INTRODUCTION

Chemoprevention (or chemoprotection) refers to the use of chemicals including phytochemicals, pharmaceuticals and minerals to retard, reduce or reverse the onset of cancer caused by exposure to xenobiotics such as drugs, heavy metals and electrophiles (Wattenberg, 1985). Among the phytochemicals examined so far, organosulfur compounds (OSCs) found in garlic have been extensively studied and are found to possess antioxidant (Imai et al., 1994), anticarcinogenic (Hatono et al., 1996), antiatherogenic (Steiner and Lin, 1998; Efendy et al., 1997), immunostimulatory (Abdullah et al., 1989; Lau et al., 1991) and antiaging properties (Moriguchi et al., 1996).

Garlic (Allium sativum), a member of the lily family, is a perennial plant that is cultivated worldwide. The garlic bulb is composed of individual cloves enclosed in a white skin. It is the bulb, either fresh or dehydrated that is used as a spice of medicinal herb (Block, 1985). Garlic has been used throughout history for the treatment of a wide variety of conditions and these have been extensively investigated (Umar et al., 1996). Its usage predates...
written history. Its use as a remedy for heart disease, tumors and headaches are documented in the Egyptian codex Ebers, dating from 1550 BC. Epidemiological studies have suggested that garlic plays a significant role in the reduction of deaths caused by malignant diseases (Agarwal, 1996). This has led many investigators to examine garlic and garlic constituents for their antitumor and cytotoxic actions both in vitro (Prasad et al., 1996; Rabinkov et al., 1998) and in Laboratory animals (Augusti and Sheela, 1996; Iqbal and Athar, 1998). Data suggested that garlic contains several potentially important agents that possess antitumor and anticarcinogenic properties (Agarwal, 1996). It contains both water soluble compounds such as L-seleocystein (SC), S-allycysteine (SAC) as well as lipid soluble compounds such as diallytrisulfide (DATS), diallyldisulfide (DADS) (Nisha and Tood, 2001). Garlic also contains high level of tellurium and selenium compounds (Ip et al., 2000; Mesheeshy et al., 1998).

Ascorbic acid (vitamin C) is essential micronutrients in primates. It is a water-soluble chain breaking antioxidant that exhibits its antioxidant property by scavenging free radicals. Ascorbic acid also recycles plasma membrane α-tocopherol via the reduction of the α-tocopheroxyl radical. Ascorbic acid has also been reported to exhibit prooxidant activities (Chen et al., 2005, 2007). It was earlier reported by Halliwell (1996) that ascorbate promotes fenton reaction in vitro and may induce non-enzymatic lipid peroxidation in various tissues. Also, Chen et al. (2008) reported that ascorbate at pharmacological doses produced sustained ascorbate radical and hydrogen peroxide in mice. These and other reports have implicated ascorbate in enhancing reactive oxygen production which may trigger-off Nrf2 protective response and this could be an important mechanism in the protective effect of ascorbic acid via the Nrf2-mediated response in exposed animals.

Cadmium is widely used in industrial chemicals (Waisberg et al., 2003). The toxicities associated with this metal ion are well known (ATSDR, 1999). It has been reported that cadmium depletes glutathione and protein bound sulphydryl groups (Waisberg et al., 2003), resulting in enhanced production of reactive oxygen species such as superoxide ion, hydroxyl radicals and hydrogen peroxide (Galan et al., 2001; Stohs et al., 1995). These reactive oxygen species play an important role related to degenerative or pathological processes such as aging (Burns et al., 2001), cancer, coronary heart disease, Alzheimer disease (Diaz et al., 1997), neurodegenerative disorders, atherosclerosis, cataracts and inflammation (Aruoma, 1998). Studies have also shown that cadmium elevates the levels of lipid peroxidation in the liver (El-Maraghy et al., 2001) and liver of mitochondrial of exposed rats (Casalino et al., 1997).

