Research Article
AMERICAN JOURNAL OF PHARMACY AND HEALTH RESEARCH
www.ajphr.com
2016, Volume 4, Issue 6
ISSN: 2321–3647(online)

Antioxidative and Free Radical Scavenging Properties of *Piper Cubeba* (Piperaceae) In Mercury Intoxicated Mice, *Mus Musculus*

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ABSTRACT

This experimental study was carried out to evaluate the protective potential of *Piper cubeba* in mercury induced changes in Hepatic antioxidant activities after short-term dose of the heavy metal in mice. Twenty four, two month old male mice were divided equally and randomly into four groups viz., I, II, III and IV. Group I served as control. Group II and III were oral dosed on mercury chloride at sub lethal dose (5mg of $HgCl_2$/ Kg body weight of animal) on every day for4 7 days respectively. After 7^{th} day the group III animals alone again fed with the methanolic extract of *Piper cubeba* fruits at a dose of 200 mg/kg body weight for another 7 days. There was significant decrease in protein metabolism, catalase (CAT), reduced glutathione (GSH) and glutathione peroxidase (GPx) in group II animals, simultaneously an increased in the level of lipid peroxidation (LPO) was also recorded in group II animals. Group IV animals did not show any significant alteration in protein metabolism and antioxidant properties of the animal. The mercury intoxicated mice again treated with methanolic extract of *P. cubeba* restoring the liver to reorganize its protein metabolism and antioxidant properties to near normal level/ a little over untreated control. The result suggested that medication of *P. cubeba* nullify the mercury toxicity in mice.

Keywords: *Piper cubeba*, Glutathione, Catalase, LPO etc.,

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Received 18 February 2016, Accepted 21 March 2016

Please cite this article as: Jagadeesan G *et al*., Antioxidative and Free Radical Scavenging Properties of Piper Cubeba (Piperaceae) In Mercury Intoxicated Mice, Mus Musculus. American Journal of Pharmacy & Health Research 2016.

INTRODUCTION

Among various kinds of pollutants heavy metals have ecotoxicological significance because of their increasing input, extended persistence (Gupta and Raghubanshi, 2002)[1], wide spread toxicity to biota and their tendency to accumulate and biologically magnified (Kapila Manoj and Ragothaman, 1999][2]. Among the effects of highly toxic heavy metals attention has been focussed on the toxic effect of mercury after Minamata Bay Tragedy (1950), outbreaks of mercury poisoning from Guatimala (1960s), Pakistan (1969) and Iraq (1971). Mercury is a widespread environmental and industrial pollution, which induces severe alterations in the tissues of both animals and men (Lund *et al.,* 1991; Mahboob *et al.,* 2001)[3,4]. Mercury is known to be the most toxic of all heavy metals (Taylor, 1979)[5].

There are many sources of mercury inputs to the biosphere. Natural sources are significant contributors. Approximately 10,000 tons of mercury and its compounds are originates from degassing of earth's crust, to this amount approximately 20,000 tons/year is added by anthropogenic activity (Hansen and Dasher, 1997)[6]. Mercury and its compounds are used in varying amounts in batteries, fluorescent and high intensity light bulbs, thermometers, thermostats, light switches, certain types of dental fillings, vaccines (Zahir *et al*., 2005)[7], skin whitening creams and soaps (Harada *et al.*, 2001)[8]. Mercury also released into the atmosphere by the burning of medical wastes, wood and fossil fuels such as coal and oil (Kureishy *et al.,* 1979)[9]. In addition to industrial activities, worldwide agriculture and mining have also contributed major amounts of mercury to soil, water and air (California EPA, 1999)[10]. It is estimated that the mercury emission will increase approximately at a rate of 5% per year (Zhang *et al.,* 2002)[11]. Under normal physiological condition animals maintain a balance between generation and neutralization of ROS (Bharathi and Jagadeesan, 2014)[12]. The mercury induced free radical stress has been cited widely in literature (Ali *et al.,* 1992; Hussain *et al.,* 1998; Berntssen *et al.,* 2003; Milaeva, 2006)[14-17]. An imbalance in the antioxidant protective mechanisms leading to oxidative stress in the cells is beings identified as a common factor in mercuric chloride exposure. Living organisms are normally protected against oxidative stress by various enzymatic and non-enzymatic compounds endowed with antioxidant activity (Bharathi *et al*., 2014)[13]. Antioxidants can bind to free radicals before the free radicals cause harm. Antioxidants are known to reduce oxidative radical induced reaction (El-Demerdash *et al.,* 2004)[18]. Antioxidants systems protect the cells against the adverse effect of ROS (Valentine *et al.,* 1998)[19]. Free radicals or ROS generated in tissues are effectively scavenged by the antioxidant

defense system that constitutes antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx). In addition to antioxidant enzymes, non-enzymatic molecules including thiols and disulfide bonding play a vital role in antioxidant defense system. Some of the antioxidants are obtained from food (Prior, 2003)[20]. Particularly enormous amount of antioxidant constituents are synthesized in some of the herbal plants (Jagadeesan and Margarat, 2010)[21].

