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STUDY OF THE TOXIC EFFECT OF DRINKING WATER CONTAMINATED WITH LEAD ON LIVER AND KIDNEY FUNCTIONS IN PREGNANT RATS: THE BENEFIC ROLE OF *Aquilaria malaccensis* L. SPICE

Derouiche Samir[1,2*], Ahmouda Ibtissam[1], Moussaoui Rokaya[1]

[1]*Department of Cellular and Molecular Biology, Faculty of the Sciences of Nature and Life, El Oued University, El Oued 39000, El Oued, Algeria*
[2]*Laboratory of Biodiversity and Application of Biotechnology in the Agricultural Field, Faculty of the Sciences of Nature and Life, University of El Oued, El-Oued 39000, Algeria*

*Corresponding Author: dersamebio@gmail.com

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Abstract

The objective of this work was to study the effect of using *Aquilaria malaccesis* spice on some biological parameters in pregnant rats exposed to lead-contaminated drinking water. Twenty (20) female rats were randomly divided into four groups (n = 5): control, pregnant rats, pregnant + Pb and pregnant +Pb+ *A. malaccensis* (Aq). Lead (200 mg/kg b.w.) as Pb $(C_2H_3O_2)_2$ was added to their drinking water for 20 days. *A. malaccensis* (heartwood powder at a dose of 10 g/kg of diet) was added to the feed during the last 10 days of lead exposure in the animals. The results showed that *A. malaccensis* was rich in various flavonoid and phenolic compounds. On the other hand, the results showed that pregnancy caused significant physiological and biochemical changes in rats**.** Our results also showed a significant increase ($p < 0.05$) of liver lead, blood glucose, lipids profile, serum uric acid, urea, transaminases, MCV, Platelet, MDA and GST levels in the pregnant rats Pb^{2+}group compared to the pregnant rats. The results obtained also revealed a significant decrease ($p < 0.05$) in the level of RBC, WBC, Hb, hematocrit, GSH and SOD activity in comparison with pregnant rats group. The treatment with *A. malaccensis* spice partially improved the biochemical and hematological parameters, which protected the tissues against radical attacks (oxidative stress) caused by lead in pregnant rats. In conclusion, this work shows that *A. malaccensis* spice has beneficial effects in reducing oxidative stress and toxic effects of lead on the liver and kidneys in rats during gestation.

INTRODUCTION

Pregnancy is a period of intense physical and physiological changes accompanied by serious health risks, even for women with no previous health problems [1]. A woman during pregnancy is exposed to many toxic substances, which affect her health and the health of the fetus. Among these substances, lead (Pb) [2] is a non-essential toxic heavy metal widely distributed in the environment. The main sources of lead emissions today are piston-engine airplanes using leaded aviation gasoline and mining and metal processing, particularly lead smelters.

Additionally to these stationary sources, there are utilities, lead-acid battery producers, and waste incinerators [3]. According to World Health Organization, lead toxicity accounts for 1.5% (900,000) of deaths annually in the world [4]. Millions of adults were exposed to high levels of lead as children. Animal studies and epidemiological evidence accrued that such exposures likely disrupted healthy development across multiple organ systems [5]. Various dysfunctions in the physiological, biochemical and behavioral systems are induced by chronic exposure to minute concentrations of lead [6].

As a xenobiotic, lead is known to induce a wide range of dysfunctions in the central and peripheral nervous systems and the hemopoietic system [7]. The study by Burroughs and Rollins showed that lead is responsible for several cardiovascular diseases such as arterial hypertension [8]. The liver and kidneys are prime targets for many xenobiotics, including lead, which is very heavily bioaccumulated in the liver (33%). There are many pregnancy complications that can be related to oxidative stress which is considered a risk factor during pregnancy [9]. Oxidative stress is responsible for various tissue damages caused by lipid peroxidation (LPO) induced by reactive oxygen species (ROS) [10]. Toxic metals increase the production of free radicals and reduce the availability of antioxidant stores and thus decreasing the capacity to respond to damage-induced stress [11]. Many studies prove that metals are able to interact with nuclear proteins and DNA, causing oxidative deterioration of biological macromolecules, eventually leading to many chronic diseases, such as atherosclerosis, cancer, and diabetes [12]. For a long time, plants have been used as a medication against several diseases, and many of them are still the basis of a system of traditional medicine in different cultures [13].

