International Food Research Journal 23(4): 1663-1668 (2016)
Journal homepage: *http://www.ifrj.upm.edu.my*

Novel cytoprotective effect for Cd-toxicity by using garlic extracts

[1]Boonpeng, S., [2*]Siripongvutikorn, S., [1]Sae-Wong, C. and [3]Sutthirak, P.

[1]Nutraceutical and Functional Food Research and Development Center,
[2]Department of Food Technology, Faculty of Agro-Industry, Prince of Songkla University,
Hat Yai, Songkhla, 90112,Thailand
[3]Aquatic Animal Biotechnology Research Center, Faculty of Sciences and Industrial Technology,
Prince of Songkla University, Mueang, Suratthani 84000, Thailand

Article history

Received: 25 May 2014
Received in revised form:
13 November 2015
Accepted: 1 December 2015

Keywords

Anti-Cd
Garlic
Pickled garlic
Cytoprotective

Abstract

Cd contamination is a very serious international problem as it can enter into the food system, become bioaccumulated, and endanger human health. Detoxifying Cd is challenging because it is usually not successful. Garlic (*Allium sativum* L.) has been used for various purposes such as cooking ingredient, nutraceutical and functional food and folklore medicine as antibacterial, antioxidant, and anti-tumorogenic agent for over 5000 years. Thais usually consume garlic in both fresh and pickled forms in their daily food. This present work aimed to seek new alternative way to reduce Cd toxicity. The anti-Cd property of aqueous garlic extracts (in both fresh and pickled form) was determined and compared with standard diallyl disulfide (DADS) by using HEK 293 cells as target cells. The result showed that pickled garlic extract exhibited cytoprotective property better than fresh garlic extract and had less toxicity compared with DADS. Moreover, the extracts and DADS clearly possessed cytoprotective effect when the cells were treated with the sample before exposed to Cd. Therefore, consumpting fresh and pickled garlic can be a good alternative treatment to protect Cd toxicity particularly in pickled form.

Introduction

Cadmium (Cd) is an inorganic toxicant affecting human health through occupational and environmental exposure. Main sources of human exposure to Cd are food, water and fumes that contaminated from heavy industries such as electroplating, plastic production, battery manufactures and pesticides (Pari *et al*., 2007). It is classified as a group I human carcinogen and has long biological half-life that importantly makes it a cumulative toxin (Waalkes, 2000). Cd acts as catalyst to increase oxidative stress in the formation of reactive oxygen species (ROS), induce lipid peroxidation, and reduce glutathione and protein-bound sulfhydryl groups (protein-SH). The kidney is target organ that damaged with both acute high-dose but more commonly, long term chronic exposures (ATSDR, 2013). Many researchers reported that Cd induced mitochondrial injury and apoptosis in vero cells (Murugavel *et al*., 2007). The most injured three human cell lines were human liver hepatoma cells (HepG2) followed by human embryonic kidney cells (HEK 293) and human astrocytoma cells (1321NI), respectively (Lawel and Ellis 2010). Actually, synthetic chemicals in form of chelating agents and antagonist are attempted to cure Cd toxicity, however some of them are undesirable side effects. Therefore, Cd intoxication therapy from medical plants is extremely interesting.

Garlic (*Allium sativum* L.) is an important cooking ingredient not only in Thai dishes but also Asian cuisines. It is also used as an herb and spice for treatment and prevention several illness as anti-acne, antimutagenic, antioxidant and detoxifying (Pari *et al*., 2007). Organosulfur compounds (OSCs), well-known bioactive compound of garlic, are classified in two groups as oil-soluble and water-soluble. Allicin which is water-soluble and easily transformed in to oil-soluble polysulfide as mostly diallyl disulfide (DADS) 67.7%, diallyl trisulfide (DATS) 14.6%, diallyl sulfide (DAS)13.3%, and diallyl tetra sulfide (DTS) 5.4% when allicin allowed to standard at room temperature for 20 h. Water-soluble compounds are odorless and also formed during aqueous garlic extraction, when the primary compound γ-glutamyl-S-allylcysteine (GSAC) is transformed into S-allylcysteine (SAC) and this reaction is catalyzed by γ-glutamyltranspeptidase (γGT). SAC along with its derivatives, S-methylcysteine (SMC) and S-allylmercaptocysteine (SAMC) are components of aqueous extracts of garlic and have reported biological activity both in vitro and *in vivo* assay.