Medicinal herbs continue to play an important role in health care (Persuto, 1996)[22]. Many herbal preparations may be therapeutically effective even for diseases in which conventional drugs are ineffective. Remedies from plant sources proved to be very popular in primary health care in India for a long time. A worldwide trend towards the use of natural phytochemical has increased (Lee *et al.*, 2003)[23]. Several medicinal herbs have been reported to have antioxidant property (Aqil *et al.*, 2006)[24]. A great number of spices and aromatic herbs contain chemical compounds exhibiting antioxidant properties are attributed to a variety of active phytochemicals including vitamins, carotenoids, terpenoids, alkaloids, flavonoids, lignans, simple phenols and phenolic acid (Liu and Ng, 2000)[25]. *Piper cubeba* commonly known as tailed pepper belong to Piperaceae family is one of the popular medicinal plant extensively used as a spice in many countries, including India, Indonesia, Arab, Europe and Morocco. It exhibits a variety of pharmacological actions. The three groups of secondary metabolites reported from the berries of *Piper cubeba* are alkaloids, lignans and terpenoids (essential oil). Piperine is an abundant alkaloid in the berries of this species (Parmar *et al.*, 1997)[26]. The antioxidant property of piperine is evidenced (Rauscher *et al.*, 2000; Vijayakumar *et al.*, 2004, 2006)[27,28]. Lignans possess a variety of biological activities like antioxidant, antitumor, antiviral activities (MacR and Towers, 1984)[29]. *Piper cubeba* is rich in phenolic content. A direct relationship between antioxidant activity and phenolic content of plant extract has been reported (Kaur, 2002; Ivanova, 2005)[30,31] Antioxidant activity of *Piper cubeba* have been reported (Karthikeyan *et al.*, 2003; Choi *et al.*, 2005; Aqil *et al.*, 2006; Yamaguchi *et al.*, 2006)[24,32-34].

Based on these reports, the present study has designed to evaluate the possible antitoxic effect of methanolic extract of *Piper cubeba* fruits against mercuric chloride induced oxidative damage of the liver by determining bio chemical and bio-enzymological parameters.

METERIALS AND METHOD

The Animal Ethics Committee, Rajah Muthiah Medical College, Annamalai University, approved the experimental protocol. Healthy and pure strain mice, *Mus musculus*, ranging in

between the body weight of 25-30 g were procured from the Department of Experimental Science, Central Animal House, Rajah Muthiah Medical College, Annamalai University, Annamalai Nagar and used in the present experimental study.

Experimental Procedure

A total of 24 female mice were used in the present experimental studies. The mice were divided into four groups, each contains 6 animals. Normal food and water given to group I mice (control group) and mercuric chloride (at a dose of 5 mg/kg body weight dissolved in double distilled water) administered orally to II and III groups respectively for 7 days. After 7th day the III group animals alone again fed with the methanolic extract of *Piper cubeba* fruits at a dose of 200 mg/kg body weight for another 7 days. To the IV group, the methanolic extract of *Piper cubeba* fruit alone given orally for 7 days. After the scheduled treatment all the mice were decapitated by cervical dislocation. Liver from autopsied animals were collected for biochemical and bio-enzymological assays.

Group I	**Untreated control**	Provided standard diet and clear water *ad libitum* and observed for 30 days
Group II	Mercuric Chloride treatment	5 mg/ animal/day. Oral administration daily upto 7 days
Group III	Post treatment of Methanolic extract of *Piper cubeba* fruit	5 mg/animal/day. Oral administration daily upto 7 days on mercury intoxicated rats
Group IV	Methanolic extract of *Piper cubeba* fruit	5 mg/animal/day. Oral administration daily upto 7 days

Preparation of plant extract

Fresh fruits of *P. cubeba* were purchased from Chidambaram traditional medical shop and identified by a Taxonomist and preserved in the Department of Botany, Annamalai University, Annamalainagar, India. After removing impurities, it is powdered in an electric blunder. 500 gm powder was kept in the Soxhlet apparatus. Soxhlation was done by using methanol as a solvent. Soxhlation was continued upto 24 hours for separating the contents present in it.