On the other hand, *Aquilaria malaccensis* is a species of tropical plants of the Thymelaeaceae family. It is one of the main sources of agar wood, which provides clues about their pharmacological properties [14]. Our objective in this work was to evaluate the toxic effects of drinking lead contaminated water in pregnant rats and to study the efficacy of herbal medicine with *Aquilaria malaccesis* as a spice.

MATERIALS AND METHODS

Chemicals

Sodium chloride (NaCl), Hydrochlorid acid (Hcl), Hydrogene peroxide (H_2O_2), Thiobarbituric acid (TBA), Methanol, Coomassie Blue, Butylate dihydroxy toluene (BHT), Trichloroacetic acid (TCA), Phosphate-buffered (KH_2PO_4, K_2HPO_4), Ethylene diamin tetra-acetic acid (EDTA) were of analytical grade.

Plant Materials and Preparation of Aqueous Extract

Aquilaria malaccensis heartwood was collected in herbalist's shops from a local market in El-Oued (Algeria). The vegetal materials (Removed from trees up to 3 meters tall and about 9 years old) were washed with water, and then dried at room temperature for 48 to 92 h, and ground into powder and stored at room temperature until use. For aqueous extract preparation, 25g of the dry plant of *A. malaccensis* were mixed with 250ml of distilled water and boiled over low heat for 2h. Then, the solution was macerated for 24 hours at room temperature, and filtered using Watman paper. Next, the filtrate was evaporated using an evaporator [15].

Qualitative and Quantitative Analyses of Phytochemical Composition

Phytochemical Screening

Phytochemical tests were performed on extracts prepared from the plant by qualitative characterization techniques using standard screening test.

Estimation of Total Phenol

The polyphenols were determined by the Folin-Ciocalteu method. This method, initially described by Slinkard and Singleton [16], makes it possible to know the total polyphenolic content of a given sample. The sample of the aqueous extract of *A. malaccensis* (0.5 ml) and 2 ml of sodium carbonate (75 g / l) were added to 2.5 ml of 10% (v / v) Folin- Ciocalteau with gallic acid as standard. After 30 min of reaction at room temperature, the absorbance was measured at 765 nm. The tests were carried out three times in order to ensure the reproducibility of the results. The total phenolic content was expressed in mg Equivalent of Gallic Acid per gram of sample.

Estimation of Total Flavonoids

Determination of the total flavonoid content of the aqueous extract of *A. malaccensis* was carried out by the method described by Lin and Tang [17]. 0.5 ml of a 2% AlCl3-ethanol solution was added to 0.5 ml of sample or standard. After 1 h at room temperature, the absorbance was measured at 420 nm. Quercetin was used as a standard for plotting the calibration curve. The tests were performed three times in order to ensure the reproducibility of the results. The results were expressed in milligram equivalent Quercetin per gram of sample.

Method of Phytochemistry HPLC Analysis

The aqueous extracts of *Aquilaria malaccensis* were filtered before injection. An HPLC system was used with a detector at λ=280nm for polyphenols and at 360nm for flavonoids The experimental conditions were as follows: The column used was 150mmx4.6mm with C18 as stationary phase, mobile phase: acetonitrile and glacial acetic acid (2%, pH=2.6, 30°C). The identification of the peaks of polyphenols and flavonoids was made using the standard achieved by pure components by comparing the retention times.

