doi.org/10.36721/PJPS.2021.34.3.SUP.1031-1037.1

Sodium arsenate induced genotoxicity, morphometric and morphological changes in mice embryo and protective role of *Moringa oleifera* extracts

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Abstract: The present study was carried out to find the comparative ameliorative role of *Moringa oleifera* leaf and flower extracts against sodium arsenate induced genotoxic, morphometric and morphological changes in mice embryo. Seven to eight week old pregnant females (*N*=44) with body weight of 20-25g at gestation day zero were divided randomly in groups (A, B, C, D, E, F, G, H, I, J and K). Group A was of control while all others were experimental groups and administered with selected doses of sodium arsenate as toxicant (6mg/kg B.W and 12mg/kg/B.W) and *Moringa oleifera* leaf and flower extracts as antidote (150mg/kg and 300mg/kg B.W). Significant ($p<0.05$) amelioration at dose 300mg/kg of *Moringa oleifera* leaf extract was observed against sodium arsenate induced morphological abnormalities like micromelia, excencephally, cryptothalmia, anopthalmia, laproschisis and morphometric changes like fetus weight, head circumference, crown rump and snout length were observed. Significant protection of DNA was showed in *Moringa oleifera* leaf extract treated groups (27.50±2.51) as compared to sodium arsenate (66.25±2.75). So concluded that sodium arsenate induced teratogenicity can be decreased using *Moringa* extract especially of *Moringa oleifera* leaf extract as it contains bioactive compounds like phenolics.

Keywords: Gestation day, comet assay, exencephally, laproschisis, crown rump.

INTRODUCTION

Metals are classified according to their density, atomic mass and atomic number (Ali and khan, 2018). Heavy metals are such pollutants which cannot be broken down by microorganisms like other organic pollutants. So these metals are accumulating in our ecosystems (Chen *et al*., 2019). Humans are exposed to heavy metals like cadmium, mercury, arsenic and lead through contaminated soil, water and surrounding (Nordberg *et al*., 2015). These metals are non biodegradable with toxic effects to biological systems and causing diseases and extinction of wild species (Ullah *et al*., 2014). It has been reported that over 200 million peoples are at risk of arsenic poisoning in 105 countries and about 100 million peoples from India, Bangladesh, Pakistan and China are at health risk of underground drinking water (Niazi *et al*., 2017 and Shakoor *et al*., 2018). In Pakistan arsenic level in the ground drinking water is above then the World Health Organization prescribed safe limits of arsenic 10 ppb (Rasool *et al*., 2016) and 20% peoples living in Punjab and 36% of Sindh province are drinking ground water containing greater then 10µg/L of As (Kazi *et al*., 2009). Hazard quotient value of cadmium and arsenic is 5.80 and 2.00 in drinking water of Khyber Pakhtunkhwa, Pakistan (Khan *et al*., 2015). Arsenic induced cardiovascular disorders, dermal effects, respiratory dysfunctions, reproductive abnormalities and neurological disturbances in childhood and youngs via contaminated water (Dauphine *et al.*, 2013). Chronic exposure to arsenic induces ROS (Respiratory oxidative stress) generation which causes DNA damage. Sources of arsenic contamination were described by Saghiri *et al.* (2016) which demonstrated, arsenic is exposed to human through drinking ground water, food and air. Human and geochemical activities are also factor of arsenic contamination and exposure.

Moringa oleifera, synonym *M. ptreygosperma* Gaertn, family *Moringaceae* is commonly called drumstick, kelor or horse radish (Anwar *et al.,* 2003). It has importance for its bactericidal and numerous uses like hepatoprotective, antioxidant, hypotensive, antimicrobial, anticarcinogenic and antidiabetic activity (Dilawar *et al.,* 2018). *Moringa*

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oleifera roots, gum, leaf, bark, fruit, flower, seed and seed oil has pharmacological benefits (Gopalakrishnan *et al.,* 2016; Falowo *et al*., 2018) and can be used for treatment of menstrual disorders, as cardioprotective, antioxidant, to reduce abortion and as fertility enhancer (Nwamarah *et al.,* 2015). Phenols reduced the free radicals by donating hydrogen to these free radicals, converting these to non-reactive species hence act as antioxidants (Nhukarume *et al*., 2010). Bioactive compounds obtained from natural products can be used against metallic toxicity as Biochanin-A (Jalaludeen *et al*., 2016). Olive oil and red lentils have antioxidant properties and were used against sodium arsenate induced hepatotoxicity and oxidative stress (Mohammadian *et al*., 2018; Kalantari *et al*., 2017). Data regarding protective role of natural products obtained from plants specially *Moringa oleifera* against arsenic induced embryotoxicity is not available. So this study was carried out to find the protective role of *Moringa oleifera* extracts against sodium arsenate induced embryo toxicity in mice.