Estimation of protein

The total Protein content of the tissue was estimated by the method of Lowry *et al.* (1951)[35]. The tissue was isolated and is quickly weighed in an electrical balance in a cold room and separately homogenized in 10% TCA. The homogenate was centrifuged at 3,500 rpm for 15 minutes. The supernatant was discarded and the residue was suspended in 1 ml of 1 N NaOH. It is centrifuged again for 3,500 rpm for 15 minutes. 0.5 ml of the solution equivalent to 10 mg of the tissue was transferred into a clean test tube and 4 ml of reagent C [Mix A (2 g of sodium carbonate in 100 ml of 0.1 N NaOH)] 50 ml and B (5 g of copper sulphate in 100 ml of 0.1% sodium potassium

tartarate) 1 ml and 0.4 ml of Folin phenol reagent (Folin phenol and water mixed in 1:1 proportion) were added. The tubes were shaken well and kept at laboratory temperature for 30 minutes. The blue colour developed was read at 620 nm against a reagent blank in a spectronic-20 spectrophotometer. Bovin serum (Sigma Chemical Co.) was used to construct the standard graph. The protein content is expressed in mg/g wet weight of the tissue.

Estimation of reduced glutathione (GSH)

Total reduced glutathione was determined by the method adopted by Beutler and Kelley (1963)[47]. The isolated tissues were homogenated in the PBS buffer solution. 0.2 ml of tissue homogenate was taken in a clean test tube and added with 1.8 ml of 0.1% EDTA solution and 3.0 ml of precipitating reagent (1.67g of metaphosphoric acid, 0.2g EDTA and 30g sodium chloride in 1 litre of distilled water) and mixed thoroughly and kept for 5 minutes before centrifugation at 3,000 rpm for 10 minutes. 1.0ml of supernatant was taken in a clean dry test tube and it was suspended in the 4.0ml of 0.3 M disodium hydrogen phosphate solution and 1.0 ml of DTNB reagents were added and read at 412 nm in an UV – spectrophotometer (Spectronic- 20)against a reagent blank. A set of standard solution containing 20-100 µg of reduced glutathione was treated similarly. The values are expressed as µg /100 mg wet weight of tissue.

Estimation of Glutathione peroxidase (GPX)

The activity of glutathione peroxide was assayed by the method of Rotruck *et al*. (1973)[36]. 0.2ml of tissue homogenate was taken in a clean test tube and the following reaction mixture was added. The reaction mixture consisted of 0.2 ml of 0.4 M phosphate buffer, 0.2 ml of 0.4 mM EDTA, 0.1 ml of 10mM sodium azide were incubated at 37°c for 2 minutes. Then 0.2 ml of 2mM reduced glutathione and 0.1 ml of 1 mM hydrogen peroxide were added to the test tubes and again incubated at 37°c exactly for 10 minutes. The reaction was arrested by the addition of 0.5 ml of 10 % TCA. The tubes were centrifuged and the supernatant was assayed for GSH by the method of Beutler and Kelley (1963) as described in section 2.12.2.3. A blank was treated similarly to which 0.2 ml of the enzyme was added after the incubation. The activity of glutathione peroxide is expressed as µg /100mg wet weight of the tissue.

Estimation of catalase (CAT)

Catalase was assayed colorimetrically with the method of Sinha (1972)[37]. Tissues were homogenized in phosphate buffer solution. 0.1 ml of the homogenate was taken in a test tube and 1.0 ml of phosphate buffer was added. 0.4 ml of 0.2 M hydrogen peroxide was added to the above mixture. After 30 and 60 seconds 2.0 ml of dichromate acetic acid reagent was added respectively. Test

tubes were kept in boiling water both (60°C) for 10 minutes. The mixture was cooled immediately in tap water and the colour developed was read at 620 nm against reagent blank in UV visible spectrophotometer (spectronic-20). Activity of catalase was expressed as a mole of hydrogen peroxide utilized/min/100 mg of protein.