*Corresponding author.
Email: *sunisa.s@psu.ac.th*
TeL: +66-74-286344; Fax: +66-74-558866

Several studies addressed that aged garlic extracts (AGE) had huge antioxidant potential and contained most of SAC and SAMC (water-soluble compounds) and a few of oil-soluble compounds (Iciek *et al*., 2009).

In Thailand, garlic is commonly consumed in forms of bulbs, crushed or chopped, and pickled. To extend shelf life and reduce the strong odor of garlic as well as generate a new product, pickling process was reported (Boonpeng *et al*., 2014). Our previous studied has shown that fresh garlic and pickled garlic extracted with distilled water possessed antioxidant activities. Moreover, anti-Cd property of pickled garlic exhibited the higher activity than fresh garlic when treated in HEK 293 cells. Both fresh and pickled garlic extracts showed higher cell survival compared with standard diallyl disulfide (DADS) (Boonpeng *et al*., 2014). The present study aimed to evaluate and prove the protective effect of garlic extracts on Cd toxicity using extracellular and intracellular assay as well as cell viability.

Material and Methods

Thai garlic grown in northern part of Thailand during February – April, 2014 was used as raw material for fresh and commercial pickled one following small and medium enterprises (SMEs) method. Both fresh and pickled garlic forms were analyzed for moisture content (AOAC, 1995), pH and aw followed the method of AOAC (1990).

Chemical

Chemical used for determination of anti-cadmium toxicity were purchased from Gibco® and Invitrogen, USA. Most chemicals used for extracellular and intracellular determination were purchased from Sigma, Germany beside QRAC, New Zealand and LAB-SCAN, Ireland.

Preparation of garlic extracts and DADS solution

Fresh garlic bulb and pickled garlic were obtained from the same field and lot in Lamphun province, the northern part of Thailand. DADS (Sigma, Germany) was used as a standard agent. Garlic extracts and DADS solution preparation were prepared according to Boonpeng *et al.* (2014). Briefly, fresh and pickled garlic extracts at the concentration of 50, 100 and 200 µg/mL and 7.31, 14.63 and 29.26 µg/mL of DADS were used based on higher than 80% of the cell viability.

DADS content determination

DADS determination was performed according to the method proposed by Wan *et al.* (2007). Fresh and pickled garlic extracts were analyzed by High Performance Liquid Chromatography (HPLC), 1100, Agilent Technologies, Germany. Experiments were performed with a column Zorbax Eclipse XDB C8 (4.6 x 150 mm. and 5 µM particle). The mobile phase consisted of water and methanol. The flow rate was 1.0 mL/min and wavelength detector was 210 nm.

Cell culture

Human embryo kidney cells (HEK 293) were purchased from American Type Culture Collection. HEK 293 cell lines were grown in Minimum Essential Medium (MEM), supplemented with 10% Fetal bovine serum (FBS) and 1% penicillin–streptomycin. The cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air for analysis.

Effect of $CdCl_2$, fresh and pickled garlic extracts and DADS on cell viability

Cell viability was determined after cells were grown following the cells culture method. Cells were plated in a 96-well plate at a density of 1 x 10^6 cells/mL and allowed the cells attached to the culture plate for 24 h before treated with the sample as follow:

Group 1: treated with the fresh and pickled garlic extracts at a concentration of 0, 50, 100, 200 µg/mL and DADS at the concentration of 7.31, 14.63 and 29.26 µg/mL for 24 h, then followed by treated with 135.8 µM $CdCl_2$ and incubated for 24 h.

Group 2: treated with 135.8 µM $CdCl_2$ together with fresh and pickled garlic extracts at the concentration of 0, 50, 100, 200 µg/mL and DADS at the concentration of 7.31, 14.63 and 29.26 µg/mL and incubated for 24 h.

Group 3: treated with 135.8 µM $CdCl_2$ for 24 h then followed by treated with fresh and pickled garlic extracts at the concentration of 50, 100, 200 µg/mL and DADS at the concentration of 7.31, 14.63 and 29.26 µg/mL and incubated for 24 h.