Animals and Experimental Design

All experimental procedures employed, including rat care and handling, were conducted in accordance with international guidelines provided by the local ethics committee (26 EC/DCMB/FNSL/EU2020) of the

department of cellular and molecular biology, Faculty of Natural Sciences and Life, El-Oued University. Female rats with weight (192.72± 3.86 g) were bought from animal's service of Pasteur institute in Algeria. They were housed in faculty of SNV, University of El-Oued (Algeria) in plastic cages divided in three groups of 5 rats of each. They kept in the animals breeding house for adaptation. The animals were adapted to laboratory condition photoperiod (12 h of night/12h of darkness), at ambient temperature of 25 ± 4 °C and humidity of (64.2 ± 14 %) for two weeks. The standard diet and water were free for the animals during the adaptation period. The experimental part was conducted in compliance with the ethical approval. Female rats were randomly divided into the following three groups (5 rats):

Group 1 (Control): Non pregnant rats served as normal control.

Group 2 (Pregnant): Pregnant rats received normal diet

Group 3 (Pregnant + Pb): Pregnancy rats received drinking water contaminated with lead acetate (200 mg/kg b.w) as $Pb(C_2H_3O_2)_2$ for 20 days.

Group 4 (Pregnant +Pb+Aq): Pregnancy rats received drinking water contaminated with lead acetate and treated with powder of heartwood of *Aquilaria malaccensis* (1% w/w in feed) for 10 days.

Blood Collection and Tissue Preparation

At the end of 3rd week of experiment, the pregnant rats were fasted for 16 h, anesthetized by chloroform inhalation then sacrificed by decapitation. Then, the rats were dissected and the embryos were examined to prove pregnancy (figure 1). Blood was collected in EDTA tubes for hematological analysis. The serum was obtained by blood centrifugation at 3000 rpm for 10 min and stored at -20 °C until the use for urea, creatinine, calcium and electrolytes levels assay. Then, the liver and kidney of rats of the different groups were rapidly excised, weighed and stored at -20° C until use for lead and oxidative stress evaluation.

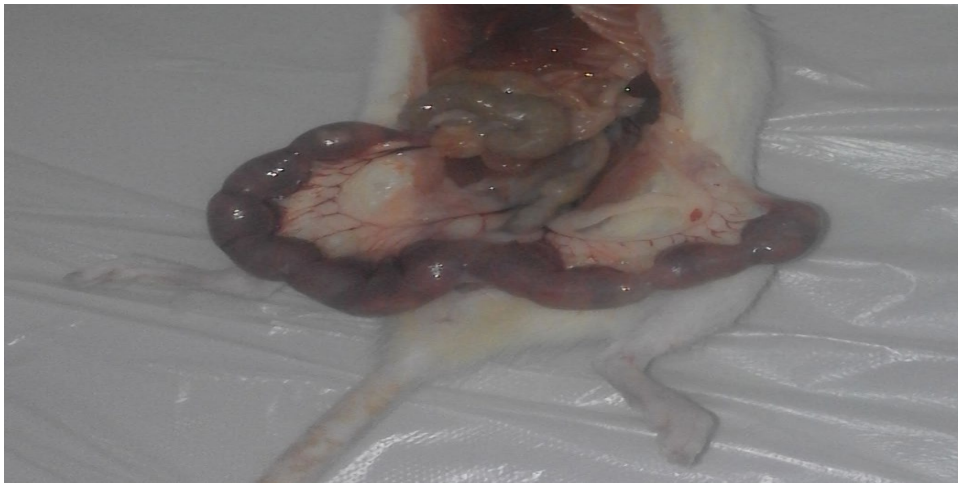

Figure 1. Dissect of pregnant rats exposed to lead

Determination of Biochemical and Hematological Markers

Serum urea, uric acid, GOT and GPT parameters levels were determined by auto-analysis (BIOLIS24j) using a commercial kit from Spinreact (Spain). Hematological analysis (FNS) was performed by the hematology autoanalyzer (Sysmex).

Determination of Liver Lead Concentration

Dry calcination of liver was carried out in a muffle furnace at a temperature of 600 ° C for 6 hours. The ash obtained was dissolved by an attack of 3ml of pure nitric acid (HNO3). The liquid obtained was filtered on filter paper in a 20 ml flask and completed to its final volume with demineralized water. For the lead assay, lead standards were prepared from a 1000 ppm stock solution, using a nitric acid (1%) solution for dilution [18].