MATERIALS AND METHODS

Chemicals

Sodium arsenate ($Na_2HAs_4O.\ 7H_2O$) was obtained from Sigma chemicals company (USA). Agarose (normal and low melting), Tris, Ethidium bromide, TCA and Triton X-100 were obtained from Merck Co. (Germany). All the chemicals were of highest analytical grade.

Plant sampling and preparation of extracts

Leaves and flowers of *Moringa oleifera* were collected from the botanical garden of C-Block, Ravi campus Pattoki, University of Veterinary and Animal Sciences Lahore, Punjab, Pakistan. Plant samples were identified by (Plant taxonomist) in Department of Botany, Government College University, Lahore (Voucher Specimen No.GC. Herb. Bot. 3725). Plant material was washed and sun dried. After drying plant material was grounded to form powder. Powder (400gm/litter) was extracted through the soxhelt apparatus using methanol (95%) as solvent. When extraction completed, solvent was evaporated using rotary evaporator apparatus, obtained residues approximately 25gm (leaf and flower) were stored at 4^0C and dissolved in distilled water just before use (Aprioku *et al*., 2018).

Phytochemical analysis of extracts

Qualitative phytochemical analysis to observe the presence of different bioactive compounds in extracts of *Moringa oleifera* was carried out using the standard method in reports of Santhi and Sengottuvel (2016). The quantitative analysis of *Moringa oliefera* leaves and flowers extracts was carried out using spectrophotometric and aluminum calorimetric method for total phenol and flavonoid contents of extracts respectively (Singleton *et al.*, 1999; Yadav and Agarwala, 2011), total alkaloids, saponins and tannins were determined using the standard methods of Horborne (1973), Obadoni and Ochuko (2001) and Van-Burden and Robinson (1981) respectively.

Sodium arsenate and test extract administration to animals at GD-8

Seven to eight week old Albino mice, *Mus musculus* of 20-25g were kept in plastic cages at 25±2°C with 12 hours, dark and light cycle at animal hose of C. Block Ravi campus Pattoki of UVAS Lahore. Mice were treated with standard diet in form of pellets (National feed N0.14). Timed mating was induced by placing 1 male and 2 female together to obtain pregnant female. The indication of vaginal plug determined the gestation day zero (Rodriguez *et al.*, 2015). For this study permission was granted from the ethical committee of University of Veterinary and Animal sciences Lahore, Pakistan (Diary N0.161.Dated 6-2-2020).

Sodium arsenate to induce embryo toxicity and test extracts (leaf and flower) of *Moringa oleifera* was used with different dose concentrations in 0.1ml of water. Ameliorant *Moringa* extracts as antidote was administered 1hour before sodium arsenate to all experimental groups. The doses were chosen according to previous reports of Hill *et al*. (2008) and Onyewuchi *et al*., (2018). The animals of control group A were administered with distilled water while sodium arsenate and test extract of *M. oleifera* leaf and flower at GD-8 as given below:

A (0.00), B (6.00, 0.00), C (12.00, 0.00) mg/kg B.W.
Where, groups D to G were sodium arsenate and *M. oleifera* flower extract treated groups.
D (6.00, 150.00), E (6.00, 300.00), F (12.00, 150.00) and G (12.00, 300.00) mg/kg B.W.
Where, groups H to K were sodium arsenate and *M. oleifera* leaf extract treated groups.
H (6.00, 150.00), I (6.00, 300.00), J (12.00, 150.00) and K (12.00, 300.00) mg/kg B.W.

Dissection of animals and sample collection at Gestation Day-18

Samples were collected from control and all experimental groups at gestation day-18. For this purpose females were anaesthetized using ether and fetuses were obtained through surgical incision. Litters were counted as normal, abnormal and deceased for gross fetus analysis. All extra embryonic membranes were cleared and fetuses were transferred to 10% Formalin for 48hrs and then kept in 70% alcohol for further studies (Carson and Hladik, 1997). While some samples were freeze for comet assay.