Extracellular and intracellular Cd accumulation

The cells were seeded in 75 cm^2 flask at a density of 1 x 10^4 cells/mL for 6 days. On the third day after cells seeding, the culture media was changed and at day 6, the cells were divided into 3 groups and treated as mentioned above. Extracellular Cd accumulation was done by taking culture media from each treatment without the adhered HEK 293 living cells on the flask surface.

Then, HEK 293 cells were harvested and washed twice with 3 mL of phosphate-buffered saline (PBS). The cells were centrifuged at 3000 g for 5 min then the pellet was resuspended in 5 ml of a cell lysis

buffer. The cell lysate was digested overnight at 37 °C with 100 ml of 10% sodium dodecyl sulfate. After digestion, 1.4 mL of saturated NaCl (6 M) was added and shaken vigorously for 15 s, followed by centrifugation at 3000 g for 20 min. The supernatant was separated and used for intracellular analysis. Total Cd content of extracellular and intracellular was done by Inductively Coupled Plasma- Optical Emission Spectrometry (ICP-OES), optima 4300DV Perkin Elmer, Massachusetts, USA.

Statistical analysis

Results were analyzed using one way analysis of variants (ANOVA) and mean comparisons were performed using the Duncan's new multiple range test (DMRI). Statistical analyses were carried out using the SPSS statistical software (SPSS, Inc., Chicago. IL).

Results

Physical quality of fresh and pickled garlic

The moisture content, water activity, and pH of fresh garlic were 64.37 ± 0.15 (g/100g), 0.99 ± 0.001 and 6.09 ± 0.05, respectively while these values of pickled garlic were 84.57 ± 0.09 (g/100g), 0.97 ± 0.001 and 3.89 ± 0.27, respectively.

DADS content

DADS content of fresh and pickled garlic extracts determined by HPLC technique, were 25.92 ± 0.25 and 10.31 ± 0.06 µg/g.

Effect of $CdCl_2$, fresh and pickled garlic extracts and DADS on cells viability

As previous studied, IC_{50} of $CdCl_2$ at 135.8 µmol/L (1.7 x 104 µg/mL) determined by MTT assay (Boonpeng et al., 2014), was used in this experiment. It was found that cells viability were highest when fresh or pickled garlic extracts or DADS were pre-introduced before exposed to $CdCl_2$ (Figure 1 A-C in deep black bar).

When the fresh, pickled garlic extract or DADS and $CdCl_2$ were added together into the cells, death cells increased particularly in DADS at the highest concentration, 29.26 µg/mL (Figure 1A-C in grey bar). The cells viability of DADS at 29.26 µg/mL was lowest ($p<0.05$) when compared with fresh and pickled garlic extracts.

Effect of fresh and pickled garlic extracts and DADS on Cd accumulation in HEK 293 cells

To prove that the accumulation of cadmium is associated with the toxicity of cadmium, extracellular

