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Research Article

Extenuation of Sodium Arsenite-Induced Oxidative Stress and Genotoxicity by Ethanol Extract of *Amaranthus hybridus* Seeds in Wistar Rats

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Abstract

In this study, we assessed the effect of ethanol extract of *Amaranthus hybridus* seeds against sodium arsenite (SA)-induced oxidative stress and genotoxicity in Wistar rats. The effects of three doses of the extract (100, 200, 300 mg/kg body weight) on sodium arsenite (2.5 mg/kg body weight) toxicity were tested. The extract and the toxin were administered for 14 days via oral gavage. Activities of superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) decreased significantly ($p<0.05$); while levels of malondialdehyde (MDA) and hydrogen peroxide (H_2O_2) increased significantly ($p<0.05$) in group treated with sodium arsenite only compared with control. However, pre-treatment with *A. hybridus* seeds extract before arsenite resulted in increased SOD, CAT and GPx activities and decreased concentrations of MDA and H_2O_2 compared with administration of sodium arsenite only. In addition, treatment with sodium arsenite induced a significant increase ($p<0.05$) in the frequency of micronucleated polychromatic erythrocytes (mPCEs) formed in the bone marrow cells compared to the control whereas, pre-treatment with *A. hybridus* seeds extract at various doses decreased the frequency of mPCEs in a dose-dependent manner when compared to the group treated with sodium arsenite alone. In conclusion, the ethanol extract of *A. hybridus* seeds protects against sodium arsenite-induced oxidative stress and genotoxicity in Wistar rats. Consumption of amaranth grain is encouraged for its nutritional and chemo-protective values.

Keyword: sodium arsenite, amaranth, genotoxicity, micronuclei, Oxidative stress

INTRODUCTION

Arsenic has been recognized as a serious public health menace particularly in arsenic-endemic environment. Its wide commercial, industrial and medicinal applications have led to an increased risk of exposure (Chen *et al.*, 2006; Hu *et al.*, 2005; Meharg, 2003; Miller *et al.*, 2002; Rossman, 2007). Major routes of exposure are through ingestion of arsenic-contaminated foods, contaminated underground water and inhalation via emissions from burning of arsenic-containing fossil fuels, glass and pesticide manufacturing processes, weathering of arsenic-containing minerals and ores, while dermal absorption is considered a minor route of exposure (Rossman, 2007). Prolonged environmental and/or occupational exposure to arsenic has been associated with diverse arrays of health problems. These include hypertension, cardiovascular disease, diabetes mellitus, renal injury, skin lesions, and cancers of the skin, lung, bladder, liver and kidney (IARC, 2004; Smith *et al.*, 1998). Plausible mechanisms of action of arsenic as a carcinogen include production of chromosomal abnormalities, tumour promotion and induction of oxidative stress (Hughes, 2002; Kitchin, 2001).

In general, the processes of mutagenesis and carcinogenesis are influenced by dietary factors. The use of certain naturally occurring plant substances enhance fortification of physiological defence mechanisms which is believed to be one of the most effective and cheap ways of prevention against environmental chemical carcinogens (Ishaq *et al.*, 2003). For instance, consumption of whole grain products has been associated with reduced risk of chronic diseases including cancer (Bonaccio *et al.*, 2012; Jones *et al.*, 2010; Lillioja *et al.*, 2013).

Amaranth (*Amaranthus hybridus*, family: Amaranthaceae) is a pseudo cereal with seeds and leafy vegetables that are rich in essential amino acids such as lysine, methionine, threonine, and tryptophan (Akin-Idowu *et al.*, 2013; Pisarikova *et al.*, 2006). It is therefore a good source of quality protein, vitamins and minerals (Islam *et al.*, 2003). It also has a substantial amount of antioxidants (Hunter and Fletcher, 2002). Amaranth seeds have been found useful in confectionaries and bakery industries (Hozova *et al.*, 1997), and feed formulations for some farm animals (Andrasofszky *et al.*, 1998; Rouckova *et al.*, 2004; Serratos, 1996). It has also found beneficial application as adjunct in diet for susceptible individuals with hypercholesterolemia (Andrea and Areas, 2002). There are, however, scanty records of studies on the effect of amaranth seeds on chemical carcinogens. The aim of this study was to assess the effects of ethanol seed extract of *A. hybridus* on sodium arsenite-induced oxidative stress and genotoxicity in male Wistar rats.

