattopadhyay (1998).*

Experimental Design:

Treatments were given through aqueous medium, exposure period was 96 h. The first group was used as negative control. The second group was exposed to a concentration of Neem leaves extract (3 ppm). The third group was exposed to a sub lethal dose of sodium arsenate (1mg/L) *(Jana and Sahana, 1989)* and the fourth group was exposed to the same concentration of Neem extract (3 ppm) together with the same sub lethal conc. of sodium arsenate (1mg/L). After termination of the exposure period 8 fishes were utilized for chromosome preparation and 8 fishes for micronucleus preparation and DNA fragmentation estimation.

Chromosome Aberrations:

Samples for studying the chromosomal aberrations were taken from the anterior kidney of fishes after the exposure period. The fish were injected with yeast suspension at dose 1 ml /100 gm b.w. *(Lee and Elder, 1980).* Twenty four hours after the first injection, specimens were injected intra muscular with 0.01 ml of 0.03 mg/g b.w. freshly prepared colchicine and squash technique for kidney tissue was used for the preparation of chromosomes *(Al-Sabti et al. 1984).* For every fish, at least 50 metaphase spreads were examined and the chromosomal aberrations were detected.

Micronucleus Preparation:

A drop of blood from the caudal vein was mixed with a drop of fetal calf serum, then directly smeared on slide and air dried. After fixation in absolute methanol for 5 min., they were stained with 5% Giemsa for 7 min. Two thousand erythrocyte per fish were analyzed to determine the frequency of micronucleated cells *(De Flora et al. 1993).*

DNA fragmentation test:

DNA fragmentation was quantified by diphenylamine (DPA) method according to *Gibb et al. (1997)* with little modifications. Samples for studying DNA fragmentation were taken from the liver of fishes. The amounts of both fragmented and intact DNA were determined by spectrophotometer at 600 nm. The fragmentation of DNA was calculated according to the equation:

$$\text{DNA fragmentation} = \frac{\text{O.D. of fragmented DNA}}{\text{O.D. of fragmented DNA} + \text{O.D. of intact DNA}} \times 100$$

Data evaluation and statistical analysis:

The reduction percentage in number of chromosome aberration or micronuclei in the group treated with the Neem leaves extract and sodium arsenate was calculated according to *Manoharan* and *Banerjee (1985)* and *Waters et al. (1990)* by the following formula:

$$\text{Reduction \%} = \frac{\text{Frequency of CA or MN in A - Frequency of CA or MN in B}}{\text{Frequency of CA or MN in A - Frequency of CA or MN in C}} \times 100$$

Where, A = Sodium arsenate
B = Neem extract mixed with sodium arsenate
C = Control

The data obtained from all the experimental groups were statistically analyzed using one way analysis of variance (ANOVA) followed by Tukey HSD post Hoc for multiple comparisons between pairs. Results are reported as mean values ± S.E. and differences were considered as significant when $P \leq 0.05$.

RESULTS

Chromosome Aberrations Assays:

The typical metaphase complements of *Clarias lazera* fish were found to consist of 56 chromosomes of different types as (submetacentric, metacentric, subtelocentric and telocentric). Table 1 and histogram 1 showed the mean ± standard errors of individuals and total chromosomal aberrations induced in all experimental groups. Various forms of chromosome abnormalities recorded were chromatid gaps, deletions, breaks, fragments, end to end associations and dicentics, whereas chromatid gaps were excluded (Figures 1 and 2). The results showed that the mean value of chromosomal aberrations induced by Neem leave extract was recorded as 4.57± 0.50 which was found to be statistically not significant when compared with the negative control value (3.12± 0.35). However, the mean value of chromosomal aberrations induced by sodium arsenate reached to 22.33 ±2.12. On the other hand, Neem extract significantly ($P \leq 0.05$) reduced the frequency of chromosomal aberrations induced by sodium arsenate, The reduction percent was was calculated to be 71.5 %.

