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Evaluation of the Activity of *Lippia alba* Leave Extract in Reducing Ochratoxin A in Albino Rats

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Authors' contributions

This work was carried out in collaboration between all authors. Author OOO designed the study, wrote the protocol and wrote the first draft of the manuscript. Author TOA performed the statistical analysis and managed the analyses of the study. Author JEA managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Ochratoxin A is a metabolite of fungal origin with the potential of inciting oxidative stress which results in disease conditions in man and animals. Plant parts from time immemorial have been used to treat disease conditions with considerable success, hence the study is aimed at determining the capacity of *Lippia alba* leaf extract to reduce the concentration of ochratoxin A in animal serum, kidney, and liver. Albino rats (60) were obtained and maintained, then divided into pre-treated set which were administered plant extract before toxin administration intraperitoneally, and post-treated set administered the toxin then treated with the plant extract. After 7 days of treatment and observation, the animals were euthanized, serum collected, and the organs harvested. Ochratoxin A concentration was determined using HPLC. Results obtained showed that serum of animals in group 5 treated with *L. alba* extract after toxin administration reduced OTA levels to 0.12 ± 0.06 ng/mL in the pre-treated set, while 0.58 ± 0.01 ng/mL was obtained in the post-treated set. The

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livers of pre-treated animals presented a 75% reduction in ochratoxin A concentration level compared to post-treated set that obtained 59.57% ochratoxin A reduction. Kidney toxins levels were lower than values obtained in the liver and serum. Though considerable reductions in ochratoxin A levels were recorded in the kidney, there was no significant difference from the control at $p<0.05$ in values obtained between the post-treated and pre-treat sets unlike values obtained in the serum and liver that presented significant differences at $p<0.05$. The study concluded that the plant possesses therapeutic capabilities which brought about the effects noted in the study. It is recommended that further study be undertaken to unravel the other potentials of the plant and mechanism of action.

Keywords: Ochratoxin A; Lippia alba; concentration; administration; intraperitoneal.

1. INTRODUCTION

Ochratoxins are poisonous mycotoxins contaminating agricultural produce and threatening food security [1]. Apart from being responsible for reducing food quality, they also bring about disease condition (ochratoxicosis) and cancerous development in man and in animals [2]. Ochratoxin A (OTA) is a hepatotoxic metabolite produced by *Aspergillus* and *Penicillium* species and defined as a carcinogen by the International Agency for Research on Cancer (IARC) [3,4]. Production of the toxin is promoted by poor storage conditions, high water activity, high temperature and humidity, pest infestation, and strain of fungal pathogen [1]. As a result of its carcinogenic potential, WHO and FAO set provisional maximum intake of 100 ng/kg body weight [5]. Exposure can be by ingestion of contaminated agricultural food and products, consumption of contaminated animal products or permeation through the skin [6,7].

Monitoring ochratoxin A levels in body system can be by measuring the toxin in human blood, breast milk and some tissues [8-10]. Half-life of OTA is increased by its covalent binding to albumin in di-anionic form thus extending its plasma half-life which results in the creation of a reservoir from where OTA is subsequently released to other target organs and cells in the body [11,12]. In human, ochratoxin A half-life is 35 days [13]. While OTA binding prolongs its half-life, it reduces its elimination, and the toxin is serum albumin, species; sex, dosage, form administered, exposure route specific [14-16]. Removal of ochratoxin A from the body is by the biliary (transfer from plasma through hepatocytes to the bile) and renal excretory systems which transport the metabolites through the kidney into urine, and thereafter excreted through the urinary and fecal routes [17]. Elimination also takes place through milk in lactating animals [18].

Ochratoxin A and its metabolites are implicated in the induction of reactive oxygen species (ROS) which aid oxidative damage within cells, while another theory proposed that, the lactone carbonyl group on OTA ring induces toxicity in the O-methylated OTA (OM-OTA), and another theory claimed that quinines attached to OTA-derived quinine (OTAQ/OTAHQ), are products of oxidative dechlorination which can induce oxidative stress within the cell [19-22].

