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Research Article

Phytochemical Constituents Analysis and Neuroprotective Effect of Leaves of Gemor (*Nothaphoebe Coriacea*) on Cadmium-Induced Neurotoxicity in Rats: An *In-Vitro* Study

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ABSTRACT
The objectives of this study were to determine phytochemical compositions and neuroprotective effects of aqueous extract of leaves of *gemor* against Cd-induced neurotoxicity *in vitro*. Neurotoxicity was induced by 3 mg/l of Cd in a form of cadmium sulphate ($CdSO_4$) in brain homogenate. Phytochemical screening of plant extract was determined using the qualitative method to measured the flavonoid, alkaloid, steroid, triterpenoid, and phenolic content. The neurotoxicity effect of Cd and neuroprotective effect of the aqueous extract of leaves of *gemor* (*Nothaphoebe coriacea*) was determined by assessing the concentration of hydrogen peroxide (H_2O_2), malondialdehyde (MDA), carbonyl compound (CC), and the activity of superoxide dismutase (SOD) and catalase (CAT) enzymes. Phytochemical screening results showed that the plant extract contained all phytochemical constituents with phenolic was the highest. Administration of Cd led to a significant elevation of H_2O_2, MDA, and CC level and significantly decreased the activity of SOD and CAT compared to control. Administration of plant extract showed significant decreases in H_2O_2, MDA and CC level and significant increase in SOD and CAT activity compared to Cd administration group. The results suggest that the administration of aqueous extract of leaves of *gemor* provide significant protection against Cd-induced neurotoxicity *in vitro*.

Keywords*:* Antibiotic, Amylase, Catalase, Kinetic Parameters, Neonatal Sepsis, Saliva

INTRODUCTION

Development of industry usually coupled with increased pollution of several persistent chemicals, including heavy metals, such as cadmium Cd[1]. Cd remains a source of concern for industrial workers and for populations living in polluted areas, especially in developed countries, such as Indonesia and especially South Kalimantan province[2]. Since Cd is not degraded in the environment, the risk of human exposure is constantly increased due to Cd also enters to the food chain[3]. If Cd enters the human body, it may affect the function of several organs including nervous system[4]. The neurotoxicity of Cd can be seen from the several symptoms including a headache and vertigo, olfactory dysfunction, parkinsonian-like symptoms, slowing of vasomotor functioning, peripheral neuropathy, decreased equilibrium, decreased the ability to concentrate, and learning disabilities[4]. The mechanisms involved in neurotoxicity of Cd are poorly understood. Oxidative stress has been proposed as a mechanism for Cd toxicity in a number of tissues such as the kidney, liver, and brain[2,5-6]. According to previous study, the administration of 3 mg/kg body weight/day Cd subcutaneously caused a significant increased in the levels of lipid peroxidation and protein carbonyls along with significant decrease in the activities of superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) in brain of rats[7]. Shagirtha *et al* study also showed that the administration of 5 mg/kg body weight Cd-chloride in saline orally for 4 weeks could significantly increased the levels of lipid peroxidation, lipid hydroperoxides, and protein carbonyl, and significantly decreased the activity of SOD, CAT, and GPx activities in brain of rats[8]. Currently, an extensive research has been done to evaluate several natural antioxidants regarding their chemo preventive effects in heavy metal-induced toxicities[9-10]. Antioxidants are becoming very popular in combating oxidative stress related diseases[8]. Among these natural antioxidants *gemor* (*Nothaphoebe coriacea*), which is a member of Lauraceae family that grows widely in several in swampy forests of Sumatra and Kalimantan, Indonesia[11]. The bark of gemor is commonly used as a material for insecticide, hio (a stick used for buddha's ritual) and glue. There is growing evidence that the parts of this plant may have great health benefits[12]. According to the previous study, aqueous extract of bark and leaves of *gemor* showed significant activity to inhibited the glucose metabolism alteration by Cd in liver homogenate of rats. This activity might be caused by