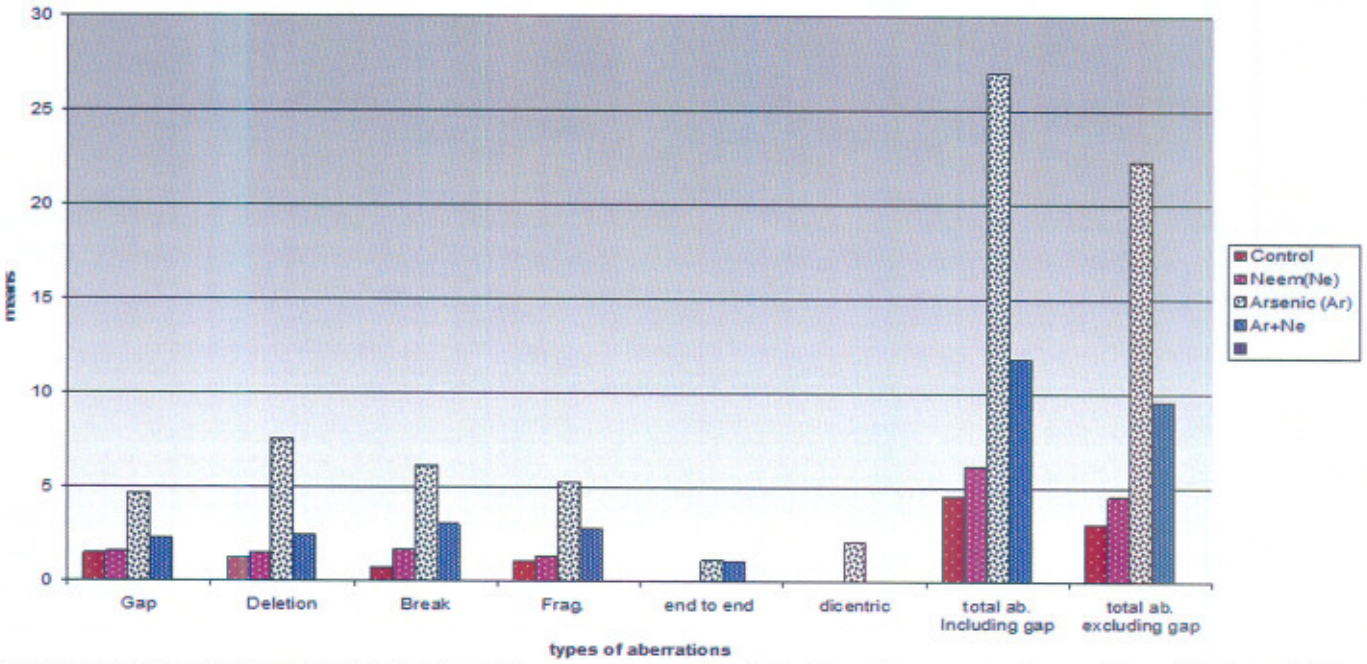
Micronucleus Assay:

The erythrocytes of *Clarias lazera* were generally observed as round with a centrally located round nucleus and a considerable amount of cytoplasm. The size and position of micronuclei in the cytoplasm showed slight variation and normally one micronucleus per cell was observed. The frequencies of micronuclei recorded in all groups are summarized in Table 2 and Histogram 2. The results showed that Neem extract induced non significant increase in the mean value of micronuclei in erythrocytes of *Clarias lazera.* Sodium arsenate induced a statistically significant increase ($P \leq 0.01$) in the frequency of micronuclei (M±SE) as compared with control value (M±SE). On the other hand, Neem leaf extract significantly ($P \leq 0.05$) reduced the frequency of micronuclei induced by sodium arsenate alone and the reduction in the micronuclei incidence was calculated to be 86.29%.

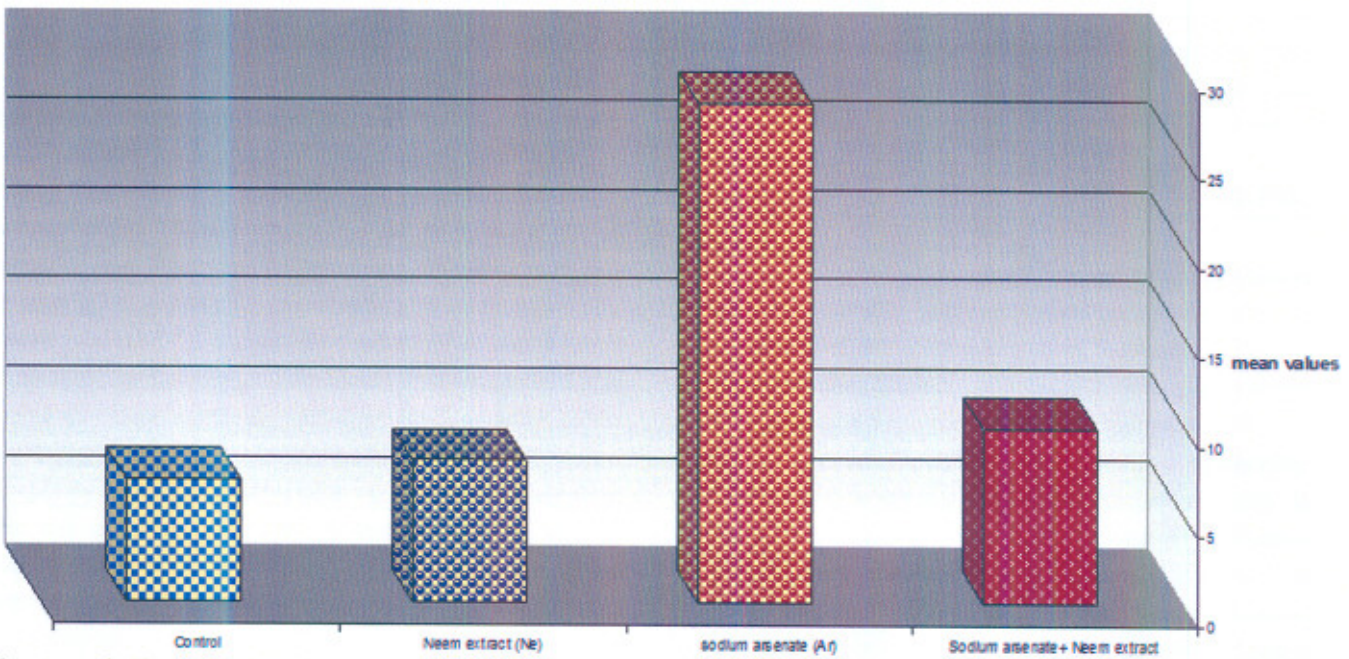
Table 1: The mean values of chromosomal aberrations induced in fish kidney cells of all experimental groups.