Efforts to develop phytochemicals that can remove, deactivate, or detoxify mycotoxins; that is accepted for use, and cost effective without producing residual toxic components in the prevention of fungi infection and mycotoxins contamination in agricultural produce have been on for a while [23]. These plant are laden with antimicrobial potential, easy to prepare and apply without huge capital investment, locally renewable, user-friendly and environmentally safe [24-27].

Lippia alba of the family Verbenacaea, is an aromatic, perennial shrub common to the tropical forests and made up of over 200 species [28]. The leave infusion has sedative activities and has been reportedly use to treat digestive upset, diarrhea, and stomach pains [29-31]. The plant has cytotoxic, antifungal, antibacterial, antiviral, sedative, febrifuge, carminative, antispasmodic and anti-inflammatory potentials which results from the presence of citral, linalool, carvone, limonene, γ-terpinene, 1,8-cineol-camphor, 1,8-cineollimonene, limonene-piperitone [32,33]. The plant also contain geranial, neral, geraniol and trans- β–caryophyllene and a host of plant oils that contain oxygenated monoterpenes and bicyclosesquiphellandrene which confers it with high antifungal activity [34,35]. The study aims to determine the ability of *Lippia alba* to reduce the concentration of ochratoxin in animal serum and organs.

2. MATERIALS AND METHODS

2.1 Collection and Preparation of Albino Rat Models for Ochratoxin A Toxicity Study

Albino rats (250 - 300 g) were obtained and treated for the study. The animals were kept in 12 h light and darkness respectively, provided feed, and administered water *ad libitum* till the end of the study. Feed administered was prepared by Grand cereals limited, Plateau State, Nigeria. The feed was confirmed to be free of mycotoxin before use. Animals were maintained following the Guide for care and use of laboratory animals in research and teaching policy of the National Academy of Science, National Institute of Health (NIH) publication 86-23 (1985 revised).

2.2 Experimental Design

Sixty (60) animals were employed for the study. The animals were divided into two (2) set of 30 animals each and each set was further divided into a group of five animals. The animals acclimatized to their new environment 14 days before the experiment started.

2.2.1 Set 1: *Lippia alba* pre-treated

These animals were administered *Lippia alba* extract at 300 mg/kg body weight for a period of three weeks before intoxication with OTA. The idea was to build the immunity of the animals. Group 1: Negative Control – Treatment with 10% DMSO alone; Group 2: Positive Control – Administration of 300 mg *L. alba* per kg body weight; Group 3: Treatment with only 2 mg OTA per 250 mg body weight; Group 4: Treatment with only 1 mg OTA per 250 mg body weight; Group 5: Treatment with 2 mg OTA/250 g bw + 300 mg *L. alba* per kg body weight; Group 6: Treatment with 1 mg OTA/250 g bw + 300 mg *L alba* per kg body weight.

2.2.2 Set 2: *Lippia alba* post-treated

Animals in this set were administered *Lippia alba* extract at 300 mg/kg body weight after intoxication with OTA depending on the group. The animals 3 h after intoxication with observation for behavioral changes were treated with *Lippia alba* extract based on body weight. The grouping was as in set 1.

2.3 Body Weight of Treated Albino Rats

The weight of the animals were taken from the beginning of the experiment till the end of the study. The kidneys and livers were weighed after sacrificing.

