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CONTRIBUTION TO THE PHYTOMEDICINAL STUDY OF THE SOLID AND AQUEOUS EXTRACT OF Anthurium schlechtendalii KUNTH ROOT AGAINST LIVER DAMAGE IN A RAT MODEL

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Abstract

Plants have been used as a herbal treatment option for liver disease in alternative medicine. The current study was carried out to investigate the therapeutic potential of Anthurium schlechtendalii Kunth roots as a hepatoprotective or remission agent for liver damage. A preclinical study was carried out through oral administration of 4-tert-octylphenol (100 mg kg-1/day) as inducer of liver disease, and solid and aqueous extracts from the plant roots. The extracts were tested at doses of 125 mg kg-1/day and 1.8 mg mL-1/day, respectively, using a rat model in a 4-week experiment. Growth parameters (initial and final body weight, food intake, liquid consumption, liver weight) and liver function markers (aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP) were determined. In addition, analysis of variance (one-way ANOVA) procedures, with a post hoc Tukey's multiple range test for comparison of means ($p < 0.05$), were performed. The study showed that, according to the lethal concentration (LC50) through the PROBIT transformation, the extract was non-toxic. The experimental model used, however, was not able to show liver function marker damage nor changes in growth parameters, so no statistically different results were found between the study groups (A, control; B, 4-tert-octylphenol; C, solid extract; D, solid and aqueous extracts; E, 4-tert-octylphenol plus solid extract; F, 4-tert-octylphenol plus solid and aqueous extracts). The method of toxic administration was not through direct oral dose but mixed in food, possibly producing a low intake of 4-tert-octylphenol thus not causing liver function marker damage. The need to continue and expand relevant research, to accumulate sufficient scientific knowledge and elucidate the efficacy of A.

schlechtendalii Kunth roots in the prevention, mitigation, or remission of liver damage, has been made evident by the results obtained.

Keywords: *Liver disease, Anthurium schlechtendalii Kunth roots, hepatoprotective action, phenolic compound, alternative medicine.*

Introduction

The liver is a complex organ that performs multiple functions. Its relationship with other organs and the metabolic pathways, as well as its detoxification capacity, derives from biotransformation of enzyme systems, such as the cytochrome P450 family (CiP450), which confers a critical role in health, and any deficiency in its physiology can cause complex disorders. Hepatotoxicity is a dysfunctional liver condition caused by drug administration, exposure to viruses and chemical agents (heavy metals, pesticides) and/or genetic factors [1,2]. Abnormal values of specific biomarkers are present under hepatotoxic conditions. As a result of an imbalance in cellular metabolism, the exposure of substances that interact with essential biomolecules leads to modifications at a structural and functional level [3]. The biochemical marker that commonly defines a hepatotoxic condition is elevation of liver enzymes such as alkaline phosphatase (ALP), aspartate aminotransferase (AST), and particularly alanine aminotransferase (ALT), reflecting hepatocellular damage [4]. Currently, liver disease is one of the leading causes of death in the world. In the European Union (EU), approximately 29 million people suffer from chronic liver disease [5]. Mexico's National Institute of Statistics and Geography reported that the incidence of hepatic diseases in Mexico increased, reaching 32,453 deaths [6]. Between 2010 and 2013, liver diseases represented the sixth highest cause of mortality in Mexico [7]. The number of xenobiotics involved in episodes of hepatotoxicity is increasing. The phenolic derivative 4-tert-octylphenol is a toxic and highly stable compound resistant to biological attack [8]. It has also been identified as an endocrine disruptor or xenoestrogen, which has implications for human health, the environment, and the food chain, particularly its hepatotoxic capacity [9,10].

Mexico is a country with outstanding biodiversity and many of the plants, one of the most important natural resources, are used in traditional medicine by Mexican herbalists [11]. *Anthurium schlechtendalii* Kunth (stone root), which belongs to the family Araceae, is mainly a tropical plant, with a great diversity of species in Asia and tropical America [12]. One hundred and twenty-one species and 18 genera of this plant found in Mexico are reported in the scientific literature. Araceae endemism in Mexico is high, mainly in the genus Anthurium, of which 26 of a total of 41 species are endemic [13,14]. Approximately 45% of the total Mexican species grow in the state of Veracruz [15].