Treatments	Structural chromosomal aberrations						Total chromosomal aberrations	
	Gap	Deletion	Break	Fragment	End to End association	Dicentric	Including gap	Excluding gap
Control	1.5±0.32[a]	1.25±0.3[a]	0.75±0.2[a]	1.12±0.99[a]	0.0±0.0[a]	0.0±0.0[a]	4.62±0.49[a]	3.12±0.35[a]
Neem extract (Ne)	1.6±0.41[a]	1. 5±0.3[a]	1.7±0.36[ab]	1.37±0.9[a]	0.0±0.0[a]	0.0±0.0[a]	6.17±0.36[a]	4.57±0.50[a]
Arsenic (Ar)	4.7±0.52[b]	7.57±1.0[b]	6.2±0.91[c]	5.28±2.3[c]	1.14±0.3[b]	2.14±0.4[b]	27.03±2.3[c]	22.33±2.12[c]
(Ar) + (Ne)	2.3±0.37[a]	2. 5±0.3 [a]	3.1±0.47[b]	2.87±1.12[b]	1.12±0.12[a]	0.0±0.0[a]	11.89±0.53[b]	9.59±0.65[b]

b and c: significant and highly significant difference from the negative control a at $P<0.05$ and $P<0.01$, respectively.



Histogram 1: Showed the mean values of different chromosomal aberrations induced by sodium arsenate alone and in combination with Neem extract in kidney cells of fresh water fish *Clarias lazera*



Histogram 2: Showed the mean values of micronuclei induced by sodium arsenate alone and in combination with Neem extract in the erythrocytes of fresh water fish *Clarias lazera*

Table 2: The frequencies of micronuclei induced in fish erythrocytes of all groups.

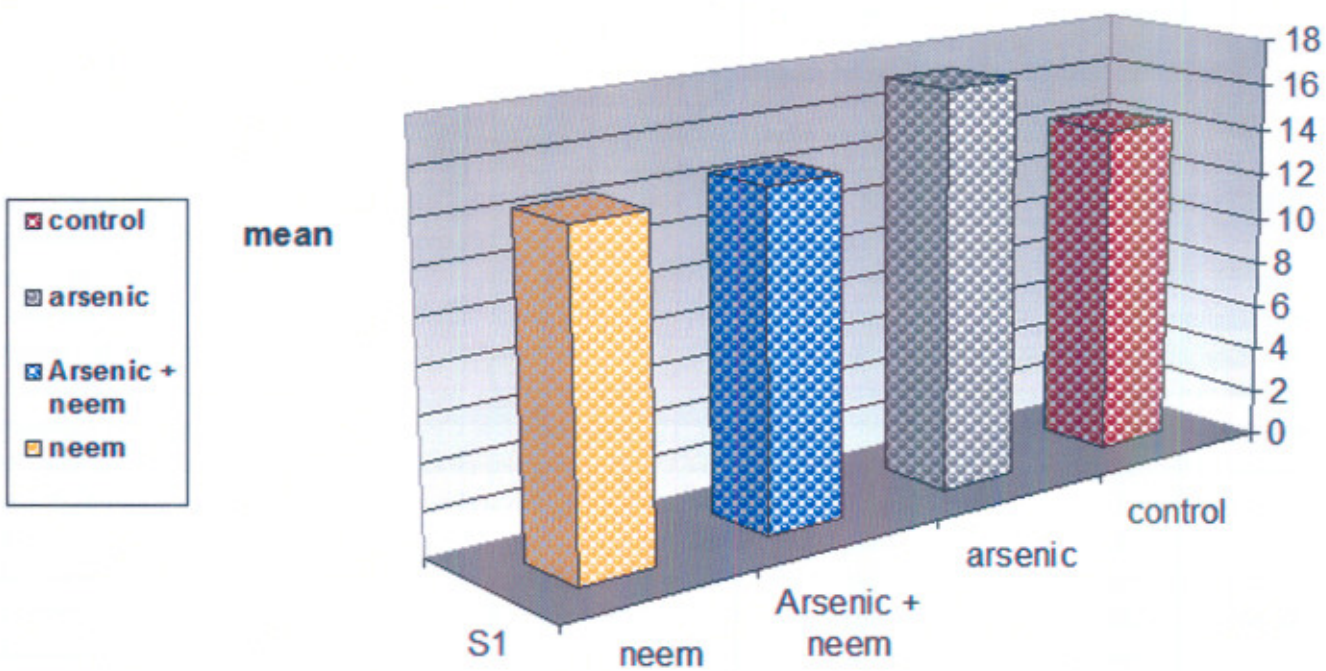
Treatments	Total Counted PCEs/ fish	Mean ± S.E.
Control	2 000	6.8 ± 0.66 [a]
Neem leaf extract	2 000	7.87 ± 0.63 [a]
Sodium arsenate	2 000	27.9 ± 2.2 [b]
Sodium arsenate + Neem leaf extract	2 000	9.75 ± 0.55 [a]