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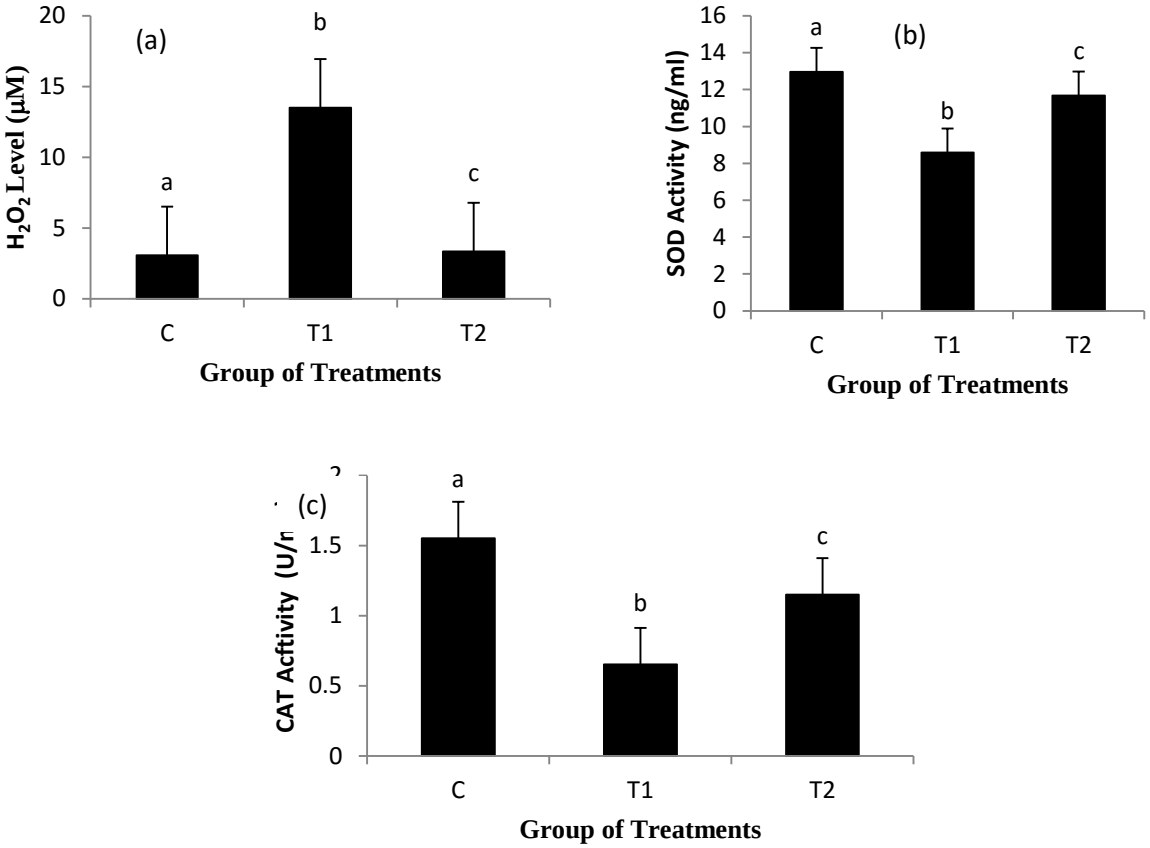

Figure 1: Effect of Cd and aqueous extracts of leaves of *gemor* on (a) H_2O_2 level; (b) SOD activity; and (c) CAT activity in brain homogenate. Values are mean±SEM of two replicates in each group of treatment. Bars of the same compartment carrying different letters of the alphabet are significantly different from each other ($p < 0.05$). C represent control group; T1 represent Cd treatment group; and T2 represent leaves of *gemor* treatment group.

phytochemical constituents in both bark and leaves of *gemor*, such as flavonoid and alkaloid[13]. Based on these facts, our study was designed to investigate the phytochemical constituents in aqueous extract of leaves of gemor, the toxic effects of Cd on the brain of rats, and to study the protective effects of aqueous extract of leaves of *gemor* on Cd-induced neurotoxicity in rats. To the best of our knowledge, this is the first evaluation of the neuroprotective effect of this plant and the results of this study may help to find a useful and natural treatment to prevent the neurotoxicity of Cd.