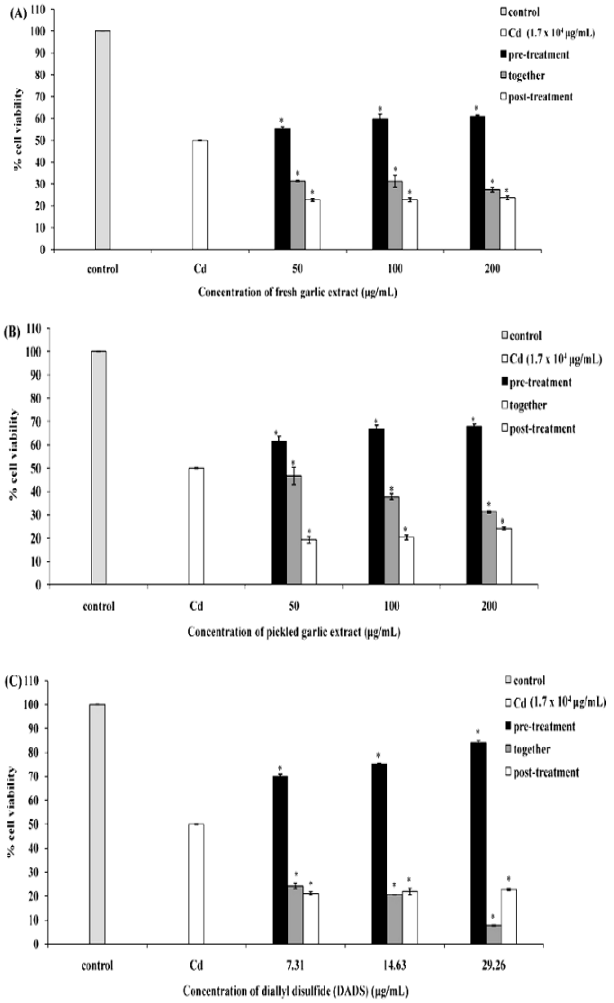

Figure 1. Anti-Cd toxicity properties on HEK 293 cells was determined using MTT assay. (A) The cells were treated with fresh garlic extracts, (B) The cells were treated with pickled garlic extracts and (C) The cells were treated with diallyl disulfides (DADS); each value is expressed as a mean ± SD (n=3). *Statistically significant in comparison to cells treated with Cd alone as $p\leq0.05$. presents the cells pre-treated with fresh or, pickled garlic extracts or DADS before exposure to Cd, ■ presents the cells treated with fresh or pickled garlic extracts or DADS together with Cd and ■ presents the cells exposed to Cd before post-treating with fresh or pickled garlic extracts or DADS

and intracellular Cd content were evaluated. For extracellular accumulation, Cd content of every treatment was not significantly different ($p>0.05$) and similar to sample treated with Cd only (Figure 2 A-C). Cd accumulated in cells or claimed as intracellular of living cells was showed in Figure 3A-C. Its content in pre-treatment group of fresh and pickled garlic extracts was lower than DADS group. As showed in Figure 3B, Cd accumulated in the cells when $CdCl_2$ and DADS treated together were lower than treated with $CdCl_2$ alone may due to cells

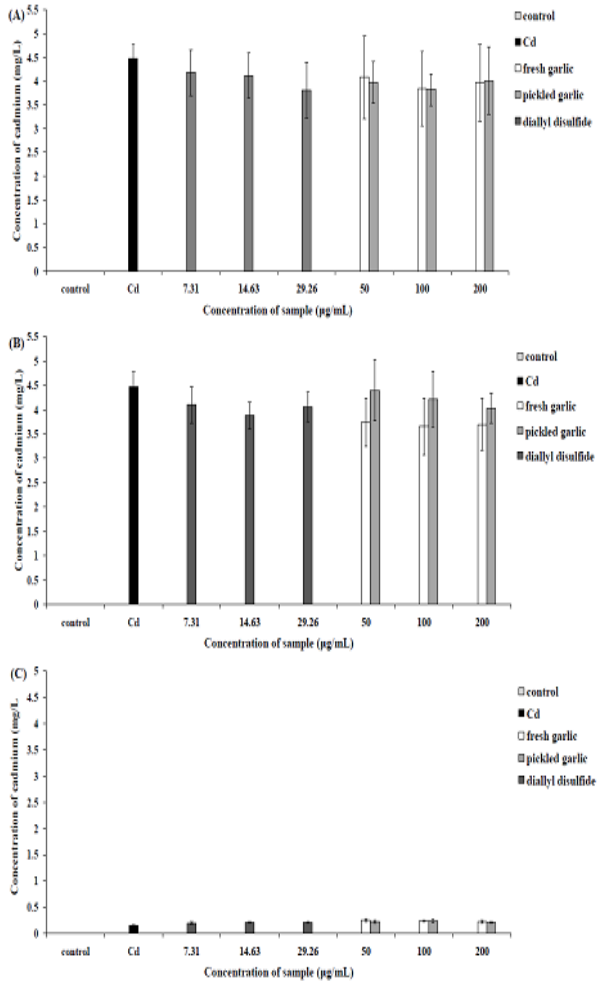

Figure 2. The extracellular Cd accumulation on HEK 293 cells was determined using ICP-OES. (A) The cells were pre-treated with fresh or pickled garlic extracts or DADS before exposure to Cd, (B) The cells were treated with fresh or pickled garlic extracts or DADS together with Cd and (C) The cells were exposure to Cd before post-treated with fresh or pickled garlic extracts or DADS; each value is expressed as a mean ± SD (n=3).