b: significant significance the negative control a at P<0.05.

Table 3: DNA fragmentation in fish tissues of all experimental groups.

Groups	DNA Fragmentation	
	Rang	M+S.E.
Control	12.4 - 17.62	14. 3 ± 0. 77 [a]
Neem leaf extract	12.45-17.65	14.5 ±0.79 [a]
Sodium arsenate	14.8-18.8	17.3+0.57 [b]
Sodium arsenate + Neem extract	12.01-17.79	14.55+0.92 [a]

b: significant significance the negative control a at P<0.05.



Histogram 3: Showed the mean values of DNA fragmentation induced bySodium arsenate alone and in combination with Neem extract in liver cells of fresh water fish *Clarias lazera*

DNA Fragmentation Assay:

The means of the percent of DNA fragmentation recorded in all groups are summarized in table 3 and represented in histogram 3. The results showed that there was non significant difference between the percent of DNA fragmentation of Neem leaf extract treated group and that of the control group. DNA fragmentation was significantly (P ≤0.05) elevated in the sodium arsenate treated group (M+SE) when compared with the negative group (M+SE). The tested dose of Neem leaf extract significantly (P≤ 0.05) reduced the percent of DNA fragmentation induced by sodium arsenate alone and the reduction in the DNA fragmentation was calculated to be 91.66 %.

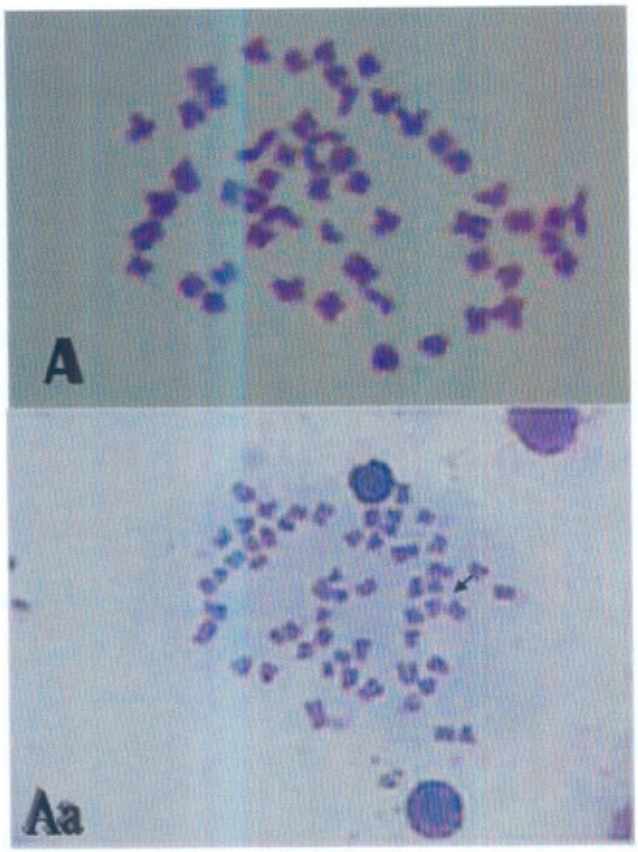


Figure 1: A . metaphase spread of normal cell from Clarias lazera fish.
Aa . metaphase spread showing end to end associations (arrow).