MATERIAL AND METHODS

Collection and Identification of Plant Materials

The fresh leaves of *gemor* was collected from Tumbang Nusa, Central Kalimantan, Indonesia. The plant was authenticated by Dr. Kade Sidiyasa, Technology Research Institute for Conservation of Natural Resources, Balikpapan, East Kalimantan. Before use, it was ensured that the leaves were free from contamination, sand and no microbial growth. The leaves were shade dried and was made into coarse powder using a commercial blender.

Preparation of Extracts

Extraction was done by decoction methods[14]. 100 g of shade-dried leaves of *gemor* was weighed and 1000 ml of distilled water was added to it respectively. This mixture was taken in 1000 ml beaker and subjected to heating continuously for 30 min at a temperature of 90°C. Then the mixture was allowed to cool on room temperature and subjected to filtration by means of vacuum filter. The filtrate so obtained is concentrated, so that all the excess solvent is evaporated in order to get the concentrated extract. The extract then used to preliminary quantitative analysis for flavonoid and alkaloid content and for experimental protocol section.

Qualitative Analysis of Phytochemical Constituents

Phytochemical screening of the crude aqueous extract of *gemor* was carried out using standard phytochemical procedure[12]:

Flavonoid: A few chop of 1% NH_3 solution is added to the aqueous extract of each plant sample in a test tube. A yellow coloration is observed if flavonoids compound is present.

Alkaloid: A 2 ml of test solution are taken with 2N HCl. The aqueous layer formed was decanted and then added with one or a few drops of Mayer's reagent. Formation of white precipitate or turbidity formed indicates the presence of alkaloids.

Steroid: A 2 ml of test solution and minimum quantity of chloroform are added with 3-4 drops of acetic anhydride and one drop of concentrated H_2SO_4. Formation of purple

Table 1: Phytochemical constituents present in the aqueous extract of *gemor*

Flavonoid	Alkaloid	Steroid	Triterpenoid	Phenolic
+	+	+	+	++

color changes into green color that indicates the presence of steroids.

Triterpenoid: 5ml of aqueous extract of each plant sample is mixed with 2ml of $CHCl_3$ in a test tube 3ml of concentrated H_2SO_4 is carefully added to the mixture to form a layer. An interface with a reddish-brown coloration is formed if triterpenoids constituent is present.

Phenolic Compounds: The extract (500 mg) was dissolved in 5 ml of distilled water. To this, few drops of neutral 5% ferric chloride solution were added. A dark green colour indicated the presence of phenolic compounds.

Experimental Protocol

The brain samples were collected from 27 old male rats (*Rattus novergicus*) with 2-3 month old, weighing 200-250 g. Then brain samples were taken by surgically procedure with ether as an anaesthesia. Then the brain fixed in phosphate buffer at pH 7.0. The brain was ground to form a liquid. Subsequently the solution was taken and centrifuged at 3500 rpm for 10 min and the top layer were taken and stored until it uses.Furthermore, the brain homogenate were prepared to experimental *in vitro* models. Samples divided into 3 groups (1 control group and 2 treatment groups). Control (C) group: brain homogenate only, Treatment 1 (T1) group : brain homogenate + 3 mg/l of Cadmium Sulphate ($CdSO_4$), Treatment 2 (T2) group: brain homogenate + 3 mg/l of $CdSO_4$ + aqueous extracts of leaves of *gemor*. Each solution then incubated at 37°C for 1 hour. After incubation, brain hydrogen peroxide (H_2O_2), MDA, and CC level, and SOD and CAT activity were estimated.All animals used and care were in compliance of the Ethics Commission of Faculty of Medicine, University of Lambung Mangkurat, Banjarbaru, South Kalimantan, Indonesia.