Estimation of lipid peroxidation (LPO)

Lipid peroxidation was assayed by the method of Nichans and Samuelson (1968)[38]. In this method, malandialdehyde and other thiobarbituric acid reaction substances (TBARS) were measured. Tissue (liver) homogenates was prepared in Tris-Hcl buffer (pH 7.5). 1 ml of the tissue homogeneous was taken in a clean test tube and 2 ml of TBA–TCA–Hcl reagent was added and then mixed thoroughly. The mixture was kept in a boiling water bath (60°C) for 15 minutes. After cooling, the mixture was centrifuged at 1000 rpm for 10 minutes and the supernatant was taken to read the absorbance of the chromophore at 535 nm against the reagent blank in a UV visible spectrophotometer (Spectronic-20) 1,1′,3,3′-tetramethoxy-propane was used to construct the standard graph. Values were expressed as nmoles/100 mg of tissue.

RESULTS AND DISSCUSSION

Biochemical analysis of the tissues provides a means to detect the effect of pollutants at an early stage of contamination. Among biochemical parameters, the protein metabolism is significant one. Protein is an essential substance needed for growth and development. It plays an important role in the process of interactions between intra and extracellular media being a part of cell membrane. All enzymes and hormones that control body activities are made up of proteins. It also provides the energy for all vital functions during the stress condition. Mammals have inbuilt detoxifying mechanisms that operate against metal ion toxicity. Some proteins play important role in the detoxification mechanism (Cohen *et al.*, 1997)[39]. The most important antioxidant protein present in the body for neutralizing the free radicals is glutathione. For efficient detoxification, the liver cells require sulphur-containing amino acids such as taurine and cysteine. Toxic substances cause reduction in the level of protein.

Table 1. Changes in the level of protein content, sulfhydryl groups and LPO contents in the liver tissue of mice, *Mus musculus*, treated with 7 days of sub-lethal dose of mercuric chloride followed by 7 days methanolic extract of *Piper cubeba* fruits treatment

(Values expressed as mg/g wet weight of the tissue)

Nature of the organ	Group A	Group B	Group C	Group D
Protein	191.17 ± 0.48	63.16 ± 0.31*	210.00 ± 0.58□	238.83 ± 0.49
% COC		-66.96	9.84	24.67

% COHgT			232.49	
Sulhydryl groups	157.20 ± 0.03	120.40 ± 0.03*	147.83 ± 0.11#	165.10 ± 0.03
% COC		-23.41	-5.96	5.03
% COHgT			22.78	
LPO	0.661 ± 0.004	3.235 ± 0.008*	1.276 ± 0.001#	0.722 ± 0.001
% COC		389.40	93.04	9.23
% COHgT			-60.56	

Mean ± S.E (Mean of six individual observation)

* Significant at $p < 0.05$ compared with control

Significant at $p < 0.05$ compared with $HgCl_2$ treated

% COC – Percentage change over control

% COHgT – Percentage change over mercury treated

Group A – Control

Group B – $HgCl_2$ treated

Group C – $HgCl_2$ followed by *Piper cubeba* fruits extract treatment

Group D – *Piper cubeba* fruits extract alone treated

Table 2: Changes in the level of enzymatic and non-enzymatic antioxidant level in the liver tissue of mice, *Mus musculus*, treated with 7 days of sub-lethal dose of mercuric chloride followed by 7 days methanolic extract of *Piper cubeba* fruits treatment

Nature of the organ	Group A	Group B	Group C	Group D
Catalase	64.03 ± 0.07	47.97 ± 0.07*	60.25 ± 0.04#	66.32 ± 0.05
% COC		-25.08	-5.90	3.58
% COHgT			25.60	
GSH	2.033 ± 0.005	0.998 ± 0.000*	1.850 ± 0.000#	2.152 ± 0.011
% COC		-50.91	-9.00	5.85
% COHgT			85.37	
GPx	135.782 ±0.009	105.483 ± 0.016*	130.66 ± 1.664*#	137.788 ± 0.051
% COC		-22.31	-3.03	1.48
% COHgT			24.82	

Mean ± S.E (Mean of six individual observation)

* Significant at $p < 0.05$ compared with control

Significant at $p < 0.05$ compared with $HgCl_2$ treated

(Values expressed as □moles of H_2O_2 utilized/min/100 mg protein)