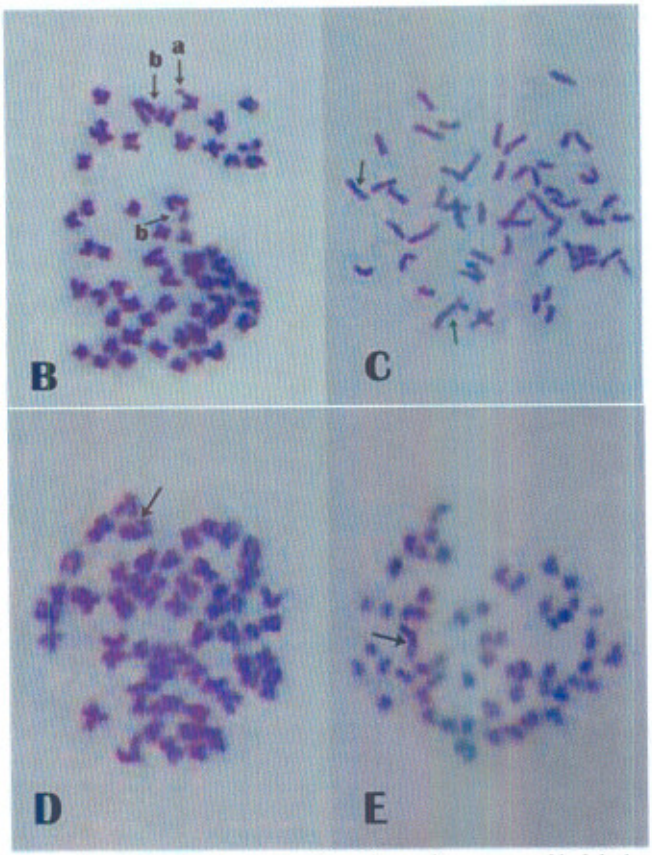


Figure 2: B . metaphase spread showing a fragment and b deletion (arrow)
C. metaphase spread showing dicentric chromosome(arrow).
D. metaphase spread showing break(arrow).
E .metaphase spread showing gap (arrow).

DISCUSSION

The aim of this work was to evaluate the antimutagenic activities of ethanolic extract of Neem leaves against the mutagenicity of sodium arsenate in *Clarias lazera* fish using chromosomal aberrations, micronucleus and DNA fragmentation tests. Results of the present study showed that sodium arsenate significantly increased the mean values of total chromosomal aberrations, number of micronucleated polychromatic erythrocytes and percentage of DNA fragmentation in fish cells after 96 h. of treatment. These results were in agreement with several previous literatures. *Patlolla and Tchounwou (2005)* found that arsenic trioxide exposure significantly increased the number of structural chromosomal aberrations; the frequency of micronucleated cells and decreased the mitotic index in treated Sprague-Dawley rats. Furthermore, *Ramirez and Garcia (2005)* indicated that arsenic induced a significant increase in micronuclei frequency in gill cells of zebra fish *(Danio rerio)*. The types of recorded chromosomal aberrations in the present study such as end to end associations and dicentric chromosome were similar to those recorded by *Chou et al. (2001)* in cell line culture. Also, *Bernstam and Nriagu (2000)* reported that arsenic induced sister chromatid exchanges and clastogenic effects in a wide range of *in vitro* systems in human and animal derived cells and also changed spindle morphology of cultured human keratinocytes. Kligerman et al. *(2003)* indicated that arsenate was able to induce genotoxic effects in human lymphocytes analysed by means of CAs, SCE, aberrant cells, replicative index and mitotic index. *Crossen (1983)* reported that the clastogenic effect of arsenic appears to be due to interference with DNA synthesis. Arsenic has also been shown to block dividing cells in the S and G2 phases *(Petres et al. 1977)*. Arsenic genotoxicity has been analysed extensively in a wide range of *in vivo* (*Basu et al. 2004* and *Martinez et al. 2005)* and *in vitro (Dopp et al. 2004)* studies and the overall conclusion is that there is a clear induction of genotoxic effects, including an increase in micronucleus (MN) frequency and a decrease in the proliferation index that reflects its toxic potential. Azadirachtin, one of the active compound present in the extract of the Neem, did not induced any significant changes in mitotic indices or in chromosomal aberrations in the bone marrow cells of treated male and female mice *(Abou-Tarboush et al. 2005)*. Also, *Stewart (1998)* reported that NeemAzal Technical and NeemAzal did not show any mutagenic activity in rats using *in vitro* and *in vivo* techniques. In the same time, *Chandra and Khuda-Bukhsh*