There are reports that traditional medicine, particularly in China, offers remission of chronic kidney disease (CKD) through phytochemicals [16]. One species that has demonstrated such properties is *Anthurium schlechtendalii Kunth*. It has been reported that added to drinking water (root infusion), it has been used for symptomatic treatments of postpartum pain and spasmodic urinary tract disorders, and to control bleeding [17,18]. There are no phytochemical or phytopharmacological studies in the scientific literature on the preventive or remission effect of *A. schlechtendalii* Kunth on liver function. Stark, et al. [19], reported that the presence of antioxidants and phenolic compounds in the root and leaf extracts of *A. schlechtendalii* Kunth could be responsible for their anti-inflammatory potential. To ascertain its use in society and the possibility that *A. schlechtendalii* Kunth could be efficacious for the treatment of the disease mentioned above, this study aimed to evaluate either the preventive or remission effect of the aqueous extract of *A. schlechtendalii* Kunth (stone root) on hepatic damage induced by 4-tert-octylphenol, or both, in a murine model.

Methods

Collection of plant material, classification and treatment: *Anthurium schlechtendalii* Kunth roots (104 kg) were collected in Actopan, Veracruz, Mexico. The taxonomic identification and classification were performed at the herbarium of the Institute of Ecology, A.C. (INECOL). The roots were cleaned and washed to remove the impurities and cut into small pieces of approximately 20 mm with a stainless-steel knife. Subsequently, the roots were dried at room temperature for one week to reduce the moisture content.

Plant extracts and drying: The plant material used was reduced to approximately half of its original weight (% moisture) to obtain the extracts, taking as a reference the percentage of moisture and solids when receiving the batch. An infusion extraction was performed using a 1:5 w/v plant–water ratio. The plant material was weighed on a portable electronic scale (Ohaus, Scout Pro SP601, China) and given an additional cleaning treatment by placing it in a plastic colander, where it was sprinkled with deionized water from a plastic wash bottle and brushing the roots to remove residual impurities as much as possible. Then, the roots were cut into small pieces of around 10 mm in length, placed in a plastic sieve strainer and rinsed with deionized water, before draining to remove as much water as possible.

The plant material was put in a 1000-mL beaker, covered with an appropriate volume of deionized water and heated for 13 min on a heating magnetic stirring plate (Gallenkamp, 400, England). The beaker was removed from the heat, and the suspension was filtered through a plastic sieve to separate the solids from the infusion. The solids and infusion were allowed to cool to room temperature. The infusion was then passed through a filtration system (Kitasato flask assembly and Büchner funnel) using a Whatman No. 2 paper supported by a water-suction vacuum pump (Cole Parmer, 7049-50, Chicago, Illinois). The obtained residue was filtered through a cellulose membrane (Millipore, Catalog No. HVLP04700) of pore size 0.45 µm, using glass microfiltration equipment pre-sterilized by a dry heat oven with the aid of a vacuum pump (Edwards, MF20, England). The aqueous extract (infusion) was lyophilized (Labconco, 4.5, Kansas, USA) at a pressure of 133 × 10-3 mbar (0.133 bars) and a temperature of -40°C. At the end of the process, the material was weighed and transferred to sterile conical tubes (Mark FaLDon).

Determination of lethal concentration 50 (LC50): The procedure used was an adaptation of the works of McLaughlin, et al. [20], Molina-Said [21] and Ajibola et al. [22]. Artemia salina eggs (from Canada) were purchased at a local aquarium in Veracruz city. Artemia salina were cultured in a cylindrical device covered in black plastic to protect it from light. The first phase of the culture was hydration. Artemia salina eggs (50 mg) were weighed and suspended for one hour in 250 mL hydration solution (desalted water, pH 7.5), with continuous aeration supplied by a fish tank pump (Elite, 802, China) in a vertical position and irradiated with white light from a 60-watt lamp. The eggs were then filtered through a 100% polyester screen cloth (SPE brand, 200 mesh count, China) and subsequently decapsulated by rinsing with a 1:5 v/v aqueous NaClO solution prepared from commercial chlorine (Clorox Brand) for no more than 3 min. Excess chlorine was removed by rinsing with deionized water. Finally, the eggs were incubated in a sterile saline solution (sea salt) at 28°C for 20 h, with a white light placed approximately 30 cm from the hatchery. To ensure hatching, air was pumped at a constant volume using a fish tank pump (Elite, model 802, China). Subsequently, the larvae were collected with a sterile Pasteur pipette, with white light from a lamp guiding the viable larvae, in order to separate them from the rest of the eggs that did not hatch. The viable larvae were maintained for 24h in saline supplemented with commercial dry yeast (10 mg/L) under the same aeration and light conditions.