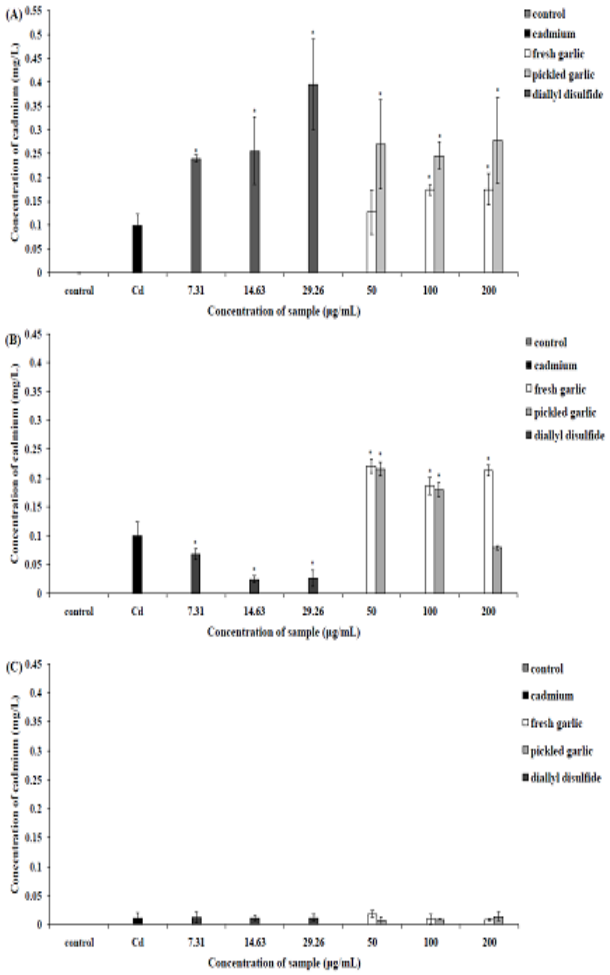

Figure 3. The intracellular Cd accumulation on HEK 293 cells was determined using ICP-OES. (A) The cells were pre-treated with fresh or pickled garlic extracts or DADS before exposure Cd, (B) The cells were treated with fresh or pickled garlic extracts or DADS together with Cd and (C) The cells were exposed to Cd before post-treated fresh or pickled garlic extracts or DADS; each value is expressed as a mean ± SD (n=3).*Statistically significant in comparison to cells treated with Cd alone.

death by Cd toxic. In Figure 3C, post-treatment of garlic extract or DADS demonstrated the lowest Cd accumulation in the cells. This pointed out that Cd is very poisonous element and contact time to remove its toxin is very short. In addition, recover or revital cell pre-exposed to Cd by using the extracts and DADS is not successful.

Discussion

Physical quality of fresh and pickled garlic

According to the results, It showed that pickling process reduced pH by producing more acid via mainly vinegar addition.

DADS content

Pickled garlic extract contained lesser DADS compared with fresh garlic extract may due to leaching effect during pickling. Actually DADS, a less polar is easily to dissolve in acetic condition containing vinegar.

Effect of $CdCl_2$, fresh and pickled garlic extracts and DADS on cells viability

Cells viability of pre-introduced DADS was highest (>70 -80%) followed by pickled (60-70%) and fresh (55-60%) garlic extracts. This result showed that pre-introduced DADS and the garlic extracts could protect cells death injured with Cd. From the result, the fresh, pickled garlic extract or DADS and $CdCl_2$ were added together into the cells, as known

that garlic contains OSCs, which may probably react with Cd. It may be justifiable assumed that cadmium sulfide (CdS) took place and synergist more toxicity than $CdCl_2$ toxicity alone (Lewis, 1992). It pointed out that sulfide compounds containing in the garlic particularly in pure DADS form interacted with Cd leading to higher toxicity. Moreover, the result also showed that cells survival was not successful when the HEK 293 cells was treated with $CdCl_2$ before using DADS and the garlic extracts (Figure 1A-C in white bar). This implied that $CdCl_2$ was very dangerous compound efficiently killing cells with less reversible process.

In general, fresh, pickled garlic extracts and DADS could be prevent HEK 293 cells dead when they were introduced before applying $CdCl_2$, Therefore, cytoprotective effect but not detoxifying property were explanation. Moreover, this finding suggested that treated DADS possessed strong anti-Cd as protective effect but also synergist toxicity of Cd in some circumstance.