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MATERIAL AND METHODS

Chemicals: Sodium arsenite was purchased from Sigma Chemical Co., St. Louis MO, USA. Sodium arsenite was administered by oral intubation to rats at a dose of 2.5 mg/kg body weight, corresponding to $1/10^{th}$ of the LD_{50} for sodium arsenite (Preston *et al.*, 1987). Other reagents and chemicals used were of analytical grade, products of Sigma Chemical Co., Saint Louis, MO, USA or BDH Chemical Ltd, Poole, England.

Plant extract: *Amaranthus hybridus* (accession number NH 84/444-4) was obtained from the germplasm of National Horticultural Research Institute (NIHORT) Ibadan. Seeds were milled into flour and extracted in 80% ethanol using a soxhlet apparatus. The extract was concentrated at reduced temperature (40 ^oC) using a rotary evaporator (Buchi, USA). The resulting molten extract was lyophilized using a freeze dryer (Freezone 4.5, Labconco, USA) at high vacuum pressure (133×10^{-3} mBar). The dried extract was stored in air tight amber bottle and kept at -20 ^oC until used.

Experimental design: Forty male Wistar rats weighing between 130-150 g were obtained from the Faculty of Veterinary Medicine, of our University. They were divided into eight groups of 5 each and housed in a well ventilated room at 27 ± 2 ^oC in the Animal House of Department of Biochemistry, University of Ibadan, Ibadan, Nigeria They were fed with commercial rats' pellets purchased from Ladokun Livestock Feeds Limited and given water *ad libitum*. After two weeks of acclimatization, animals were randomly divided into eight treatment groups of five animals each as shown in Table 1. Experimental animals were treated and sacrificed following standard rules laid down by the University Ethics Committee on the treatment of experimental animals.

Table 1
Experimental design showing the distribution and treatment of rats

Groups	Treatment	Dosage (mg/kg body weight)
I	Distilled water	
II	SA	2.5
III	*A.hyb*	100
IV	*A.hyb*	200
V	*A.hyb*	300
VI	*A.hyb* + SA	100 + 2.5
VII	*A.hyb* + SA	200 + 2.5
VIII	*A.hyb* + SA	300 + 2.5

A. hyb = Grain extract of *Amaranthus hybridus,* SA= Sodium arsenite. All rats were kept in well ventilated room at 27 ± 2 ^0C and fed with commercial rats' pellets, product of Ladokun Livestock Feeds Limited, and given water *ad libitum*

Termination of the experiments and collection of animal tissues: The experiment was terminated and the rats were sacrificed by cervical dislocation twenty-four hours after administration of the last sodium arsenite regime. Two hours prior to the sacrifice, the animals were intraperitoneally injected with 0.04% colchicine. Livers were harvested, rinsed in ice cold 1.15% KCl solution and weighed. Liver samples were minced and homogenized using a potter-Elvehjem homogenizer; the homogenate was centrifuged at 20,000 x g for 20 min at 4 ^oC (HERMLE LABNET Z 323K). The resulting supernatant was used for the antioxidant enzymes assays. Femoral bone marrow from each animal was collected and used for micronucleus assay.

Micronucleus (MN) assay
The bone marrow cells were prepared according to the method of Heddle and Salmone (1981) as modified by Macgregor *et al*. (1987). Briefly, femur bones from each animal were removed and stripped of muscles. The bone marrow was flushed out on to a microscope glass slide to which a drop of fetal serum albumin was added. The slides were air dried and fixed in absolute methanol for 5 min. They were subsequently stained in 0.4% May-Grunwald stain, allowed to dry and furthermore stained in 5% Giemsa stain (dissolved in 0.01M phosphate buffer pH 6.8) for 30 min, thereafter rinsed in distilled water, air dried and fixed in xylene for 20 min. They were air dried again and subsequently mounted in DPX (BDH) and covered with cover glass. The fixed cells on the mounted slides were scored under light microscope to detect the presence of micronucleated polychromatic erythrocytes.

Assessment of lipid peroxidation: Extent of lipid peroxidation was determined by measuring the formation of thiobarbituric acid reactive substances (TBARS) as described by Varshney and Kale (1990). An aliquot of the liver microsomal sample was mixed with 1.6 ml of Tris-KCl buffer to which 0.5 ml of 30 % TCA was added. Then 0.5 ml of 0.75 % TBA was added and placed in a water bath for 45 minutes at 80 ^oC. This was then cooled in ice and centrifuged at 3000 x g for 10 min. The clear supernatant was collected and absorbance measured against a reference blank at 532nm. The TBARS (malondialdehyde or MDA) concentrations of the samples were calculated according to the method of Adam-Vizi and Seregi (1982). Lipid peroxidation, expressed as, units/ g tissue was calculated using the molar extinction coefficient of MDA, which is 1.56×10^5 $M^{-1}cm^{-1}$.