A solution of 40 mg/L $K_2Cr_2O_7$ was used as a positive control to guarantee total mortality of the nauplii. The LC50 of $K_2Cr_2O_7$ has been reported to be between 12.50 µg/mL and 12.60 µg/mL [21,23]. The concentration of the positive control was diluted by 50% due to the addition of other

components. Ten wells were used for the positive control test. Nauplii suspension (100 µL) was added to each well in a 96-well flat-bottom polystyrene tray (12 columns × 8 rows) with low evaporation caps (Corning, Costar 3595, USA) (10 ± 1), and 100 µL of a solution of the lyophilized aqueous extract of *Anthurium schlechtendalii* Kunth, corresponding to each concentration (10 µg/mL, 100 µg/mL, 1000 µg/mL, 10,000 µg/mL), were also added. The wells were filled with 200 µL (negative control, positive control, and extract concentrations). Each assay was performed with three replicates for each concentration of the lyophilized aqueous extract from the roots and 200 µL saline water was added to the microplate wells to minimize evaporation. Living and dead nauplii were counted after 24h of exposure to the above treatments to determine LC50. A PROBIT test was performed on the SPSS Version 20 program to verify the dose–response.

Animals and treatment: Thirty male Wistar rats (9–10 weeks old, weighing 240 ± 10 g) were purchased from Harlan Teklad, Mexico and individually placed in stainless steel boxes in a temperature-controlled room (26 ± 2 °C) with 60% relative humidity and a 12-h light–dark cycle (7 am,7 pm). The animals had free access to a standard (commercial) diet in powdered form and potable water throughout the experimental period. The standard diet contained 0.75% phosphorus, 0.95% calcium, 0.20% magnesium, 23% crude protein, and 3.3 IU/g vitamin D3 (5012-Rat Diet, Lab Diet USA). The animal experiments were approved by the Animal Ethics Committee, Chemical–Biological Area University of Veracruz.

Random assignment of the experiment: After the adaptation period (7 days), 30 animals were weighed and randomly allocated into six groups (A, B, C, D, E, F) of 5 animals each. The cages had been previously numbered (1–30) and were represented in the draw with these numbers inside spheres inside a rotating drum. In another rotating drum were five spheres for each of the six groups with their corresponding letters. A number and a letter were selected for each animal to generate a specific key. Then, the following experimental groups were organized to determine the prevention or remission of liver damage: Control (A), received a standard diet and water ad libitum; B, received a standard diet supplemented with 4-tert-octylphenol (100 mg/kg body weight) and water ad libitum; C, received a standard diet supplemented with solid extract and water ad libitum; D, received a standard diet supplemented with solid and aqueous extracts; E, received a standard diet supplemented with 4-tert-octylphenol (100 mg/kg body weight), solid extract and water ad libitum; and F, received a standard diet supplemented with 4-tert-octylphenol (100 mg/kg body weight) plus solid and aqueous extracts. The experimental diet was a standard powdered diet plus a solid (125 mg/kg/day) and/or aqueous (1.8 mg/mL/day) root extract of *A. schlechtendalii* Kunth for each experimental diet. The animals received the respective experimental diet and the aqueous extract for 4 weeks, prepared once a week and refrigerated until use. The preparation of the diets and aqueous extract and the feeding of the animals were done by someone other than the researcher to ensure that the researcher did not know which animals made up each study group and thus avoid error of prejudice. Bodyweight, food intake, and fluid intake were recorded daily. At the end of the study, the animals were euthanized by decapitation, and their blood was collected. Liver function tests were performed. The liver of each animal was also extracted for further analysis.

Assays: Alkaline phosphatase (ALP), aspartate aminotransferase (AST), and alanine aminotransferase (ALT) were determined. They were analyzed by colorimetric and enzymatic methods on a Vitro 250 kit from Johnson & Johnson, using dry chemical reagents from Rochester, New York, USA.

Statistical analysis: The data were expressed as mean ± standard deviation (X ± SD). Statistical significance was determined with analysis of variance procedures (one-way ANOVA) and post hoc Tukey's multiple range tests. Data were analyzed using IBM SPSS Statistics, Version 20, 2011 [24].