Morphometric and morphological studies

To observe the morphological abnormalities of fetuses induced by sodium arsenate, like craniofacial, tail, chest cage, arms and legs parts and contrary to this protective

role of *Moringa* extracts against arsenic was deeply studied and fetuses with more morphological defects were selected, recorded and tabulated for further morphometric studies which include fetus weight, head circumference, nasal length, tail length, crown rump length, fore and hind limbs length. Vernier caliper and microscope was used for this purpose. Head circumference was measured using online Elipse circumference calculator. Formula used for this calculation is given below:

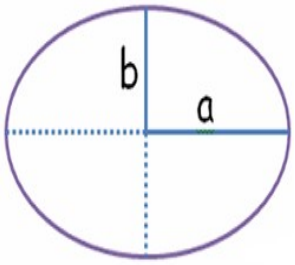
Circumferance=

$2\pi\sqrt{1/2(a^2+b^2}$

$\pi = 3.14$

a= width of head (half diameter)

b= half of length (head)



(a= width, b= front)

DNA damage assessment

Genotoxic effects (DNA damage) of sodium arsenate and curative potential of *Moringa oleifera* leaf and flower extracts were studied through comet assay. Briefly, 1% normal melting agarose was used to precoat the slides and dried. In 2ml lysis solution embryonic tissues were chopped to form tissue homogenate. Filtrate obtained from homogenate was centrifuged at 3000 rpm for 20 minutes. Supernatants were transferred to new eppendroffs and pellets were discarded. Centrifuged for 5-7min. at 3000rpm with addition of 1% PBS. 10 to 20µl of pellet was mixed with 80 to 100 µl of LMA (Low Melting Agarose), added on precoated slides and after covering with cover slip placed on ice slab. After removing cover slips, slides were placed in lysis buffer for two hour than placed slides in electrophoresis tub, added chilled alkaline buffer. After 20 minutes, Slides were electrophoresed for 30 minutes. Slides were washed with distilled water to neutralize the buffer, stained with 80 microlitter of 1X Ethidium bromide. The comet slides were pictured by using fluorescent microscope (Olympus, Japan). Comet scores were calculated from 0-4. 0 (zero) for no damage and 4 for maximum damage (Cigerci *et al*., 2015).

STATISTICAL ANALYSIS

SAS software (version 9.1) was used as a statistical tool for analysis. One way analysis of variance (ANOVA) was used to compare the means between control and experimental groups. Duncan multiple range test (DMRT) for the multiple mean comparison was used in case of significant results (Duncan, 1955). For all the analysis we considered 5% significance level, where as $p<0.05$ was considered significant.

RESULTS

Ameliorative property of *Moringa oleifera* is due to its bioactive compounds such as alkaloids, phenols, flavonoids, saponins, steroids, glycoside, tannins and terpenoids. The results of qualitative and quantitative phytochemical analysis of *Moringa oleifera* leaf and flower extracts are shown in table 1 and table 2.

Morphometric studies

Morphometric parameters were significantly ($p<0.05$) different in sodium arsenate administered groups (table 3 B and C) as compared to control group A. Significant reduced fetus weight, fore and hind limb length, head circumference, snout length and crown rump length were observed in sodium arsenate treated groups at dose of 6mg/kg B.W. and 12mg/kg B.W. While all other groups administered with different doses of *Moringa oleifera* leaf and flower extracts (as ameliorant) + sodium arsenate (as toxicant) showed effective protective role of *Moringa oleifera extrcts* against sodium arsenate specifically *Moringa oleifera* leaf extract (table 3) showed ameliorative property and significantly reduced the embryo toxic effects of sodium arsenate in all morphometric studied parameters.

Morphological studies

Morphologically controls group (A) showed normal growth of body organs with normal body size (fig. 1). Considerable abnormalities were observed in only sodium arsenate treated groups B and C (fig. 1). Deformities at a dose of 6mg/kg/ B.W. of sodium arsenate include macrotia, macroglossia, micromelia, laproschisis and cryptothalmia (fig. 1). Whereas sodium arsenate at 12 mg/kg/ of body weight showed neck fissure, exencephally, club feet, open eye, micromelia, kinky tail and macroglossia (fig. 1). While all other groups treated with different doses of sodium arsenate and *Moringa oleifera* leaf and flower extracts as antidote showed variable degree of abnormalities except group I (fig. 1) of *Moringa oleifera* leaves extract which at 300mg/kg effectively reduced the toxicity of sodium arsenate.