Therefore, the present study was carried out to evaluate the antioxidant effects of heated garlic extract, a natural source of antioxidant, when compared with ascorbic acid, a conventional antioxidant, in cadmium-induced rat liver damage and to define a role for the involvement of Nrf2-dependent antioxidants in this protection.

MATERIALS AND METHODS

Plant materials
Garlic (Allium sativum) bulbs were purchased from a local market in Akure, Ondo State, Nigeria. The garlic juice was prepared by the method of Kasuga et al. (2001).

Animals
Adult male albino rats of wister strain, weighing 160-220 g were purchased from Department of Animal Science, University of Ilorin, Nigeria. They were kept under standard laboratory conditions with standard pelleted diet and water ad libitum. The animals were allowed to acclimatize for four weeks before the experiment.

Reagents
All chemicals used were of analytical grade and are products of Sigma Chemical Co. (St. Louis, MO, USA). SOD assay kit (Product code 19160) was obtained from Fluka (Gillingham, UK). Catalase activity assay kit (Product number CAT 100) was obtained from Sigma, Poole, Dorset, UK. Thiobarbituric acid was obtained from Sigma (UK). Antibodies were purchased from Santa Cruz Biotech. Inc. (Santa Cruz, CA, USA). Goat anti-rabbit antibody (horseradish peroxidase conjugated) was obtained from Bio-Rad (Herts, UK). Nitrocellulose membranes were obtained from Amersham Biosciences. Dichlorofluorescein diacetate (DCF-DA) was purchased from Sigma-Aldrich (UK).

Dosage and treatment
Heated Garlic Juice (HGJ) and ascorbic acid were given as oral dose of 100 mg kg⁻¹ day body weight each for 4 weeks. Acute intraperitoneal dose of 4 mg kg⁻¹ of cadmium was given for 3 days at the last week of treatment. The rats were sacrificed 24 hr after the last dose of cadmium had been administered.

Experimental procedures
The rats were divided into six groups of 5 animals each. Treatments were done as below:

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Group 1: Normal control
Group 2: Animals received heated garlic juice 100 mg kg\(^{-1}\) body wt orally, daily for 4 weeks.
Group 3: Animals received ascorbic acid 100 mg kg\(^{-1}\) body wt orally, daily for 4 weeks.
Group 4: Animals received acute dose of cadmium 4 mg kg\(^{-1}\) body weight intraperitoneally.
Group 5: Animals received cadmium 4 mg kg\(^{-1}\) body weight intraperitoneally for 3 days + pretreatment with heated garlic juice 100 mg kg\(^{-1}\) body wt orally, daily for 4 weeks.
Group 6: Animals received cadmium 4 mg kg\(^{-1}\) body weight intraperitoneally for 3 days + pretreatment with ascorbic acid, 100 mg kg\(^{-1}\) body weight orally, daily, for 4 weeks.

**Preparation of samples**

For biochemical estimation, liver tissue was taken after sacrificing the animals by cervical dislocation. The liver was then washed, weighed and homogenized in ice cold 0.9% normal saline solution. The homogenate was centrifuged at 4,000 x g for 15 min at 25°C and the supernatant was used for the studies.

**Lipid peroxidation assay**

The level of malondialdehyde (MDA) in the homogenate was assayed by the thiobarbituric acid reactive species (TBARS) method (Varshney and Kale, 1990). The assay involves the monitoring of the pink coloured complex formed in a reaction between MDA and thiobarbituric acid (TBA). The reaction mixture consists of 500 μl homogenate, 1,000 μl TBA reagent (0.375% TBA, 0.3 M Sodium acetate and 0.15% SDS, pH 3.8), 50 μl 2 mM butylated hydroxytoluene (BHT). After incubating for 60 min at 90°C, the mixture was cooled and centrifuged at 5,000 x g for 10 min. The absorbance of 1 ml of the supernatant was read at 532 nm and the amount of MDA formed was extrapolated from the standard curve and expressed as μmol MDA/mg protein. Protein was determined by the Biuret method of Gornall et al. (1949) using bovine serum albumin (BSA) as standard.