Determination of superoxide dismutase (SOD) activity: Hepatic SOD activity was determined by the method described by Misra and Fridovich (1972). The ability of superoxide dismutase to inhibit the auto-oxidation of adrenaline in an alkaline medium (pH 10.2) to adrenochrome makes this reaction a basis for a simple assay for SOD. Epinephrine was added to the assay mixture containing tissue supernatant and the change in extinction coefficient was followed at 480 nm in a spectrophotometer.

Determination of catalase (CAT) activity : Catalase activity was determined by the method of Aebi (1984). The method involves spectrophotometrically monitoring the rate at which H_2O_2 is decomposed by the enzyme catalase at 240 nm.

Determination of glutathione peroxidase (GPx) activity: Hepatic GPx activity was determined spectrophotometrically according to the method of Rotruck *et al*. (1973). The total reaction mixture contained 0.5 ml 0.1 M phosphate buffer (pH 7.4), 0.1 ml 2.5 mM H_2O_2, 0.2 ml 4.0 mM reduced glutathione (GSH), and 0.1 ml 10 mM sodium azide. To this reaction mixture, 0.6 ml of the sample was added and the whole mixture was incubated at 37 ^oC for 3 min, after which 0.5 ml of trichloroacetic acid (TCA) was added and thereafter centrifuged at 3000 x g for 5 min. To 1 ml of the supernatant

from above, 2 ml of K_2HPO_4 and 1.0 ml of 5'-5'-dithiobis-2-dinitrobenzoic acid (DNTB) was added and the absorbance was read at 412 nm against a blank.

Determination of hydrogen peroxide (H_2O_2) level: The level of hydrogen peroxide generated was determined by the method Wolf (1994). This is based on the principle of ferrous oxidation with xylenol orange reagent. The colour development was measured spectrophotometrically at 560 nm.

Protein estimation: The protein content of the liver homogenate was determined according to the method of Lowry *et al*. (1951) using bovine serum albumin as a standard.

Statistical analysis: Data were analysed by Statistical Program for the Social Sciences SPSS version 17 (SPSS, Chicago, IL, USA) and presented as mean ± standard deviation (S.D). Duncan's multiple range tests was used to compare the significance of the differences at 5 % level

RESULTS

Administration of sodium arsenite alone in rats caused a significant ($p<0.05$) decrease in activities of antioxidant enzymes, superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) when compared with the control group (Table 2). On the other hand, significant ($p<0.05$) increase in levels of SOD, CAT and GPx activities were observed in groups pre-treated with extract of *A. hybridus* seeds before sodium arsenite when compared to the group administered the arsenite alone. The increase in enzymes activities were extract dose dependent and is much pronounced in group administered the extract at 300 mg/kg body weight. In order to assess effects of sodium arsenite and/or amaranth extract on lipid peroxidation, a biomarker of oxidative stress, MDA and H_2O_2 levels were measured in the treated groups. It was observed that sodium arsenite treatment caused significant ($p<0.05$) increase in MDA and H_2O_2 levels compared with the control. However, pre-treatment with amaranth seed extract before sodium arsenite led to decrease in sodium arsenite-induced MDA and H_2O_2 (Table 2).

Furthermore, to assess the effect of the extract of amaranth seeds in sodium arsenite-induced genotoxicity, we measured the relative number of micronucleated polychromatic erythrocytes (mPCEs) in the bone marrow cells of rats. There was a significant ($p<0.05$) increase in the number of mPCEs scored in the group of rats treated with sodium arsenite alone. A significant ($p<0.05$) reduction in the number of induced mPCEs was observed in groups pre-treated with amaranth seed extract. The reduction was in a dose-dependent fashion (Table 3).

DISCUSSION

Oxidative stress has been widely reported as a mechanism of arsenic-induced toxicities and carcinogenesis (Hughes *et al.*, 2011; Kitchin, 2001). In the present study, oxidative stress set in when animals were treated with sodium arsenite as reflected in the results (Table 2). This is possibly due to depletion of anti-oxidative defence system in the body of experimental rats. Reactive oxygen species (ROS) are often generated as by-products of certain physiological processes and through exposure to xenobiotics and environmental chemical carcinogens.