Results

Solid and aqueous extracts of *A. schlechtendalii* Kunth: About 8 kg of the plant material with a

relative moisture content of 50% were extracted by aqueous infusion in a 1: 5 w/v ratio to obtain the solid extract after lyophilization to give 39.2 g. About 35 g of this solid extract were allocated to be administered to the study groups that should have the solid extract included in their diet. On the other hand, 7.8 kg of the same batch of plant material, from which 50% of the original moisture had also been removed, were used to obtain the aqueous extract, generating 32 liters, which were administered to murine whose diet should include this type of extract.

Toxicity test of *A. schlechtendalii* Kunth extracts: Table 1 shows the different concentrations of the extracts of *A. schlechtendalii* Kunth and the response obtained in the toxicity test with Artemia salina. The nauplii were evaluated within 24 h of being in direct contact with the extracts, and the larvae were observed using a stereoscopic microscope (Vickers; M17/428N; UK); nauplii that had no movement were considered dead. The data obtained from the toxicity test with Artemia salina to determine the lethal concentrations (LC50) of the extracts of *A. schlechtendalii* showed that the toxicity of the extracts had concentrations between 10000 and 1000 µg/mL, since, for the upper limit of concentrations, all the Artemia salina were dead (100%), while, for the lower limit, a low percentage (7.5%) of larvae was still found (Table 1). According to the results obtained, the extracts showed no toxicity for the first three concentrations tested on Artemia salina. Data analyzed using the PROBIT Transformation (Probability Unit) based on living and dead organisms yielded an LC50 of 1441.6 µg/mL.

Growth parameters, food and fluid intake, and liver weight in rats fed with *A. schlechtendalii* Kunth roots and treated with or without 4-tert-octylphenol: Table 1 shows the parameters of growth, food and fluid consumption, and liver weights for control and experimental groups, whose diets were supplemented with *A. schlechtendalii* kunth, with or without the administration of 4-tert-octylphenol. The treatment with *A. schlechtendalii* Kunth roots and administration of 4-tert-octylphenol for 4 weeks had no significant effect on body weight for the control and experimental groups when their respective initial and final values were compared. In addition, the parameters of food and fluid intake, as well as liver weight, reflected the same trend, with no statistically significant difference between the control and experimental groups.

Biochemical determinations: The results of the biochemical parameters in rats whose diets were supplemented with A. schlechtendalii Kunth roots and treated with or without 4-tert-octylphenol are shown in Table 2. A 4-week study of the liver enzymes aspartate aminotransferase (AST), alanine aminotransferase (ALT), and alkaline phosphatase (ALP) showed no statistically significant differences between the control and experimental groups.

Discussion

Medicinal plants are natural resources with enormous preventive value against diseases. They are everywhere around us and should be used regularly as part of our lifestyle since prevention is better than cure. The aim of this study was to evaluate the therapeutic effect of *A. schlechtendalii* Kunth (stone root) on the model of hepatic damage induced by 4-tert-octylphenol. *A. schlechtendalii* Kunth is used in alternative herbal treatment of chronic renal failure in some communities in the southeast of Veracruz, but there is no scientific evidence supporting its therapeutic use as a hepatoprotective or remission agent of hepatic or renal damage. In addition, it is necessary to confirm its non-hepatotoxicity since the liver is the most important organ for detoxification in the human body. There are no studies in the literature on the toxicity of *A. schlechtendalii* Kunth extracts, or its hepatoprotective or remission effect on hepatic damage induced by 4-tert-octylphenol. It is now known that extracts of plants with potential pharmacological action can be tested for in vitro toxicity using a useful, economical, and traceable bioassay called lethality test, with Artemia salina [22,25].

Larvae of Artemia salina are aquatic invertebrates that play a fundamental role in the flow of energy within the food chain. They can be used to determine the toxicity of a substance by evaluating its average lethal concentration (LC50). This technique has a high correlation with specific toxicity tests [20], because it is sensitive to a wide range of compounds [26–28]. In this study, the results of the analysis of the PROBIT transformation, based on living and dead organisms, yielded an LC50 of 1441.6 µg/mL. According to the criteria of Lagarto-Parra, et al. [29], the aqueous extract of *A. schlechtendalii* Kunth is not toxic.