**ROS measurement**

The level of ROS production was assessed using dichlorofluorescein diacetate (DCF-DA) according to the method described by Hempel et al. (1999). The 500 μl of homogenates were incubated with 500 μl of 2 mg/ml DCF-DA for 30 min at 37°C. 100 μl of 1 M NaOH was added and the fluorescence intensity of 1 ml of the mixture was read with a fluorescence reader (FL6000) at excitation of 488 nm and emission of 512 nm. The results were expressed as relative fluorescence unit (RFU)/mg protein.

**Enzyme assays**

*Superoxide dismutase (SOD) activity*

Superoxide dismutase (SOD) activity was determined by the method of Misra and Fridovich (1972) using the SOD assay kit (Sigma). The reaction consists of 200 μl of homogenate, 400 μl of water soluble tetrazolium salt (WST) and 200 μl of enzyme working solution (containing superoxide anion). The mixture was incubated at 37°C for 20 min and absorbance read in Labsystem iEMS spectrophotometer. SOD activity was expressed as units/mg protein.

*Catalase activity*

Catalase activity was measured by following decomposition of H\(_2\)O\(_2\), according to the method of Sinha (1972) using the catalase assay kit (Sigma). The reaction mixture consists of 50 μl of homogenate, 450 μl of assay buffer (50 mM potassium phosphate buffer, pH 7.0) and 50 μl of 20 mM H\(_2\)O\(_2\). The decrease in absorbance was monitored at 30 sec interval at 25°C at wavelength of 240 nm. Results were expressed as units/mg protein.

**Gel preparation and Western blot analysis**

Western blot analysis was carried out on the liver homogenate. The samples were prepared in 2X lysis buffer [Tris-Cl (pH 6.8), sodium dodecyl sulfate (SDS) (2 g), glycerol (9 ml), mercaptoethanol (5 ml), bromophenol blue (0.1%) and distilled water] and heated at 100°C for 5 min. The samples were centrifuged at 1,000 x g for 1 min and equal amount of proteins were loaded on 10% polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were then transferred to nitrocellulose membrane (Hybond ECL) at 200 mA for 2 hr and incubated in specific antibodies for 1 hr. The membranes were washed 4 times in 1X TBSTween [10X TBS (100 ml), Tween20 (2 ml) and distilled water] and then incubated with secondary antibody [Goat anti-Rabbit IG-horseradish peroxidase conjugate antibody (Bio-Rad)] for 1 hr at room temperature. GAPDH was used for the normalization of protein loading. The membranes were washed 3 times with 1X TBSTween and once with 10X TBS and the blot were developed using ECL luminol chemiluminescence solutions. The proteins were quantified with an image reader LAS 3000 and the band intensities were determined by image J software.

**Statistical Analysis**

Data were analyzed using one way analysis of variance.
(ANOVA) and unpaired student’s t-test by prism software. Comparison between groups was done using Dunnett’s post test. Significant difference were set at $p^* < 0.5$; $p^{**} < 0.01$; $p^{***} < 0.001$ compared to control.

**RESULTS**

**Effects of Heated garlic juice and ascorbic acid on membrane lipid peroxidation after cadmium exposure**

In order to assess the protective effects of heated garlic juice (HGJ) and ascorbic acid (AA) on Cadmium-induced liver damage in exposed rats, rats were pre-treated orally with 100 mg/kg body weight of either HGJ or AA for 4 weeks before 3 days intraperitoneal treatment with 4 mg/kg body weight of CdCl$_2$. Levels of MDA were then determined by the thiobarbituric acid reactive substance (TBARS) as an indices of membrane lipid peroxidation. The results show significant 3 and 1.54-fold decrease in MDA level in the presence of HGJ and AA respectively when compared with control (Fig. 1). The presence of Cd caused a 1.42-fold increase in MDA levels when compared with control (Fig. 1). The results also show that HGJ significantly (1.44-fold) decrease MDA level in the presence of Cd when compared to rats exposed to Cd alone (Fig. 1). Also MDA level was significant (1.66-fold) decrease in the liver of AA pre-treated rats when compared to rats exposed to Cd alone (Fig. 1). These set of data seem to suggest that AA seem more potent in preventing lipid peroxidation than HGJ.