Effect of fresh and pickled garlic extracts and DADS on Cd accumulation in HEK 293 cells

This Cd accumulation in the extracellular was significantly higher than intracellular ($p<0.05$) about 10 times. This may be due to the limitation of Cd entering into the cells as affected of homeostasis (Kerkhove *et al.*, 2010). On the other hand, it may be due to living cells possessed their cytoprotective effect via glutathione pathway (Lawal and Ellis, 2011).

Furthermore, dose dependent effect of DADS resulted in a dramatically increasing intracellular Cd content (Figure 3A). This result could suggest that organosulfur compounds particularly DADS increased cells strength via glutathione pathway (Lawal and Ellis, 2011) then $CdCl_2$ could be triggered with metallothionine (MT). Therefore, Cd toxicity was reduced so the cells could resist to higher Cd accumulation. In addition, Cd content in the cells treated with the garlic extracts and DADS compared with treated Cd alone may directly react with Cd and had less toxicity (Boonpeng *et al*., 2014).

Cd content in the cells was decreased when increased DADS concentration. It confirmed that DADS interacted with Cd and caused higher toxicity when cells were treated together with $CdCl_2$. In addition, higher cells viability and Cd content in intracellular cells treated the garlic extracts together with $CdCl_2$ were found. Pickled garlic extract possessed higher anti-Cd toxicity compared with fresh garlic extract may explain by acidic environment of solution (pH 3.71) to facilitate zinc and copper to easier dissolve and block Cd for entering into the cells (Mike, 2012). Shaikh *et al.* (1995) addressed that the most potent antagonist of Cd accumulation are essential zinc and copper metal. In fact, entering of metal whether essential and toxic metal into cells compose of two step processes; first step, by diffusion through nonspecific ion pore or specific ion channels like as Cd^{2+}, Na^+ and K^+. Second step, the metal might be internalized in protein bound form through receptor-mediated endocytosis. As both essential and toxic metal use the same pathways therefore, toxicity of heavy metal could be reduced through some active essential metal from competition mechanism. In addition, Oustan *et al*. (2011) reported that acetic acid a major component in vinegar exhibited high Cd removal as its chelating ability.

In order to data obtained from Figure 1A and take to compare with Figure 3A found that cells viability and Cd-accumulation of the cells treated with DADS and the garlic extracts were highest. It could be confirmed that organosulfur compounds containing in the sample played an important role to protect Cd-toxicity by glutathione pathway (Imai *et al*., 1994; Ana *et al.*, 2012). Colín-González *et al.* (2012) reported that SAC was mainly composed in aged garlic extracts (AGE) which claimed to scavenge hydrogen peroxide (H_2O_2), hydroxyl radical (·OH) and superoxide anion ($O_2^{\cdot-}$). Furthermore, AGE prevented cell death treated Cd in 1321N1 and HEK 293 cells.

In addition, other antioxidant properties such as metal chelating activity of acetic acid and maillard reaction product from pickled garlic extract (Oustan *et al*., 2011) and free radical scavenging activity of many organosulfur agents may also help to reduce cell injured by treated Cd (Suru, 2008). However, these compounds also are more toxic when they were applied together with $CdCl_2$. Moreover, the cells were exposed to the $CdCl_2$ and death, using DADS and the garlic extracts to recover or revival the cells did not help.

Conclusion

Garlic extracts, fresh and pickled form and DADS showed cytoprotective ability of HEK 293 cells treated with $CdCl_2$. The possible effects of garlic extracts and DADS to protect HEK 293 cells were induced GSH and GST production for reduce toxicity. Moreover, the garlic extracts and DADS had more protective effect than curing effect. Therefore, the garlic consumption in daily food can protect or reduce toxic from Cd or heavy metal like Cd which contaminated in food or the environment. The more

cytopreventive effect, the more Cd content found intracellular. There is possible that consuming both fresh and pickled garlic as daily food may protect or reduce toxicity of Cd or other heavy metals contaminated in food or drink.

Acknowledgement

This research was financial supported by Aquatic Animal Biotechnology Research Center, Faculty of Science and Industrial Technology, Prince of Songkla University, Surat Thani Campus, and Graduate School of Prince of Songkla University, Hat-Yai campus.