Hydrogen Peroxide (H_2O_2) concentration analysis

H_2O_2 concentration was assessed by the calorimetric method with some modifications[15]. The method is based on the fact that dichromate in acetic acid is reduced to chromic acetate when heated in the presence of H_2O_2 with the formation of perchromic acid as an unstable intermediate. To measured the levels of hydrogen peroxide in brain homogenate, 5 ml of phosphate buffer was taken and 1 ml of brain homogenate was added. Then, 1 ml of the homogenate and phosphate buffer mixture was taken and put into a tube that contain 2 ml of dichromate/acetic acid. Then heat the solution in aboiling water for 10 min and measured the absorbance at 570 nm using a spectrophotometer. Used a standar curve to determined how much H_2O_2 is left in the homogenate while the reaction was stopped by acetic acid. The level of H_2O_2 was expressed as μmol.

SOD activity analysis

The activity of superoxide dismutase (SOD) was measured according to Misra and Fridovich[2]. The oxidation of epinephrine was followed in terms of the production of adrenochrome, which exhibits an absorption maximum at 480 nm. The reaction mixture contains 0.1M epinephrine, 0.1mM EDTA and carbonate buffer (pH 10.2). The temperature was maintained at 30ºC and the absorbance measured at 480 nm using a spectrophotometer. One unit of SOD activity was defined as the enzyme amount that inhibits the rate of epinephrine oxidation by 50%[16].

CAT Activity analysis

CAT activity was measured by the method of Aebi[17]. Supernatant (0.1 ml) was added to a cuvette containing 1.9 ml of 50 mM phosphate buffer (pH 7.0). The reaction was started by the addition of 1.0 ml of freshly prepared 30 mM H_2O_2. The rate of decomposition of H_2O_2 was measured spectrophotometrically from changes in absorbance at 240 nm. The activity of CAT was expressed as u/mg protein.

MDA concentration analysis

MDA levels were assessed by studying thiobarbituric acid reactive substances (TBARS) by the method previously described by Buege and Aust.[5] Aliquots of the supernatant were added to a pyrex tube that contained 10% of trichloracetic acid and 0.67% of thiobarbituric acid and incubated at 100ºC for 15 min. The mixture was allowed to cool on ice for 5 min. This was followed by the addition of 1.5 ml of n-butyl-alcohol; the mixture was vigorously agitated for 40 s and centrifuged at 1000 rpm for 15 min. The TBARS value was assessed by the spectrophotometer at the absorbance of 532 nm and calculated using the coefficient 1.56 × 105 mol/cm, expressed as μmol MDA. Commercially available MDA was used as a standard.

CC concentration analysis

Sample derivatization. Two 1-μl aliquots are needed for each sample to be assayed. Samples are extracted in a final concentration of 10% (w/v) TCA. The precipitates are treated with 500 μl of 0.2% DNPH or 500 μl of 2 M HCl. Samples are incubated at room temperature for 1 h with vortexing at 5-min intervals. The proteins are then precipitated by adding 55 μl of 100% TCA. The pellets are centrifuged and washed three times with 500 μl of the ethanol: ethyl acetate mixture. The pellet is then dissolved in 600 μl of 6 mol guanidine hydrochloride. The CC level was determined by reading the absorbance at the optimum wavelength 390 nm[2].

Statistical evaluation

Results are expressed as mean ± S.E. One-way analysis of variance (ANOVA) followed by Tukey's test was used to analyze the results with $p < 0.05$ considered significant.

RESULTS AND DISCUSSION

Table 1 shows the results of the phytochemical screening of the aqueous extract of leaves of *gemor*. The results revealed the presence of phytochemical constituents with phenolic is the highest and followed by flavonoid, alkaloid, steroid, and triterpenoid. Phenolic compounds are considered secondary metabolites and these phytochemical compounds derived from phenylalanine and tyrosine occur ubiquitously in plants and are