Oxidative Markers Measurement

The method of malondialdehyde (MDA) assay was based on the reaction between the carbonyl compounds of malondialdehyde with thiobarbituric acid according the method of Yagi [19]. The level of reduced Glutathion (GSH) was determined according the Weak and Cory [20] by measuring the optical density results from the formation of 2-nitro-5-mercocapturic acid from the reduction of dithio-bis-2-nitrobenzoic acid, which is called Ellman reagent with SH groups present in GSH. Gluthation transferase (GST) activity was measured spectrophotometrically based on the formation kinetics of a complex between a GST substrate: 1-chloro-2-4-dinitrobenzene (CDNB) and GSH according to

the method of Habig et al. [21]. The assay method of superoxide dismutase (SOD) activity using the NBT by the superoxide anion ($O2^{\cdot}$), was used as a basis for detecting the presence of SOD by measuring the absorbance at 560 nm [22].

Statistical Analysis

The statistical evaluation was carried out with Student's T test using Minitab 17.1 statistical package. The values were given as mean and standard deviations (ES) for four groups. Statistical significance was defined as $P < 0.05$.

RESULTS

Phytochemical Screening and Phenolic Compounds

The results of phytochemical tests of plant extract presented in Table 1 clearly show that the extracts of *A. malaccensis* are rich in secondary metabolites including flavonoids and saponins. As shown by the results of the quantitative analysis (Table 1), flavonoids and phenols contents in A. *malaccensis* extract were very significant.

Table 1. Qualitative and quantitative analysis of phytochemical composition of aqueous extract of *A. malaccensis*

	Compounds	*A. malaccensis*
Qualitative Analysis	Flavonoids	+
	Tannins	-
	Alkaloids	+
	Saponines	+
	Glycosides	+
	Terpenoides	+
Quantitative Analysis	Total phenolic (mg GAE/g extract)	54.6±0.94
	Flavonoids (mg QE/g extract)	5.28±0.07

(+ presence - absence)

Polyphenol and Flavonoids HPLC Analyses

The HPLC chromatogram analysis (Figures 2 and 3) shows that *Aquilaria malaccensis* is rich in polyphenols and flavonoids in different amounts. The results show the presence of important compounds such as Apigenin, Epicatechin and Naringenin for polyphenols and other compounds such as Quercetin, Kampferol and Rutinhas for flavonoids in *A. malaccensis* aqueous extract.

Biochemical Markers

Concerning the biochemical markers (Table 2), results obtained showed that a signficant rose ($p < 0.05$) of blood glucose, triglyceride cholesterol, urea and creatinine but no significant variation ($p > 0.0\ 5$) in the liver lead concentration, GOT and GPT levels in the serum of Pregnant rats group compared to the controls. Our results also showed a significant increase ($p < 0.05$) in blood glucose, lipids profile, serum GPT and GOT activities, liver lead, urea, and uric acid, levels in the Pregnant Pb group compared to the pregnancy rats. Treatment with *A. malaccensis* significantly decreased ($p < 0.05$) most of the previous biochemical parameters compared to the Pregnant Pb group.

Hematological markers

Results in Table 3 show a significant decrease ($p<0.05$) in red blood cells (RBC), hematocrit and platelet level and a significant increase ($p<0.05$) in mean corpuscular volume (MCV) but no significant variation ($p \geq 0.0\ 5$) in white blood cells (WBC) and hemoglobin (HB) levels in pregnant rats group compared to the controls. On the other hand, the results show that exposure of pregnant rats to lead acetate caused a significant decrease ($p < 0.05$) in WBC, RBC, white blood cells, hemoglobin and hematocrit levels but a significant increase in MCV and platelet levels when compared to pregnant group. On the other hand, treatment of pregnant rats + Pb group with A. malaccensis spice significantly improved ($P < 0.05$) all previous hematological markers in comparison with the pregnant + Pb group.