**Effects of Heated garlic juice and ascorbic acid on ROS production after cadmium exposure**

In order to assess the modulating effect of HGJ and AA on the oxidative stress induced by Cadmium in the liver of the exposed rats, the level of ROS was determined in AA and HGJ pre-treated rats before cadmium exposure. The results show that there was significant decrease in ROS production in the presence of either HGJ or AA when compared with control (Fig. 2). There was a significant decrease in ROS level in the liver of rats pre-treated with either HGJ (1.8-fold) or AA (2-fold) when compared with Cd. This indicates that HGJ and AA have the potential of attenuating ROS production by Cd.

**Effects of Heated garlic juice and ascorbic acid on Superoxide dismutase activities after cadmium exposure**

In order to evaluates the effects of HGJ and AA on antioxidant enzymes activities after Cd exposure, rats were pre-treated with oral dose of 100 mg/kg body weight of either HGJ or AA for 4 weeks before 3 days exposure to 4 mg/kg body weight of CdCl$_2$, given intraperitoneally. Superoxide dismutase (SOD) activities were then determined in the liver of the treated rats after Cd exposure. The results show significant increase of 2.07 and 3.5-fold

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**Fig. 1.** Levels of MDA in rats liver pre-treated with either HGJ or AA before Cd exposure. Rats were pre-treated with 100 mg/kg body weight of either HGJ or AA for 4 weeks before 3 days intraperitoneal exposure to 4 mg/kg body weight CdCl$_2$. Lipid peroxidation was determined by TBARS method. Data represents the mean activity ($n = 5$) relative to total cell protein ± S.D. $***p < 0.001$, $**p < 0.01$, $*p < 0.05$ (one way ANOVA, Dunnett’s post test). # represents significant different between pre-treated and unpre-treated rats (unpaired student t-test).

**Fig. 2.** Reactive oxygen species production in the liver of rats pre-treated with either HGJ or AA before Cd exposure. Rats were pre-treated with 100 mg/kg body weight of either HGJ or AA for 4 weeks before 3 days intraperitoneal exposure to 4 mg/kg body weight CdCl$_2$. ROS level was measured by DCF-DA. Data represents the mean activity ($n = 5$) relative to total cell protein ± S.D. $***p < 0.005$, $**p < 0.01$, $*p < 0.05$ (one way ANOVA, Dunnett’s post test). # represents significant different between pre-treated and unpre-treated rats (unpaired student t-test).
in the liver of HGJ and AA treated rats respectively when compared with control (Fig. 3). However, the presence of Cd causes a significant decrease (1.77-fold) in SOD activities when compared with control (Fig. 3). Also the results show a 2.3-fold increase in SOD activity in HGJ pre-treated rats before Cd exposure when compared with rats exposed to Cd alone (Fig. 3). Similarly, there was 4.23-fold increase in SOD activity in AA pre-treated rats when compared with rats exposed to Cd alone (Fig. 3). These set of data seems to suggest that AA is more effective in enhancing SOD activities than HGJ in the presence of Cd.

**Effects of Heated garlic juice and ascorbic acid on Catalase activities after cadmium exposure**

SOD is an incomplete antioxidant enzyme that requires the presence of catalase for the complete removal of superoxide anions. Therefore, in order to evaluate whether HGJ and AA have any effects on the activities of catalase after Cd exposure, rats were pre-treated orally with either 100 mg/kg body weight of either HGJ or AA for 4 weeks before 3 days treatment with 4 mg/kg body weight of CdCl₂. The results show significant increase of 1.82 and 1.68-fold in catalase activities in the liver of rats treated with HGJ and AA respectively when compared with control (Fig. 4). The results also show a 1.37-fold decrease in catalase activity in the presence of Cd (Fig. 4). The results also show that HGJ pre-treatment caused 3.6-fold significant increase in catalase activities when compared with rats exposed to Cd alone (Fig. 4). Similarly, a 5.2-fold significant increase in catalase activity was observed in the liver of AA pre-treated rats when compared with rats exposed to Cd alone (Fig. 4). These set of data suggest that both HGJ and AA protects liver against Cd toxicity by enhancing catalase activities.