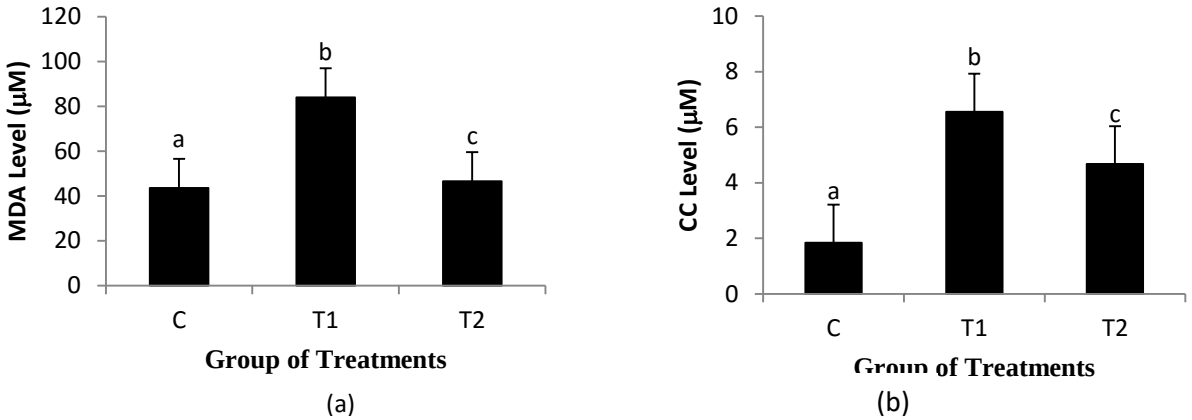

Figure 2: Effect of Cd and aqueous extracts of leaves of *gemor* on (a) MDA; and (b) CC level in brain homogenate. Values are mean±SEM of two replicates in each group of treatment. Bars of the same compartment carrying different letters of the alphabet are significantly different from each other ($p < 0.05$). C represent control group; T1 represent Cd treatment group; and T2 represent leaves of *gemor* treatment group.

diversified[18]. Phenolic compounds possess anti-inflammatory, anti-carcinogenic, anti-atherosclerotic, and other properties that may be related to their antioxidant activities[19]. To evaluate the neurotoxicity effect of Cd, we added Cd in the form of $CdSO_4$ into brain homogenate. Cd is a toxic compound and ingestion of any significant amount of it causes immediate poisoning and damage to several organs, including central nervous system[4,20]. Cd-induced neurotoxicity has been known to progress through induction of reactive oxygen species and consequent inhibition of antioxidant enzymes[21]. It can be seen from figure 1a, b, and c. Figure 1a, b, and c represent the influence of Cd and aqueous extract of leaves of *gemor* on an H_2O_2 level, and SOD and CAT activity in the brain of rats. Results showed that treatment with Cd (T1 group) led to higher H_2O_2 level and led to lower SOD and CAT activity. Statistical analysis test results showed that H_2O_2 level was significantly higher and SOD and CAT activity were significantly lower in T1 group in comparison with another group of treatments ($p<0.05$). The data from figure 1a, b and c suggest that Cd exposure led to increase H_2O_2 concentration and decrease SOD and CAT activity in brain homogenate. Results of this study indicated that Cd exposure induced the formation of H_2O_2. Cd, unlike other heavy metals, is unable to generate free radicals by itself, however, reports have indicated superoxide radical, hydroxyl radical and nitric oxide radicals could be generated indirectly. Previous report showed a generation of non-radical H_2O_2 which by itself became a significant source of free radicals via the Fenton chemistry[2,22]. Cd could replace other metals such as, iron and copper, which in turn to release and increase the concentration of unbound iron or copper ions. These free ions participate in causing oxidative stress via the Fenton reactions. Recently, Watjen and Beyersmann showed evidence in support of the proposed mechanism[23].Results of this study also indicated that Cd exposure caused the depletion of SOD and CAT activity. SOD and CAT are metalloproteins accomplishing their antioxidant functions by regulating the intracellular concentrations of ROS[24]. SOD acts as the first line of defense against ROS, dismutating superoxide to H_2O_2[2,22]. CAT protects the cells from toxic effects of ROS by converting H_2O_2 to water and molecular oxygen[20]. CAT and SOD are metalloenzymes; the former is heme-containing while the later require zinc for structural stability and copper for enzymatic activity. The Cd-induced inhibition of metalloenzymes has been explained to result from the displacement of these metals from the active site of these enzymes[20]. The increasing of the H_2O_2 level and the decreasing of SOD and CAT activity can promote the lipid peroxidation process and the formation of CC. It can be seen from the results of this present study (figure 2a and b). Figure 2a and b shows that Cd exposure led to higher MDA and CC levels. Statistical analysis test results show that MDA and CC levels were significantly higher in the T1 group in comparison with another group of treatments ($p<0.05$). The data from figure 2a and b suggest that Cd exposure led to increase MDA and CC concentration in brain homogenate. Results of this study suggest that Cd exposure induced the formation of MDA and CC.MDA is a product of lipid peroxidation and the most honest marker of oxidative stress and lipid peroxidation[25]. This compound is a reactive aldehyde and is one of the many reactive electrophilic species that causes toxic stress in cells and form covalent protein adducts which are referred to as advanced lipoxidation end products (ALE)[26]. CC is a compound that produced as a consequence of oxidative stress. In many cases, CC is considerable evidence is now emerging that it is the presence of these carbonyls rather than the initial oxidative insult that leads to the cellular damage observed[27].To evaluate the neuroprotective effect of *gemor*, we investigated the effect of aqueous extract of leaves of *gemor* against Cd-induced neurotoxicity. Figure 1a, b, and c, and 2a and b represent the effect of plant extract treatment against Cd-induced neurotoxicity. From figure 1a, b, and c shows that plant extract treatments led to lower H_2O_2 level and higher SOD and CAT activity. Statistical analysis test results shows that H_2O_2 level was significantly lower and SOD and Cat activity were significantly higher in the T2 group in comparison with another group of treatments. Also, from figure 2a, and b