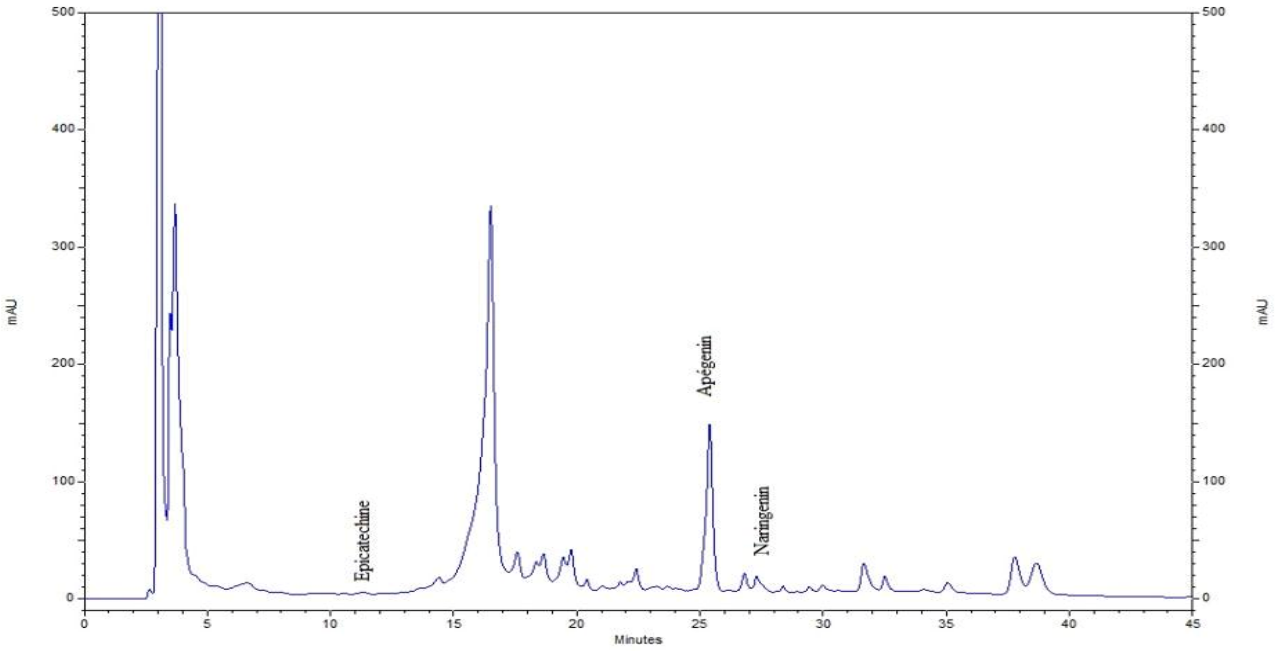

Figure 2. Chromatogram of polyphenols for the *Aquilaria malaccensis* aqueous extract