(2004) showed the ameliorating potential of azadirachtin against the CdCl2 induced clastogenic changes in fish *O. mossambicus* by various endpoints. These previous results supported the results of the present study. The present study clearly showed that the Neem extract has an antimutagenic and anticlastogenic potential in the fish tissue against the action of sodium arsenate that measured as chromosomal aberrations, micronuclei and DNA fragmentation. The results also were in a good agreement with those of *Farah, et al. (2006)*, who reported that the treatment with ethanolic Neem leaf extract significantly reduced the frequency of micronuclei and chromosome aberrations induced by pentachlorophenol (PCP) and 2,4-dichlorophenoxyacetic acid (2,4-D) in fresh water fish, *Chana punctatus*. Furthermore, ethanolic Neem leaf extract also reduced MNNG induced macronuclei and lipid peroxides and enhanced GSH- dependent antioxidant activity in mice *(Subapriya et al. 2004)*. Also, aqueous extract of Neem leaf significantly reduced the genotoxicity of N-methyl-N'-nitro-N-nitrosoguanidine in bone marrow of rat *(Arivazhagan et al. 2003)*. In the present study, the fish were simultaneously treated with Neem extract and sodium arsenate in the aqueous medium, that may be simply preventing the uptake of sodium arsenate from the water and reducing their subsequent toxicity. It has been reported previously that some drugs, dietary components and endogenous biochemical can function as antimutagen by altering the rates of mutagen absorption and uptake *(Waters et al. 1990)*. However, the exact mechanism by which Neem leaf extract reduced the genotoxicity of sodium arsenate is not understood. The protective effect of Neem extract could be ascribed as it is known that Neem leaf contains a number of potent antioxidants and anticarcinogens including carotenes, terpenoids, limonoids, quercetin, sterols and flavonoids glycosides that might play a role in inhibiting the genotoxic effect of sodium arsenate *(Subramanian and Nair 1972, Govindachari 1992 and Schaaf et al. 2000)*. The chemopreventive potential of Neem leaf extract, including inhibition of cytochrome p450 depending activity, may be related to one or more of the phytochemical constituent that are potentially anticarcinogenic *(Van Der Nat et al. 1991, Beecher 1995 and Siess et al. 1995)*. Therefore, based on the results of this study it can be concluded that simultaneous treatment with Neem leaf ethanolic extract under certain circumstances exert significant antimutagenicity against sodium arsenate mutagenicity in fish. The obtained data indicated that natural products such as Neem extracts may yield a wealth of commercially viable antimutagenic agents. The precise characterization of the antimutagenic activity of Neem extracts and exactly identifing the active compounds and their modes of action are recommended. Further studies on a variety of vertebrate models are needed for determining the extent to which Neem leaf extract can be regarded as antimutagen for human being.

REFERENCES

Abou-Tarboush, F. M., El-Ashmawi, H. M., Husain, H. E., et al. 2005. Clastogenic effect of azadirachtin of Neemix-4.5 on SWR/J mouse marrow cells. The Egyptian Journal of Hospital Medicine **18**:23--28.

Al-Sabti, K., Fijan, N.and Kurelec, B. 1984. Frequency of chromosomal aberrations in the rainbow trout (Salmo gairdnieri, Rich.) exposed to detergent and benzene. Veterinary Arhives **54**:83-89.

Arivazhagan, S., Nagini, S., Santhiya, S. T. and Ramesh, A. 2003. N-methyl-N'-nitro-N-nitrosoguanidine (MNNG)-induced *in vivo* clastogenicity: Protective effects of aqueous neem leaf extract. Die Pharmazie **58**(10):750--752.

Awasthy, K. S. 2001. Genotoxicity of a crude leaf extract of neem in male germ cells of mice. Cytobios **106** Suppl 2:151--164.

Awasthy, K. S., Chaurasia, O. P.and Sinha, S. P. 1999. Prolonged murine genotoxic effects of crude extracted from neem. Phytotherapy Research : PTR **13**(1):81--83.

Basu, A., Ghosh, P., Das, J. K., et al. 2004. Micronuclei as biomarkers of carcinogen exposure in populations exposed to arsenic through drinking water in West Bengal, India: A comparative study in three cell types. Cancer Epidemiology, Biomarkers and Prevention : A Publication of the American Association for Cancer Research, Cosponsored by the American Society of Preventive Oncology **13**(5):820-827.

Basu, A., Mahata, J., Gupta, S. and Giri, A. K. 2001. Genetic toxicology of a paradoxical human carcinogen, arsenic: A review. Mutation Research **488**(2):171-194.

Beecher, C. 1995. Potential chemopreventive compounds in the diet. In Chemoprevention of cancer, edited by D. W. Nixon. Boca Raton, FL, USA: CRC Press pp 21--62.

Bernstam, L. and Nriagu, J. 2000. Molecular aspects of arsenic stress. Journal of Toxicology and Environmental Health. Part B, Critical Reviews **3**(4):293--322.

Biswas, K., Chattopadhyay, I., Banerjee, R. K. and Bandyopadhyay, U. 2002. Biological activities and medicinal properties of neem *(Azadirachta indica)*. Current Science **82**(11):1336--1345.

Brockman, H. E., Stack, H. F. and Waters, M. D. 1992. Antimutagenicity profiles of some natural substances. Mutation Research **267**(2):157--172.

Chandra, P. and Khuda Bukhsh, A. R. 2004. Genotoxic effects of cadmium chloride and azadirachtin treated singly and in combination in fish. Ecotoxicology and Environmental Safety **58**(2):194--201.