Administration of solid extract (group C) and solid and aqueous extracts (group D) for 4 weeks resulted in lower, but not statistically significant figures for liver function markers (AST, ALT, ALP) compared to control group (A). This confirms that the extracts of *A. schlechtendalii* Kunth roots were not toxic under the dosage and administration time conditions applied in the study. However, hepatotoxicity, or liver function damage according to AST, ALT, ALP figures, could not be observed in group (B) treated with the toxic 4-tert-octylphenol, neither in those groups administered with 4-tert-octylphenol plus solid extract (E) or 4-tert-octylphenol plus solid and aqueous extracts (F). These three enzymes are released from the hepatocytes into the blood stream when liver damage occurs, so a possible cause of the lack of increase in these markers is the toxic administration method. In this study, it was not through direct oral dose but mixed in food, possibly producing a low intake of 4-tert-octylphenol. Therefore, the experimental model used was not able to show liver function marker damage nor changes in growth parameters.

As far as is known, this is the first report on the effect of A. schlechtendalii Kunth roots extract toxicity, and their hepatoprotective capacity against hepatic damage induced by 4-tert-octylphenol in a rat model. In relation to the beneficial capacity of the extracts of A. schlechtendalii Kunth, particularly on hepatic function, it is necessary for future studies to define, with changes in the experimental conditions, whether or not this capacity exists against the hepatic damage induced by 4-tert-octylphenol.

Conclusion

Under the experimental conditions of the study, and according to the lethal concentration (LC50) given by the PROBIT transformation, the extract of the roots of *A. schlechtendalii* Kunth, a plant used in traditional medicine, was not toxic. The specific finding of this study is that, under the conditions tested in the preclinical experiment [with rats), 4--tert-octylphenol hepatotoxicity could not be induced in the animal groups treated (B, E, F). The administration path used has been proposed as a possible cause. The need to continue and expand research to accumulate sufficient scientific knowledge to elucidate the efficacy of the *A. schlechtendalii* Kunth roots as a preventive, mitigating, or remission agent of liver damage has been made evident by the results achieved.

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Table 1. Evaluation of the toxicity of the aqueous extract of *A. schlechtendalii* through the lethality test of Artemia salina.

Extract (µg/mL)	Initial Count of Artemia salina	Dead Artemia salina	Mortality (%)
10,000	99	99	100
1000	98	7.33	7.5
100	101	1.3	1.3
10	95	0.66	0.7
0	100	0	0.0

Each assay was performed with three replicates for each concentration of the *Anthurium schlechtendalii* kunth extract.

Table 2. Growth parameters, food and liquid consumption, and liver weight for the control and experimental groups supplemented with A. schelechtendalii Kunth roots, with or without the administration of 4-tert-octylphenol.

Parameters						
Parameters	A	B	C	D	E	F
Initial body weight (g)	219 ± 8	214 ± 11	215 ± 11	217 ± 10	211 ± 8	218 ± 5
Final body weight (g)	290 ± 25	289 ± 8	276 ± 10	287 ± 13	273 ± 5	278 ± 14
Food intake (g)	20 ± 0.3	20 ± 0.0	20 ± 0.1	20 ± 0.5	20 ± 0.4	20 ± 0.3
Liquid consumption (mL)	34 ± 8	38 ± 7	37 ± 8	41 ± 4	43 ± 6	46 ± 4
Liver weight (g)	8 ± 1	7 ± 1	8 ± 0.5	8 ± 0.5	8 ± 0.4	8.0 ± 0.7

Values are mean ± SD (n = 5 rats), (A) Control group; (B) 4-tert-octylphenol; (C) Solid extract group; (D) Solid and aqueous extract group, (E) 4-tert-octylphenol plus solid extract; (F) 4-tert-octylphenol plus solid and aqueous extracts.

Table 3. Biochemical measurements in rats treated with *A. schlechtendalii* Kunth, with or without 4-tert-octylphenol administration.

Parameters	A	B	C	D	E	F
Aspartate aminotransferase AST (U/L)	232 ± 59	224 ± 43	212 ± 42	212 ± 80	242 ± 44	248 ± 40
Alanine aminotransferase ALT (U/L)	64 ± 20	65 ± 15	58 ± 8	62 ± 21	60 ± 7	80 ± 22
Alkaline phosphatase ALP (U/L)	143 ± 19	134 ± 40	134 ± 30	128 ± 34	134 ± 23	132 ± 20

Values are mean ± SD (n = 5 rats), (A) Control group; (B) 4-tert-octylphenol; (C) Solid extract group; (D) Solid and aqueous extract group, (E) 4-tert-octylphenol plus solid extract; (F) 4-tert-octylphenol plus solid and aqueous extract