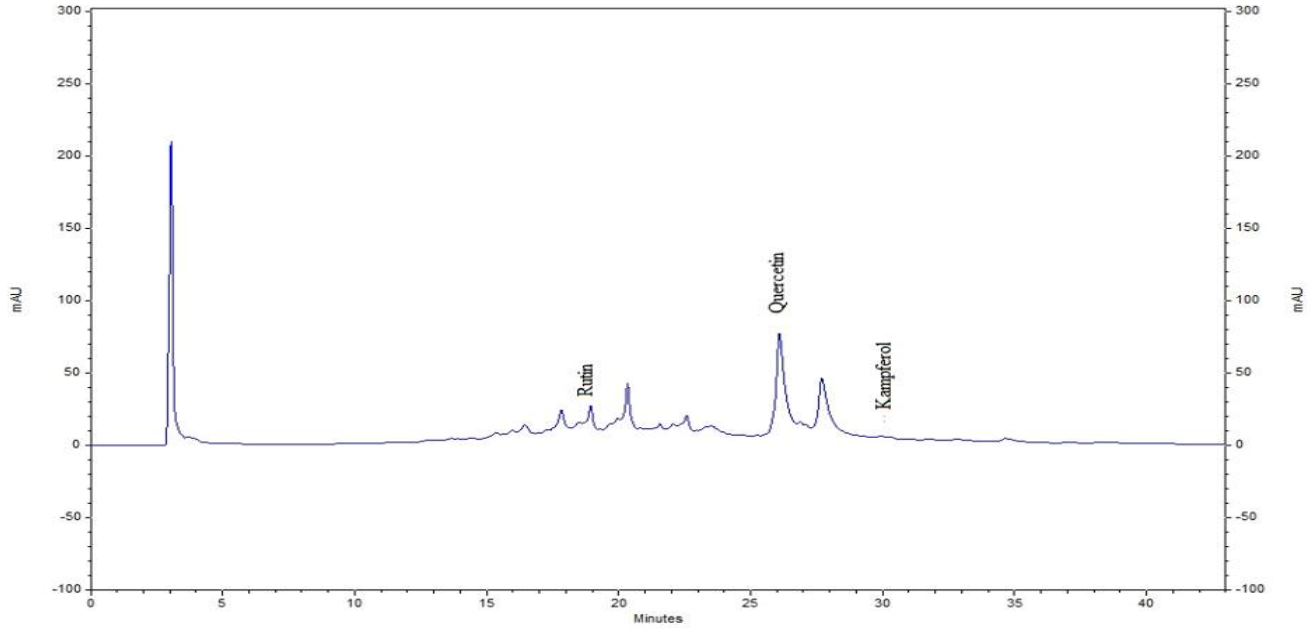

Figure 3. Chromatogram of flavonoids for the *Aquilaria malaccensis* aqueous extract

Table 2. Liver lead and serum biochemical levels in control and experimental groups

Parameters	Control (n=5)	Pregnant (n=5)	Pregnant + Pb (n=5)	Pregnant+Pb+Aq (n=5)
Liver lead (µg/g tissue)	0.020±0.009	0.031±0.002	0.152±0.007^{***a1}	0.098±0.001^{**b1}
Fasting blood glucose (g/l)	0.95±0.02	1.41±0.05*	1.60±0.09^{*a1}	1.45±0.07^{*b1}
Hepatic glycogen (µg/g)	16.12±0.5	15.03±0.6	45.20±0.9^{***a3}	12.30±0.8^{*a1b3}
Triglycerids (mg/dl)	0.25±0.08	0.4±0.06*	0.45±0.08^{*a1}	0.32±0.04^{a1b1}
Total cholesterol (mmol/l)	0.41±0.03	0.51±0.07*	3.94±0.02^{***a3}	0.49±0.03^{*b3}
Serum urea (g/l)	0.35 ±0.01	0.52±0.08*	0.68±0.03^{***a1}	0.54±0.05^{**b1}
Serum uric acid (mg/l)	12.60±1.94	14.5±0.2*	19±0.8^{**a2}	18.03±0.6^{**a1}
Serum GOT (U/l)	205.80±10.30	190±12	450±25^{***a3}	220±19^{b3}
Serum GPT (U/l)	29.50±4.33	33±1.32	59±3.23^{***a2}	58±4.62^{***a2}

The results are presented by mean± SEM. n=number of observations. Significant difference from control: *(p<0.05), **(p<0.01), ***(p<0.001). Significant difference from Pregnant group: a1(p<0.05), a2(p<0.01), a3(p<0.001) Significant difference from Pregnant +Pb group: b1(p<0.05), b2(p<0.01), b3(p<0.001)