HGJ and AA alter the expression of antioxidant enzymes in the presence or absence of Cd

Nrf2 is a transcription factor that regulates the expression of antioxidant enzymes thereby attenuating ROS level in oxidative stress. In this study, the level of Nrf2 and Nrf2-regulated genes; NQO1 and HO-1, were determined by western blots in the liver of rats after pre-treatment with either HGJ (100 mg/kg) or AA (100 mg/kg) before Cd (4 mg/kg) exposure. The results show that AA pre-treatment significantly enhanced the induction of Nrf2 and NQO1 proteins when compared with Cd (Figs. 5A and B). No significant changes were seen in HGJ pre-treated rats when compared with control (Figs. 5A and B). On the other hand, the pre-treatment with HGJ caused a significant increase in HO-1 expression when compared with Cd (Figs. 5A and B). The presence of either HGJ, AA and Cd alone however caused a significant increase in the antioxidant enzymes when compared with control.

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**Fig. 3.** Levels of superoxide dismutase activities in rats liver pre-treated with either HGJ or AA before Cd exposure. Rats were pre-treated with 100 mg/kg body weight of either HGJ or AA for 4 weeks before 3 days intraperitoneal exposure to 4 mg/kg body weight CdCl₂. SOD activities were measured. Data represents the mean activity (n = 5) relative to total cell protein ± S.D. ***p < 0.005, **p < 0.01, *p < 0.05 (one way ANOVA, Dunnett’s post test). † represents significant different between pre-treated and unpre-treated rats (unpaired student t-test).

**Fig. 4.** Levels of catalase activities in rats liver pre-treated with either HGJ or AA before Cd exposure. Rats were pre-treated with 100 mg/kg body weight of either HGJ or AA for 4 weeks before 3 days intraperitoneal exposure to 4mg/kg body weight CdCl₂. catalase activities were measured. Data represents the mean activity (n = 5) relative to total cell protein ± S.D. ***p < 0.005, **p < 0.01, *p < 0.05 (one way ANOVA, Dunnett’s post test). † represents significant different between pre-treated and unpre-treated rats (unpaired student t-test).
These data suggest an involvement of Nrf2 and Nrf2-regulated enzymes in mediating HGJ and AA protection against Cd toxicity in the exposed rat liver.

DISCUSSION

In this study we examined the protective effects of heated garlic juice (HGJ) against cadmium-induced toxicity in liver of exposed rats and these effects were compared with that of a known antioxidant, ascorbic acid (AA). Examination of Cd toxicity, using MDA levels as an index of oxidative stress, show that both HGJ and AA prevent Cd-induced membrane lipid damage. Many studies have reported increase lipid peroxidation in liver of rats exposed to Cd (El-Maraghy et al., 2001; Casalino et al., 1997; Müller, 1986). The present study was in agreement with these earlier studies as increase MDA was observed in the liver of rats after 3 days exposure to 4 mg/kg body weight Cd. The increase MDA levels observed in the presence of Cd may be as a result of enhanced oxida-