DNA damage evaluation

Dose dependent response was observed when sodium arsenate as toxicant was given with different doses (table 4 B and C) as compared to control group A, significantly breaks the DNA strands and causes the DNA damage. Significant ($p<0.05$) increased DNA damage 48.25±1.50 and 66.25±2.75 was found at dose of 6mg and 12 mg/kg/ body weight of sodium arsenate as compared to control 18.00±0.81. Statistically significant difference ($p<0.05$) was found in all experimental groups when compared with control while *Moringa oleifera* leaves extract at dose of 300mg/kg B.W. reduced the genotoxic effects of sodium arsenate effectively 27.50±2.51 when administered with sodium arsenate as antidote (table 4).

Table 1: Qualitative phytochemical analysis of methanolic leaf and flower extracts of *Moringa oleifera*

Phytochemicals Test	Leaves extract	Flowers extract
Alkolides (Mayers Test)	+	+
Flavonoids (H_2SO_4 test)	+	+
Steroids (Liebermann Burchard test)	+	+
Terpenoids (Salkowski test)	+	+
Phenols (Ferric Chloride test)	+	+
Saponins (Frothing test)	+	-
Tanins (Lead acetate test)	+	+
Glycosides (Nitroprusside test)	+	+

+ = Present, - = Absent

Table 2: Quantitative phytochemical analysis of *Moringa oleifera* leaf and flower extracts

Phytochemicals	Leaves extract	Flowers extract
Alkolides %	6.1	5.4
Flavonoids (mg/mL)	14.2	11.9
Phenols (mg/mL)	38.5	25.9
Saponins %	9.3	0.00
Tanins (mg/mL)	8.4	8.1

Table 3: Morphometric analysis of fetuses recovered at GD-18 after acute exposure (single dose) to sodium arsenate and *Moringa oliefera* (leaf and flower extracts) groups at GD-8.

Doses groups mg/kg/B.W	Fetus weight (mg)	Hind limb (mm)	Fore limb (mm)	Tail length (mm)	Snout length (mm)	Crown rump length (mm)	Head circumference (mm)
Control	1353.23±60.43^{a}	9.05±0.75^{a}	8.18±0.30^{a}	12.98±0.81^{a}	4.56±0.51^{a}	16.12±0.71^{a}	19.55±2.16^{a}
SA 6mg	720.00±11.38^{h}	6.32±0.17^{f}	5.54±0.11^{f}	7.36±0.18^{h}	3.35±0.15^{e}	8.44±0.13^{f}	15.49±0.10^{f}
SA 12mg	539.37±27.40^{i}	5.57±0.15^{g}	4.45±0.17^{g}	6.72±0.51^{i}	3.51±0.17^{d}	8.00±0.10^{g}	13.06±0.08^{g}
SA+MOFE 6mg+150mg	821.66±12.88^{g}	6.43±0.20^{f}	5.66±0.03^{ef}	8.29±0.11^{g}	3.61±0.32^{d}	8.48±0.27^{f}	16.55±0.10^{e}
SA+MOFE 6mg+300mg	1029.02±14.96^{c}	8.50±0.05^{c}	7.44±0.0^{b}	11.02±0.12^{c}	4.31±0.15^{b}	11.23±0.41^{d}	19.06±0.10^{b}
SA+MOFE 12mg+150mg	883.82±8.96^{f}	6.59±0.05^{e}	5.97±0.17^{d}	9.34±0.01^{f}	4.14±0.01^{c}	9.68±0.39^{e}	16.78±0.25^{e}
SA+MOFE 12mg+150mg	899.93±6.82^{e}	7.45±0.13^{d}	7.00±0.10^{c}	10.65±0.15^{d}	4.25±0.02^{bc}	9.69±0.21^{e}	17.22±0.02^{d}
SA+MOLE 6mg+150mg	723.59±23.07^{h}	6.72±0.31^{e}	5.68±0.36^{e}	9.70±0.17^{e}	3.52±0.27^{d}	9±0.27^{e}	17.28±0.33^{d}
SA+ MOLE 6mg+300mg	1044.84±4.11^{b}	8.66±0.48^{b}	7.44±0.02^{b}	11.84±0.80^{b}	4.30±0.07^{b}	12.75±0.17^{b}	19.21±0.14^{ab}
SA+ MOLE 12mg+150mg	899.30±5.59^{e}	7.49±0.21^{d}	7.02±0.12^{c}	10.66±0.15^{d}	3±0.16^{d}	12.74±0.16^{b}	18.53±0.32^{c}
SA+ MOLE 12mg+300mg	934.29±26.82^{d}	8.45±0.00^{c}	7.44±0.49^{b}	11.02±0.13^{c}	4.22±0.04^{bc}	12.47±0.22^{c}	19.08±1.92^{b}