Table 3. Hematological markers levels in control and experimental groups

Parameters	Control (n=5)	Pregnant (n=5)	Pregnant + Pb (n=5)	Pregnant+Pb+Aq (n=5)
WBC (x10^3/µl)	6.60 ± 0.20	7.22±0.63	3.44±0.42^{***a3}	5.90±0.71^{*a1b2}
RBC (x10^6/µl)	0.3±7.17	6.98±0.11*	6.41±0.09^{*a1}	7.33±0.05^{a3b3}
Hemoglobin (g/dl)	14.15 ± 0.12	14.12±0.52	11.23±0.91^{*a3}	14.21±0.74^{a1b2}
Hematocrit (%)	42.15±0.69	38.52±0.34*	35.46±0.81^{*a1}	40.71±0.42^{a1b2}
MCV (fl)	53.2±0.72	55.35±0.92*	56.03±0.93^{*a1}	54.50±0.87^{*b1}
Platelet (x 10^3/µl)	1.20 ± 0.020	0.71±0.05***	0.85±0.08^{***a1}	0.62±0.71^{***a1b3}

The results are presented by mean± SEM. n=number of observations. Significant difference from control: *(p<0.05), **(p<0.01), ***(p<0.001). Significant difference from Pregnant group: a1(p<0.05), a2(p<0.01), a3(p<0.001) Significant difference from Pregnant +Pb group: b1(p<0.05), b2(p<0.01), b3(p<0.001)

Liver and Kidney Oxidative Stress Markers

Our results (Table 4) show a significant increase (p<0.05) in liver MDA and liver GST levels, a significant decrease in liver GSH and kidney SOD levels and no significant variation (p≥0.0 5) in the other tissue markers in pregnant rats group compared to the controls. In addition, the results also show a significant increase (p <0.05) in MDA level and GST activity, as well as a significant decrease in GSH concentration and SOD activity in liver and kidney of pregnant rats exposed to Pb when compared to pregnant group. Conversely, the treatment with A. *malaccensis* spice caused a significant improvement (p <0.05) in all the parameters studied at the hepatic and renal levels in comparison with the Pregnant +Pb group.

Table 4. Oxidative stress markers in liver of control and experimental groups

Parameters		Control (n=5)	Pregnant (n=5)	Pregnant + Pb (n=5)	Pregnant+Pb+Aq (n=5)
MDA (µmol/mg prot)	Liver	0.18±0.037	0.5±0.03***	0.56±0.02^{***a1}	0.47±0.05^{***a1b1}
	Kidney	0.31±0.041	0.37±0.08	0.45±0.05^{*a1}	0.22±0.05^{*a3b3}
GSH (nmol/mg prot)	Liver	220±1.7	100.22±1.02***	55.81±0.92^{***a3}	63.04±0.62^{***a3b1}
	Kidney	0.48±0.90	0.49±1.33	0.38±0.64^{*a1}	0.47±1.03^{b3}
GST (nmol/min/mg prot)	Liver	0.14±1.28	0.72±0.005**	2.28±0.003^{***a3}	0.201±0.001^{*a3b3}
	Kidney	0.375±0.078	0.24±.0003	4.02±0.01^{***a3}	0.62±0.008^{**a1b1}
SOD (nmol/min/mg prot)	Liver	0.25±0.002	0.32±0.006	0.16±0.003^{a3}	0.20±0.001^{a2b1}
	Kidney	1.22 ± 0.13	0.33±0.004***	0.07±0.005^{***a3}	0.14±0.004^{***a3b3}

The results are presented by mean± SEM. n=number of observations. Significant difference from contro: *(p<0.05), **(p<0.01), ***(p<0.001). Significant difference from Pregnant group: a1(p<0.05), a2(p<0.01), a3(p<0.001) Significant difference from Pregnant +Pb group: b1(p<0.05), b2(p<0.01), b3(p<0.001)

DISCUSSION

Today, herbal treatments are returning to the fore, as the effectiveness of drugs decreases over time, leading scientific researchers to conduct in-depth studies on the chemical composition of the plant's secondary metabolites and their therapeutic actions [23]. Qualitative analysis of the aqueous extracts of *Aquilaria malaccensis* revealed the presence of flavonoids, terpenoids, saponins, alkaloids and Glycosides. The secondary metabolites produced by this plant have several interesting biological activities, and are source of active pharmacological principle against several pathologies [24].