Reference

A.O.A.C. (Association of official analytical chemists). 1990. Official methods of analysis of AOAC. 15th edn. Allengton: Washington D.C.

A.O.A.C. (Association of official analytical chemists). 1995. Official methods of analysis of AOAC. 16th edn. Allengton: Washington D.C.

Internet: Agency for Toxic Substances and Disease Registry in USA 2013. Case Studies in Environmental Medicine. Download from *http://www.atsdr.cdc.gov/csem/* on 13/5/2013.

Ana, L. C. G., Ricardo, A. S., Carlos, A. S. I., Maria, E. C. C., Abel, S. and Perla, D. M. 2012. The antioxidant mechanisms underlying the aged garlic extract- and S-allylcysteine-induced protection. Oxidative Medicine and Cellular Longevity 2012:1-16.

Boonpeng, S., Siripongvutikorn, S., Sae-Wong, C. and Sutthirak, P. 2014. The antioxidant and anti-Cadmium toxicity properties of garlic extracts. Food Science and Nutrition 2(6): 792-801.

Colín-González, L., A., Santana, A., R., Silva-Islas, A., C., Chánez-Cárdenas, E., M., Santamaría, A. and Maldonado, D., P. 2012. The antioxidant mechanisms underlying the aged garlic extract-and S-allylcysteine-induced protection. Oxidative Medicine and Cellular Longevity 2012: 1-16.

Iciek, M., Kwiecień, I. and Wlodek, L. 2009. Biological properties of garlic and garlic-derived organosulfur compounds. Enviromental and Molecular Mutagenesis 50(3): 247-265.

Imai, J., Ide, N., Nagae, S., Moriguchi, T., Matsuura, H. and Itakura, Y. 1994. Antioxidant and radical scavenging effects of aged garlic extract and its constituents. Planta Medica 60(5): 417-420.

Lawal, O. A. and Elizabeth, M. E. 2010. Differential sensitivity and responsiveness of three human cell line HepG2, 1321N1 and HEK 293 to cadmium. The Journal of Toxicological Sciences 35: 465-478.

Lawal, O. A. and Elizabeth, M. E. 2011. The chemopreventive effects of aged garlic extract against cadmium-induced toxicity. Environmental Toxicology and Pharmacology 32(2): 266-274.

Lewis, J. R. 1992. Condensed chemical dictionary. 12th edn. United States of America: New York.

Internet: Mike, L. 2012. Effect of pH on heavy metal concentration. Downloaded from *http://humate.com/wp-content/uploads/* on 6/1/ 2015).

Murugavel, P., Pari, L., Sitasawad, S. L., Sandeep, K. and Santhosh, K. 2007. Cadmium induced mitochondrial injury and apoptosis in vero cell: Protective effect of diallyl tetrasulfide from garlic. The International Journal of Biochemistry and Cell Biology 39(1): 161-170.

Oustan, S., Heidari, S., Neyshabouri, R. M., Reyhanitabar, A. and Bybordi, A. 2011. Removal of heavy metals from a contaminated calcareous soil using oxalic and acetic acids as chelating agents. International Conference on Environment Science and Engineering 8: 152-155.

Pari, L., Murugavel, P., Sitasawad, S. L. and Kumar, S. 2007. Cytoprotective and antioxidant role of diallyl tetrasulfide on cadmium induced renal injury: An in vivo and in vitro study. Life Sciences 80(7): 650-658.

Shaikh, A. Z., Blazka, E. M. and Endo, T. 1995. Metal transport in cells: cadmium uptake by rat hepatocytes and renal cortical epithelial cells. Environmental Health Perspective 103: 73-75.

Suru M. S. 2008. Onion and garlic extracts lessen cadmium-induced nephrotoxicity in rats. Biometals 21: 623-633.

Waalkes, M. P., Rehm, S. and Cherian, M. G. 2000. Repeated cadmium exposures enhance the malignant progression of ensuing tumors in rats. Toxicological Sciences 54(1): 110-120.

Wan, X., Polyakova, Y. and Row, H. K. 2007. Determination of diallyl disulfide iin garlic by reversed-phase high performance liquid chromatography. Analytical Science and Technology 20(5): 442-447.