shows that plant extract treatments led to lower MDA and CC levels. Statistical analysis test results show that MDA and CC levels were significantly lower in the T2 group in comparison with another group of treatments. The results clearly indicate that the addition of aqueous extract of leaves of *gemor* has neuroprotective effect by improved the oxidative stress status in brain homogenate. The neuroprotective activities of leaves of *gemor* might be has been attributed to the presence of phytochemical constituent. Results from table 1 clearly indicated that the extract of leaves of *gemor* contains several phytochemical constituents with the highest is phenolic. Phenolic compounds of plants are also very important because their hydroxyl groups confer several protecting ability[18,28]. The protecting ability of phenolic compounds based on several mechanisms, as follow:

Phenolic hydroxyl groups that are prone to donate a hydrogen atom or an electron to an ROS. In this mechanism, phenolic compound can act as a scavenger for ROS such as radical superoxide, H_2O_2, and radical hydroxyl. Furthermore, phenolic can break the free radical reaction and improved the oxidative stress status[29-30].

Phenolic have a high tendency to chelate metals. Phenolics possess hydroxyl and carboxyl groups, able to bind particularly to metals. From this point of view, the phenolic can bind particularly to Cd. This binding then will reduce the neurotoxicity effect of Cd[31].

Phenolic may inactivate iron ions by chelating and additionally suppressing the superoxide-driven Fenton reaction, which is believed to be the most important source of ROS in Cd-induced neurotoxicity[31].

Phenolic antioxidants inhibit lipid peroxidation by trapping the lipid alkoxyl radical. This activity depends on the structure of the molecules, and the number and position of the hydroxyl group in the molecules[28].

Phenolics (especially flavonoids) are able to alter peroxidation kinetics by modifying the lipid packing order. They stabilize membranes by decreasing membrane fluidity (in a concentration-dependent manner) and hinder the diffusion of free radicals and restrict peroxidative reaction[31].In conclusion, the present study demonstrated that Cd-induced the formation of H_2O_2, MDA, and CC, and depressed the activity of SOD and CAT in the brain of rats. This indicated that Cd-induced neurotoxicity via an oxidative mechanism. Also, the present study demonstrated that the administration of aqueous extract of leaves of *gemor* can improve the oxidative stress status in the brain of rats. The administration of extract inhibited the formation of H_2O_2, MDA, and CC, and increased the activity of SOD and CAT in brain of rats.This indicated that the extract may have neuroprotective effect against Cd-induced neurotoxicity.

CONFLICT OF INTEREST

We declare that we have no conflict of interest.

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