Values bearing the same letters are insignificant and vice versa. Whereas ($p<0.05$). Where, SA = Sodium arsenate, *MOFE* = *Moringa oleifera* flower extract and *MOLE* = *Moringa oleifera* leaf extract

DISCUSSION

Sodium arsenate is a teratogen. Trivalent arsenic is more toxic than the pentavalent arsenate (Hill *et al*., 2008). Mortality rate increased when pregnant females were exposed to inorganic arsenicals (Rahman *et al*., 2010). Significant elevation of axial skeletal abnormalities and decreased weight of fetuses were observed, when in utero exposed to arsenic, with no maternal toxicity (Hill *et al.,* 2008). Arsenic induces the DNA damage in human. This damage of DNA is due to ROS (Reactive Oxygen Species) produced by arsenic (Shi *et al*., 2004). *Moringa oleifera* is used as curative in many, pharmacological and medicinal purposes. Traditionally it is used for the treatment of more than 300 diseases in Ayurvedic medicine. This all is due to its bioactive compounds which includes phenolics and antioxidants (Alegbeleye, 2018).

Table 4: Curative effect of *Moringa oliefera* leaf and flower extracts against sodium arsenate induced DNA damage in mice embryo

Treatment Groups	Doses (mg/kg/B.w)	DNA Damage (Mean ± SD)
Control	-	18.00 ± 0.81^{f}
Sodium arsenate	6	48.25 ± 1.50^{b}
Sodium arsenate	12	66.25 ± 2.75^{a}
SA+MOFE	6+150	38.00 ± 2.94^{cd}
SA+MOFE	6+300	29.00 ± 3.36^{e}
SA+MOFE	12+150	37.75 ± 1.25^{cd}
SA+MOFE	12+300	34.00 ± 4.24^{d}
SA+MOLE	6+150	40.00 ± 2.16^{c}
SA+MOLE	6+300	27.50 ± 2.51^{e}
SA+MOLE	12+150	36.75 ± 2.06^{cd}
SA+MOLE	12+300	34.00 ± 4.24^{d}

Values bearing the same letters are insignificant and vice versa. Where, ($p<0.05$)