Table 2
Changes in the activities of SOD, CAT, GPx, MDA, H_2O_2 and protein in liver homogenate of rats treated with *A. hybridus* and sodium arsenite

Treatment	Distilled water alone	∝SA alone	100mg/kg *A.hyb*	200 mg/kg *A.hyb*	300mg/kg *A.hyb*	100mg/kg *A.hyb*+SA	200mg/kg *A.hyb*+SA	300mg/kg *A.hyb*+SA
Group	**GRPI**	**GRPII**	**GRPIII**	**GRPIV**	**GRPV**	**GRPVI**	**GRPVII**	**GRPVIII**
SOD	4.22 ± 0.408	2.82 ± 0.23*	3.03 ± 0.66	3.63 ± 1.13	3.85 ± 0.56	3.13 ± 0.09#	3.57 ± 0.14#	4.10 ± 0.70#
CAT	123.83 ±20.50	71.38 ± 8.33*	94.33 ± 5.67	100.27 ±1.96	109.64 ±5.28	73.66 ±10.24#	79.85 ± 4.47#	94.03 ± 6.78#
GPx	539.75 ± 55.70	398.44 ± 20.25*	498.40 ± 29.27	514.28 ±36.87	575.91 ±25.32	410.20 ± 23.34	434.04 ±23.38#	460.53± 15.29#
MDA	4.71 ± 1.48	11.15 ± 0.85*	5.41 ± 0.42	4.65 ± 0.26	4.26 ± 0.56	8.36 ± 0.29	6.09 ± 0.65#	5.34 ± 0.55#
H_2O_2	29.87 ± 6.70	41.42 ± 5.89*	32.99 ± 2.79	29.09 ± 6.84	27.26 ± 4.72	34.48 ± 2.75	34.29 ± 1.02	32.83 ± 1.84
Protein	0.66 ± 0.06	0.60 ± 0.05	0.50 ± 0.04	0.51 ± 0.04	0.54 ± 0.03	0.46 ± 0.03#	0.45 ± 0.07#	0.40 ± 0.12#

*∝SA = sodium arsenite; Values are expressed as means ± standard deviation; n = 5 for each treatment. * = Mean values significant different ($p<0.05$) from control, Group I. # = Mean values are significant different ($p<0.05$) from Group II treated with SA alone. Superoxide dismutase (SOD) activity is expressed as units/mg protein; catalase (CAT) activity as µmol H_2O_2 consumed/mg protein; glutathione (GPx) in µg/mol/mg protein; malondiadehyde (MDA) as units/g tissue x 10^{-6} and Hydrogen peroxide (H_2O_2) as µmol/L*

Table 3.
Micronucleated polychromatic erythrocytes (mPCEs) count of rats administered ethanol extract of *A. hybridus* and sodium arsenite per 1000 PCEs.

Treatment[∞]	Group	Number of mPCE/1000PCEs
Distilled water alone	I	5.56 ± 0.24
SA alone	II	11.31 ± 0.53*
100mg/kg *A. hyb.*	III	5.71 ± 0.38
200mg/kg *A. hyb.*	IV	5.22 ± 0.18
300mg/kg *A. hyb.*	V	5.09 ± 0.28
100mg/kg *A. hyb* + SA	VI	8.64 ± 0.44[#]
200mg/kg *A. hyb* + SA	VII	7.13 ± 0.32[#]
300mg/kg *A. hyb* + SA	VIII	6.11 ± 0.28[#]

[∞] *A. hyb* (*A. hybridus*), SA (sodium arsenite). Values are means ± standard deviation. * = Mean values are significant different ($p<0.05$) from control, Group I. [#] = Mean values are significant different ($p<0.05$) from Group II treated with SA alone.

Oxidative stress ensues when the biological system's capacity to readily detoxify the generated reactive intermediates is overwhelmed. SOD and CAT have antitoxic effects against the superoxides and hydrogen peroxide (H_2O_2) respectively. SOD catalyses the dismutation reaction of superoxides to H_2O_2 while catalase breakdown H_2O_2 formed to water and molecular oxygen (Aebi, 1984). Vahter (2002) and Hughes (2002) separately reported that a methyltransferase is required for the enzymatic methylation of arsenic in which S-adenosylmethione (SAM) serves as a methyl donor. This implies that decrease in synthesis of SAM may bring about reduced arsenic methylation and subsequently increased toxicity, probably due to accumulation of highly reactive and toxic arsenic metabolites (monomethylarsonous acid-MMA^{III} and dimethylarsinous acid-DMA^{III}). Vahter and Marafante (1987), pointed out that intake of diets low in methionine or protein resulted in reduced arsenic methylation. The result from the present study showed that treatment of rats with sodium arsenite led to reduced levels of SOD and CAT activities in the rats. There were significantly increase in SOD and CAT activities in the groups pre-treatment with amaranths seed extract before sodium arsenite compare with sodium arsenite treatment alone. This is possibly due to remarkable essential amino acids composition and antioxidant contents of the seeds (Akin-Idowu *et al.*, 2013; Hunter and Fletcher, 2002).