% COC – Percentage change over control

% COHgT – Percentage change over mercury treated

Group A – Control

Group B – $HgCl_2$ treated

Group C – $HgCl_2$ followed by *Piper cubeba* fruits extract treatment

Group D – *Piper cubeba* fruits extract alone treated

In the present study, the animals were treated with median-lethal dose of mercuric chloride the liver tissue shows the decreased level of protein with simultaneous increase in amino acid level indicate the enhanced catabolic activity of protein. The amino acids produced by the increased proteolysis enter the TCA cycle in the form of keto acid (Sahib *et al.,* 1978)[40]. This may be to meet the increased energy requirement of animal during the stress condition. Vigorous activities were also observed in mice when treated with mercury. Normally the animals are using the protein contents as a source of energy to meet their extra energy requirement during stress. This may be another reason for decreased protein content. Mercury binds to sulfhydryl groups of proteins and disulfide groups in amino acids that blocks related enzymes and hormones (Markovich and James, 1999)[41]. This also may leads to the decreased level of protein content in the liver tissue.

Free radicals cause oxidative destruction of polyunsaturated fatty acids in the biological membrane and is termed as lipid peroxidation. LPO alter physiological and biochemical characteristics of biological systems (Prakash and Rao, 1995)[42]. Lipid peroxidation can be considered as an evidence of oxidative cell injury in tissues (Sheridan *et al.,* 1996)[43]. The extent of tissues damage has been assessed by the measurement of LPO products and antioxidant status (Gutteridge, 1995)[44].

In the present investigation, an enhanced level of lipid peroxidation was observed in liver tissue of $HgCl_2$ intoxicated mice. Glutathione is a tripeptide made up of the amino acids glutamate, cysteine and glycine. Glutathione is an important antioxidant and the conjugating agent. It protects the tissues from the damaging effects of toxic compounds and promotes their elimination. Glutathione is considered as the most important antioxidant in our cells because of its detoxification and free radical protection abilities. Once they have performed their function as an antioxidant, scavenging free radicals, they are reduced to an inactive state. The regeneration into original form requires adequate supply of reduced glutathione (GSH). In the present study, a declined level of GSH is noticed in $HgCl_2$ intoxicated liver tissue. This might be due to the binding with mercury. A single Hg ion can bind to GSH and cause irreversible excretion of upto two GSH molecules (Zalups and Lash, 1996)[45]. Another possible reason is the oxidation of GSH to GSSH during the elimination of free radicals.

Mercury inhibits the activities of the free radical quenching enzymes catalase and glutathione peroxidase (Benov *et al.,* 1990)[46]. The enzymatic pathway of antioxidant system consists of SOD,

CAT and GPx. GPx and catalase are two major enzymes engaged in H_2O_2 dehydration (Aqil *et al.*, 1996)[24].

In the present investigation, a decrease in the level of catalase and glutathione peroxidase was noticed in mice treated with sublethal dose of mercuric chloride. The lower glutathione peroxidase activity could be due to a decline in GSH concentration. From the present study, it can be suggested that $HgCl_2$ treatment significantly reduces GSH content and antioxidant enzymes and thus accelerates lipid peroxidation, resulting in tissue damage.

In the present investigation, the ability of methanolic extract of *Piper cubeba* fruits to detoxify the impact of $HgCl_2$ was evaluated. An enhanced level of protein content was noticed in mercury intoxicated mice, after the *Piper cubeba* treatment indicates a normal protein metabolism in animals. The level of total sulfhydryl groups are reached near to normal in *Piper cubeba* extract treated mice. The present study shows that *Piper cubeba* extract treatment significantly inhibits lipid peroxidation and cellular injuries that protect the tissues against mercury induced oxidative damage. In the present investigation the reduced glutathione, catalase and glutathione peroxidase levels of brain, liver and kidney were significantly decreased by mercury treatment and a restored level of GSH, GPx and catalase is observed in mice treated with *Piper cubeba* extract treatment.

CONCLUSION

In conclusion, the present study demonstrates that $HgCl_2$ induced toxicity involves oxidative damage in various tissues. The *Piper cubeba* extract improved the tissue antioxidant status by increasing the levels of non-enzymatic antioxidant GSH and the activities of free radical-detoxifying enzymes such as catalase and glutathione peroxidase in liver tissue. Thus the present study demonstrated first time, the metallo-protective role of *Piper cubeba*. However, the precise mechanism by which *Piper cubeba* exerts antioxidant action remains to be defined. The present investigation has also opened avenues for further research especially with reference to the development of potent phytomedicine for mercury toxicity from *Piper cubeba* fruit extract.

ACKNOWLEDGEMENT

The authors are thankful to Professor and Head, Department of Zoology, Annamalai University for providing necessary lab facilities to carry out the work successfully

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