Fig. 5. Western blots analysis of the expression levels of antioxidant enzymes in liver of rats homogenates pre-treated with either HGJ or AA before Cd exposure. Rats were pre-treated with 100 mg/kg body weight of either HGJ or AA for 4 weeks before 3 days intraperitoneal exposure to 4 mg/kg body weight CdCl₂. Protein expression levels of (A) Nrf2, NQO1 and HO-1 were determined by western blots analysis. (B) Histogram for the expressions of these proteins. Data represents the mean band intensities of three different experiments (n = 3) relative to β-actin ± S.D. ***p < 0.005, **p < 0.01, *p < 0.05 (one way ANOVA, Dunnett’s post test). # represents significant different between pre-treated and unpre-treated rats (unpaired student t-test).
tive stress due to increase production of reactive oxygen species (Stohs et al., 1995). Lipid peroxidation is known to induce cellular damage and it is responsible for reactive oxygen species induced organ damage (Halliwell and Gutteridge, 1989). The present study shows that the increase MDA caused by Cd may be due to increase ROS production. The increase in lipid peroxidation in the liver caused by cadmium was significantly prevented by pre-treatment with ascorbic acid and HDJ. This may be due to the decrease in ROS observed in the presence of HGJ and AA. However, the results show that AA seems more effective than HGJ in preventing Cd-induced lipid peroxidation.

It has earlier been reported that heated garlic juice is less effective than aged garlic extract in preventing lipid peroxidation (Borek, 2001). HGJ contains mainly alliin due to the inactivation of enzyme allinase as a result of the heating process and this allinase was reported to be responsible for converting alliin to allicin, the precursor of most bioactive compounds in age garlic extract (Banarjee and Maulik, 2002). Therefore, the presence of alliin in HGJ may account for the less effectiveness of HGJ in preventing lipid peroxidation caused by Cd when compared with AA.

The most abundant reactive oxygen species (ROS) generated in living cells are superoxide anion and its derivatives, particularly highly reactive and damaging hydroxyl radicals, which induce lipid peroxidation of cell membrane lipids (Hemnani and Parihar, 1998). Increase in superoxide dismutase (SOD) activity of organs appears to be beneficial in the event of increase free radical generation. However, it has been reported that a rise in superoxide dismutase activity without a concomitant rise in the activity of catalase might be detrimental (Herman, 1991). Thus a simultaneous increase in catalase activity without a concomitant increase in SOD activity may be essential for a beneficial effect of increase in SOD activity. Therefore, the increase in SOD and catalase activities in liver following pretreatment with heated garlic juice and ascorbic acid has a special significance in relation to cellular defense against oxidative stress. Cd has been reported in many studies to inhibit the activities and expression of most antioxidant enzymes such as SOD and catalase (Casalino et al., 1997; Hussain et al., 1987; Liu et al., 2002; Shukla et al., 1989). The reduction in SOD and catalase activities observed in the presence of Cd may be responsible for the increase ROS and MDA. This means that the enhancement of SOD and catalase activities by HGJ and AA pre-treatment may account for the reduction in MDA level due to the enhanced removal of the superoxide anion and hydrogen peroxide. It has earlier been reported that heated garlic juice reduces tumor growth in mice (Kasuga et al., 2001) and this may be due to the enhanced expressions and activities of SOD and catalase antioxidant enzymes observed in this study.

Nrf2 is a transcription factor that regulates the expression of antioxidant enzymes in response to stress (Nguyen et al., 2009). Nrf2 is sequential in the cytosol by keap1 repressor, but in the presence of oxidative stress, the Nrf2 dissociate from its keap1 repressor and migrate to the nucleus where it bind to the antioxidant response element (ARE) to activate transcription of protective enzymes (Motohashi and Yamamoto, 2004). Since Cd generates ROS (Liu et al., 2009), it is possible that the increase ROS induced the release of Nrf2 from keap1 repressor resulting in enhanced production of Nrf2-dependent enzymes such as NQO1 and HO-1. It has earlier be shown that Cd-induced the migration of Nrf2 from cytosol into the nucleus in 1321N1 cells via PKCδ leading to increase expression of HO-1 and NQO1 (Lawal and Ellis, 2011). Therefore the increase in Nrf2, NQO1 and HO-1 protein expressions seen in this study may be due to the increase ROS production by Cd. The increase protein levels of Nrf2 and NQO1 in AA and HO-1 in HGJ pre-treated rats may be responsible for the decrease MDA and ROS levels observed in these rats after Cd exposure.

The result of this study suggests that both ascorbic acid and heated garlic juice are potent in preventing cadmium-induced liver damage in rats and ascorbic acid seems to be more effective.

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