Glutathione peroxidase (GPx) catalyses the conversion of reduced GSH to oxidised GSSG form, its synthesis or activity can be used as an index of GSH/GSSG redox status. About 90% of the total GSH content exists in the reduced form under normal physiological conditions, while around 10% is in the oxidised form. However, under the condition of oxidative stress, the concentration of GSSG is much higher than that of GSH. Our results (Table 2) revealed a significant ($p<0.05$) decrease in GPx activity in group treated with sodium arsenite alone. When the rats were pre-treated with amaranth seed extract before sodium arsenite administration, significant increase in GPx activities were observed in a dose dependent manner. This effect may be due to decreased bio-activation of sodium arsenite to highly reactive and toxic species by the extract. Glutathione (GSH), a vital component of body's natural defence system is often referred to as master antioxidant of the body. This tripeptide molecule has sulfhydryl (SH) group on the cysteine portion and this account for its strong electron donating characteristic or free radicals quenching ability. GSH donates reducing equivalent and conjugates with arsenic metabolites (AS-Arsenate and MMA^{V}-monomethylarsonic acid) (Delnomdedieu *et al.*, 1994; Scott *et al.*, 1993; Styblo *et al.*, 1995) in a reaction catalysed by glutathione-s-transferase, and thus prevent them from forming toxic intermediate metabolites but rather are readily excreted in the urine (Vahter, 1999).

Lipid peroxidation, a biomarker of oxidative stress, is one of the most investigated outcomes of ROS effects on membrane lipids. Free radicals are generated from arsenic bio-activation and attack on the lipids present in the cell leading to cellular damage. The findings in the present study that sodium arsenite caused increase in the levels of MDA and H_2O_2 in the treated group support a link between sodium arsenite-induction of oxidative stress and lipid peroxidation. However, pre-treatment with amaranth seed extract led to decrease in sodium arsenite-induced MDA and H_2O_2 (Table 2), an indication of the extract capacity to reduce lipid peroxidation. The increased H_2O_2 levels coupled with decreased SOD and CAT activities observed in liver homogenate of the rats treated with sodium arsenite appear to result from accumulation of superoxides and hydrogen peroxide radicals and might actually be the primary cause for increase lipid peroxidation as seen in the increased MDA level after arsenite treatment. Yamanaka *et al*. (1990) also reported that free radical species were generated following arsenic treatment, and this was as a result of reaction between molecular oxygen and dimethylarseine. Our results are also in agreement with other previous reports (Bashir *et al.*, 2006; Gopalkrishnan and Rao, 2006; Usoh *et al.*, 2005) of significant decrease in the liver SOD, CAT and increase in MDA level following administration of sodium arsenite.

In addition, reactive oxygen species that are generated directly or indirectly from arsenic bio-activation mediate DNA damage. Yamanaka *et al*. (2004) reported that DMA^{3+} is more genotoxic than inorganic arsenic *in vitro*. It follows perhaps that DMA^{3+} is the causative intermediate species in arsenic carcinogenesis (Obinaju, 2009). Estimated number of micronuclei in the bone marrow cells of differentially treated groups is a reliable and convenient index of both chromosome breakage and chromosome loss. Micronuclei originated from acentric fragments that were not included into the two daughter cell nuclei at the tail end of mitosis (Fenech 2007; 2010). Scavenging and prevention of formation of reactive species, and chemical or enzymatic activation of anti-oxidation process are some suggested mechanisms by which anti-mutagenic and anti-genotoxic agents mediate their action (Ishaq *et al.*, 2003). Tice and co-workers (1997) suggested the status of hepatic methyl donor as a significant factor in arsenic-induced genotoxicity. The significant reduction in the number of sodium arsenite-induced mPCEs by extract of amaranth seeds in the present study may be attributed to the antioxidant content and sulphur containing amino acid, methionine, which can interact with DMA^{3+} and somewhat suppress its toxic effect. In conclusion, the present study showed that *A. hybridus* grain extract has some protective effect against sodium arsenite-induced oxidative stress and showed good capacity to mitigate sodium arsenite genotoxicity in Wistar rats

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