Chattopadhyay, R. R. 1998. Possible biochemical mode of anti-inflammatory action of *Azadirachta indica* A. Juss. in rats. Indian Journal of Experimental Biology **36**(4):418--420.

Chou, W. C., Hawkins, A. L., Barrett, J. F., et al. 2001. Arsenic inhibition of telomerase transcription leads to genetic instability. The Journal of Clinical Investigation **108**(10):1541--1547.

Crossen, P. E. 1983. Arsenic and SCE in human lymphocytes. Mutation Research **119**(3):415--419.

De Flora, S., Vigano, L., D'Agostini, F., et al. 1993. Multiple genotoxicity biomarkers in fish exposed in situ to polluted river water. Mutation Research **319**(3):167--177.

Del Razo, L. M., Arellano, M. A. and Cebrian, M. E. 1990. The oxidation states of arsenic in well-water from a chronic arsenicism area of Northern Mexico. Environmental Pollution **64**(2):143--153.

Dopp, E., Hartmann, L. M., Florea, A. M., et al. 2004. Uptake of inorganic and organic derivatives of arsenic associated with induced cytotoxic and genotoxic effects in Chinese hamster ovary (CHO) cells. Toxicology and Applied Pharmacology **201**(2):156--165.

Dulout, F. N., Grillo, C. A., Seoane, A. I., et al. 1996. Chromosomal aberrations in peripheral blood lymphocytes from native Andean women and children from northwestern Argentina exposed to arsenic in drinking water. Mutation Research **370**(3--4):151--158.

Eisler, R. 1988 Arsenic hazards to fish, wildlife and invertebrates: A synoptic reviewBiological Report No 85U.S. Fish and Wildlife ServiceWashington, DC1-12.

Farah, M. A., Ateeq, B. and Ahmad, W. 2006. Antimutagenic effect of neem leaves extract in freshwater fish, *Channa punctatus* evaluated by cytogenetic tests. The Science of the Total Environment **364** (1--3):200--214.

Galvão, L. A. C. and Corey, G. 1987. Arsénico. Serie Vigilancia.8th ed. Organización Panamericana de la Salud y Organización Mundial de la Salud: Centro Panamericano de Ecología Humana y Salud.

Gibb, R. K., Taylor, D. D., Wan, T., et al. 1997. Apoptosis as a measure of chemosensitivity to cisplatin and taxol therapy in ovarian cancer cell lines. Gynecologic Oncology **65**(1):13--22.

Gonsebatt, M. E., Vega, L., Salazar, A. M., et al. 1997. Cytogenetic effects in human exposure to arsenic. Mutation Research **386** (3):219-228.

Govindchari, T. R. 1992. Chemical and biological investigations in Azadirachta indica. Current Science **63**:117--122.

Grover, I. S. and Bala, S. 1992. Antimutagens. In Perspectives in cytology and genetics, edited by G. K. Manna and S. C. Roy. Kalyani, India: AICCG Publication167--188.

Guha, B.and Khuda Bukhsh, A. R. 2002. Efficacy of vitamin-C *(L-ascorbic acid)* in reducing genotoxicity in fish *(Oreochromis mossambicus)* induced by ethyl methane sulphonate. Chemosphere **47**(1):49--56.

Guha, B.and Khuda Bukhsh, A. R. 2003. Ameliorating effect of beta-carotene on ethylmethane sulphonate-induced genotoxicity in the fish *Oreochromis mossambicus*. Mutation Research **542**: 1-13.

ICAR. 1993 World Neem Conference Souvenir ICARBangalore, India.

Jana, S.and Sahana, S. S. 1989. Sensitivity of the freshwater fishes *Clarias batrachus* and *Anabas testudineus* to heavy metals. Environment and Ecology **7**(2):265--270.

Kligerman, A. D., Doerr, C. L., Tennant, A. H., et al. 2003. Methylated trivalent arsenicals as candidate ultimate genotoxic forms of arsenic: Induction of chromosomal mutations but not gene mutations. Environmental and Molecular Mutagenesis **42**(3):192-205.

Lee, M. R. and Elder, F. F. 1980. Yeast stimulation of bone marrow mitosis for cytogenetic investigations. Cytogenetics and Cell Genetics **26**(1):36--40.

Lerda, D. 1994. Sister-chromatid exchange (SCE) among individuals chronically exposed to arsenic in drinking water. Mutation Research **312**(2):111--120.

Liou, S. H., Lung, J. C., Chen, Y. H., et al. 1999. Increased chromosome-type chromosome aberration frequencies as biomarkers of cancer risk in a blackfoot endemic area. Cancer Research **59**(7):1481--1484.