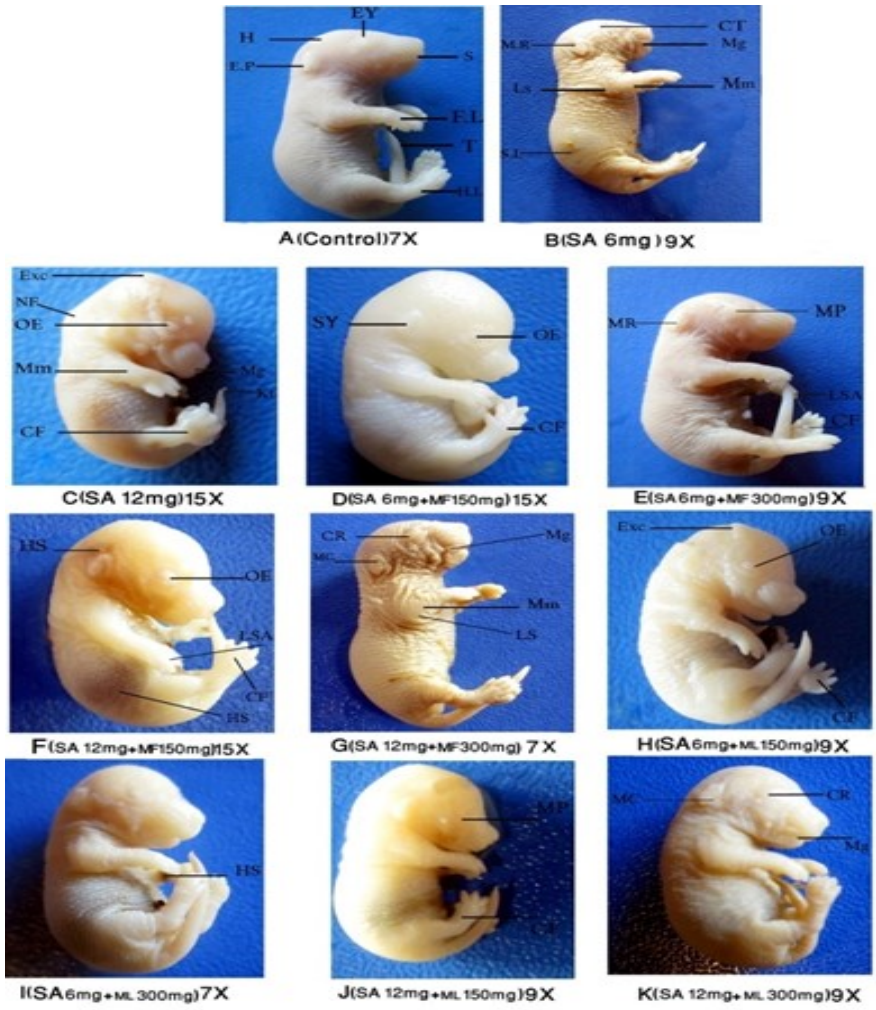


Fig. 1: Morphological features of mice fetuses recovered at GD-18 administered with different doses of sodium arsenate and *Moringa* leaf and flower extracts given above.
H: Head, Ey: eye, F.L: fore limb, H.L: hind limb, S: snout, T: tail, HS: hemorrhagic spot, KT: kinky tail, NF: neck fissure, OE: open eyelid, Mm: micromelia, SL: skin lesion, CF: club feet, Mg: macroglossia, Ls: laproschisis, Mc: macrotia, SL: snout large, Cr: cryptothalmia, Sy: synotia, LSA: low set arm, MP: micropthalmia, Exc: exencephally.

So in Present study it was observed that oral administration of sodium arsenate on GD-8, significantly decreased the litter size, fetus weight, crown rump, snout length and head circumference which is in accordance with the study of Arshad *et al*., (2017). They studied the toxic effects of sodium arsenate on mice embryo and found decreased fetus weight, crown rump length and snout length. Wlodarczyk *et al*. (2014) also had same results when exposed female rats to inorganic arsenicals and observed weight loss and reduced crown rump and increased mortality rate. While *Moringa oleifera* leaf and flower extracts treated groups specifically *MOLE* showed increased litter and fetus size, weight, increased crown rump length and birth rate which is similarity with studies of Zeng *et al*. (2019) they studied the effective potential of *Moringa oleifera* on reproductive performance of mice and found greater litter and fetus size with increased weight of fetus. Morphological defects like macropthalmia, micromelia, open eyelids and excencephally was observed in sodium arsenate treated groups which is compliance with Robinson *et al*. (2011). However in our findings *Moringa oleifera* leaves extract of dose 300 mg/kg B.W mitigated all these morphological abnormalities significantly. Significant DNA damage was observed through comet assay in sodium arsenate treated groups B & C (table 4) as compared to control group while *MOLE* and *MOFE* treated groups specifically *Moringa oleifera* leaf at a dose of 300mg/kg B.W. Showed significant prevention against sodium arsenate induced genotoxicity (DNA damage) which is in accordance with the study of Sikder *et al*. (2013). They evaluated protective effect of *MOLE* against oxidative stress induced DNA damage. Antioxidants can protect DNA from free radical mediated toxicity and *Moringa oleifera* in its leaf and flower have antioxidant property due to its polyphenolic component rich in leaf (Wangasteen *et al*. 2004).

CONCLUSION

The ameliorative and curative potential of *Moringa oleifera* flowers and *Moringa oleifera* leaves extract against the sodium arsenate induced embryo toxicity like genotoxicity (DNA damage), morphometric and morphological changes is due to its bioactive compounds (Phenolics and tannins) specifically of *Moringa oleifera* leaves extract which suggested that it could be used as ameliorant against teratogens like arsenic.

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