The results obtained show a very significant increase in the level of Pb in the liver during gestation, which reflects the binding of this metal to these target tissues. The liver is an important target for lead. Lead has a high affinity for the protein thiol groups of hepatic cell membranes, which leads to hepatic lyses and necrosis. In contrast, treatment with *A. malaccensis* reduced the level of lead in the liver. These results are in agreement with the previous study showing a chelating effect of *A. malaccensis* against lead in the liver in rats [25]. Indeed, this reduction of lead means that this plant has protective effects and is a beneficial agent against the bioaccumulation of lead even during the gestation state of the rats and therefore can be considered as a Pb chelating agent.

Concerning the effect of lead on hepatic and renal functions, the results of our study show that, during gestation, the poisoning by lead acetate in rats caused hepatotoxicity and nephrotoxicity, as revealed by increased levels of urea and uric acid and blood transaminases. These results are in agreement with previous works which have shown that kidney cells were no longer able to control the process of urinary excretion, because of their high sensitivity to lead [26]. In addition, there have been reports of increased serum transaminases due to hepatic dysfunction after exposure to several toxic metals [27]. Leakage of transaminases into the bloodstream may be the result of liver dysfunction [28]. On the other hand, the results of herbal medicine showed that using *A. malaccensis* spice restored liver and kidney functions. The protective effect of *A. malaccensis* was reflected by the decrease in serum urea and uric acid concentrations and transaminase activity in pregnant rats treated with this plant compared to PR+Pb, which shows the ability of this plant to protect against hepatic and renal damage induced by Pb during gestation. These results regarding the protection of rats and their fetuses during gestation are in agreement with a previous study [29]. On the other hand, plants inhibit hepatic damage caused by Pb. This is due to decreased accumulation of free radicals, the protection against oxidative stress and to anti-inflammatory phytochemicals such as Rutin [30].

The results of the effect of lead on oxidative stress markers show that lead affects these markers by increasing the level of tissue MDA, and the activity of hepatic and renal GST and by decreasing GSH and tissue SOD in pregnant rats. Radical phenomena play an important role in the reproduction, the nesting of the fertilized egg and the development of the embryo. But imbalance between their intense production during gestation, and their elimination can generate oxidative stress [31]. Oxidative stress has been suggested as one of the main mechanisms of Pb toxicity. Lead may induce oxidative damage possibly due to inhibition of 5-aminolevulinic acid (ALA) dehydratase leading to accumulation of aminolevulinic acid, a potential endogenous source of free radicals or due to the direct interaction of Pb with biological membranes, inducing lipid peroxidation [32]. Furthermore, during the gestation period of the rats, treatment with *A. malaccensis* improved the state of oxidative stress by restoring the specific parameters of this state. Polyphenols and flavonoids in *A. malaccensis* may be responsible for antioxidant activity and are considered good metal ion chelators. In addition, the activity of phenolic acids depends on the number and position of the (OH) group [33], and to the fact that flavonoids inactivate and stabilize free radicals depending on their highly reactive hydroxyl group (OH). They are also capable of chelating metal ions (released from their binding or transport proteins) [34]. In addition, the antioxidant effect of the plant may be due to the presence of many antioxidant compounds such as phenolic derivatives, terpenoids and flavonoids present in *A. malaccensis*. Flavonoids are the most important secondary metabolites of plants modulating lipid peroxidation involved in atherogenesis, thrombosis and carcinogenesis [35]. The phenolic groups of polyphenols can accept an electron to form relatively stable phenoxyl radicals, which disrupts the chain oxidation reactions of cellular components [36].

CONCLUSION

Lead is a strong toxic agent in the liver and kidney, causing significant damage during pregnancy by inducing oxidative stress in rats. *A. malaccensis* spice in food was able to moderate this toxicity by decreasing oxidative stress and restoring the biochemical and hematological parameters which protect rats and their fetuses during pregnancy.

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CONFLICT OF INTEREST

All authors have approved the manuscript with no conflict of interest.

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