Maki Paakkanen, J., Kurttio, P., Paldy, A. and Pekkanen, J. 1998. Association between the clastogenic effect in peripheral lymphocytes and human exposure to arsenic through drinking water. Environmental and Molecular Mutagenesis **32**(4):301--313.

Manoharan, K. and Banerjee, M. R. 1985. beta-Carotene reduces sister chromatid exchanges induced by chemical carcinogens in mouse mammary cells in organ culture. Cell Biology International Reports **9**(9):783--789.

Martinez, V., Creus, A., Venegas, W., et al. 2005. Micronuclei assessment in buccal cells of people environmentally exposed to arsenic in northern Chile. Toxicology Letters **155**(2):319--327.

Mo, J., Xia, Y., Ning, Z., et al. 2009. Elevated human telomerase reverse transcriptase gene expression in blood cells associated with chronic arsenic exposure in inner Mongolia, China. Environmental Health Perspectives **117**(3):354--360.

Ohe, T., Watanabe, T.and Wakabayashi, K. 2004. Mutagens in surface waters: A review. Mutation Research **567**(2--3):109--149.

Ostrosky Wegman, P., Gonsebatt, M. E., Montero, R., et al. 1991. Lymphocyte proliferation kinetics and genotoxic findings in a pilot study on individuals chronically exposed to arsenic in Mexico. Mutation Research **250**(1 -2):477--482.

Patlolla, A. K. and Tchounwou, P. B. 2005. Cytogenetic evaluation of arsenic trioxide toxicity in Sprague-Dawley rats. Mutation Research **587**(1 -2):126--133.

Petres, J., Baron, D. and Hagedorn, M. 1977. Effects of arsenic cell metabolism and cell proliferation: Cytogenetic and biochemical studies. Environmental Health Perspectives **19**:223--227.

Ramirez, O. A. and Garcia, F. P. 2005. Genotoxic damage in zebra fish *(Danio rerio)* by arsenic in waters from Zimapan, Hidalgo, Mexico. Mutagenesis **20**(4):291--295.

SAB DART ID Committee Draft. 1996. Evidence on developmental and reproductive toxicity of inorganic arsenic.

Salehzadeh, A., Akhkha, A., Cushley, W., et al. 2003. The antimitotic effect of the neem terpenoid azadirachtin on cultured insect cells. Insect Biochemistry and Molecular Biology **33**(7): 681-689.

Schaaf, O., Jarvis, A. P., van der Esch, S. A., et al. 2000. Rapid and sensitive analysis of azadirachtin and related triterpenoids from Neem *(Azadirachta indica)* by high-performance liquid chromatography-atmospheric pressure chemical ionization mass spectrometry. Journal of Chromatography. A **886**: 89-97.

Siess, M. H., Leclerc, J., Canivenc-Lavier, M. C., et al. 1995. Heterogenous effects of natural flavonoids on monooxygenase activities in human and rat liver microsomes. Toxicology and Applied Pharmacology **130**(1):73--78.

Stewart, R. R. 1998. Toxicological results of NeemAzal-technical and NeemAzal-formulations. In Practice oriented results on use and production of Neem-ingredients and pheromones, edited by H. Kleeberg pp 21--26.

Subapriya, R., Kumaraguruparan, R., Abraham, S. K.and Nagini, S. 2004. Protective effects of ethanolic neem leaf extract on N-methyl-N'-nitro-N -nitrosoguanidine-induced genotoxicity and oxidative stress in mice. Drug and Chemical Toxicology **27**(1): 15--26.

Subapriya, R., Kumaraguruparan, R., Chandramohan, K. V. and Nagini, S. 2003. Chemoprotective effects of ethanolic extract of neem leaf against MNNG -induced oxidative stress. Die Pharmazie **58**(7):512--517.

Subapriya, R. and Nagini, S. 2003. Ethanolic neem leaf extract protects against N-methyl -N'-nitro-N -nitrosoguanidine-induced gastric carcinogenesis in Wistar rats. Asian Pac.J.Cancer.Prev. **4**(3):215--223.

Subramanian, S. S. and Nair, A. G. R. 1972. Melicitrin, a new myricitin glycoside from the flowers of Melia azadirachta. Indian Journal of Chemistry **10**:452.

Tepsuwan, A., Kupradinun, P. and Kusamran, W. R. 2002. Chemopreventive potential of neem flowers on carcinogen-induced rat mammary and liver carcinogenesis. Asian Pacific Journal of Cancer Prevention **3**:231--238.

Van der Nat, J. M., van der Sluis, W. G., de Silva, K. T.and Labadie, R. P. 1991. Ethnopharmacognostical survey of *Azadirachta* indica A. Juss (Meliaceae). Journal of Ethnopharmacology **35**(1):1-24.

Waters, M. D., Brady, A. L., Stack, H. F. and Brockman, H. E. 1990. Antimutagenicity profiles for some model compounds. Mutation Research **238**(1):57--85.