2.4 Production of Ochratoxin A for Animal Treatment

Production of crude ochratoxin A for animal treatment was carried out using *Aspergillus ochraceus* isolated from cocoa bean seeds. Production was done using the modified methods of Wangikar et al. [36] and Stoev [37]. *A. ochraceus* was grown on CYA and after 7 days growth at 25ºC, 1 mL of the conidial suspension was inoculated into a mixture of 100 g shredded rice and maize (60:40) in 1000 mL conical flask moistened to 30% (v/w) with distilled water, the mixture is then incubated for 14 days at 25ºC. Spores of the fungi were killed by autoclaving for 1 h at 121°C after incubating. Ochratoxin A was extracted from the matrix using the procedure of Association of Analytical Chemist [38]. The matrix was homogenized in sodium bicarbonate solution (3%), centrifuged at 10,000 g for 20 min and filtered through No 1 filter paper, after which the filtrate was extracted in chloroform and 0.1 M phosphoric acid for 20 min, the solvent was then evaporated and residue dissolved in chloroform again. Ochratoxin A was eluted with methanol and analysed by TLC and HPLC. The crude product was further purified using silica gel thin-layer chromatography in ethyl acetate-methanol-acetic acid (94.5:5:0.5). Ochratoxin A was crystallized from benzene and found to be 96.7% pure and identical with standard ochratoxin A.

2.5 Extracts and Ochratoxin A Preparation and Administration to Albino Rats

Extract of plant was administered at a concentration of 300 mg/kg body weight of each animal at 7.00 am by oral administration. Administration of ochratoxin A (2 mg/250 g body weight) was through the intraperitoneal route, and preparation of the toxin was by dissolving in sterile Dimethyl sulphoxide (DMSO) and volume and dose adjusted to animal weight. OTA treatment was once, and animals were fasted for 12 h though access to water was allowed.

2.6 Serum and Organ Collection in Treated Albino Rats

After 7 days of study, the animals were weighed and then euthanized. Blood specimens were collected from the vein in the tail of animals in each group and pooled together into plain tubes. The animals were then sacrificed by cervical dislocation and the liver and kidney removed; washed with water and blotted with filter paper then weighed individually. A portion of the liver and the kidney were fixed in 4% formaldehyde, while the remaining portion were kept for biochemical tests.

2.7 Extraction and Quantification of Ochratoxin A in Serum

Ochratoxin A in serum was extracted and analyzed using the modified methods of Grajewski et al. [39] for the analysis. To 1 mL serum was added 5 mL (ratio 1:1) mixture of 0.2 M magnesium chloride and 0.1 M hydrochloric acid and 3 mL chloroform, shaken for 30 min at 200 rpm then centrifuge at 4500 rpm for 15 min at 10ºC. The water phase was removed and the solution dried at 40ºC using nitrogen, then re-dissolved in 300 µL methanol left for 3 min and 2 mL phosphate buffered saline (PBS) added. The diluted extract was passed through immuno-affinity column (R-Biopharm Rhone Limited, Glasgow, USA) by gravity or at a flow rate of 2 to 3 mL/min. Twenty milliliter (20 mL) PBS was passed through the column at a flow rate of 5 mL/min then dried by passing air through the column, and desorption solution (acetic acid: methanol (2:98 v/v)) 1.5 mL passed through the column to elute the bound OTA. One hundred microliter of the eluate was injected into the HPLC system to quantify ochratoxin A concentration.

2.8 Quantification of Ochratoxin in Kidney and Liver Specimens

Ochratoxin A extraction from kidney and liver tissues followed the modified methods of Corcuera et al. [40]. Frozen specimen of organ was grounded and to 1 g was added 4 mL cold sodium phosphate buffer and homogenized. Acetonitrile acidified with 1% formic (3 mL) was added to 1 mL of the homogenate to precipitate protein and then centrifuge at 4500 g for 20 min at 4ºC. The diluted extract was applied to an IAC after which the eluate was collected for HPLC analysis.

3. RESULTS

3.1 Ochratoxin A Concentrations in Rat Sera after Intoxication

Ochratoxin A concentrations in the serum as shown in Table 1 were lower in group 6 with reduction rates of 58.1% - 78.9% as compared with group 5 animals of albino rat with values between 50.8% and 88.2%. Values were statistically different at $p<0.05$ for the different treatments administered and ochratoxin A concentration tested when compared to the control group. Group 5 and 6 animals in the pre-treated set showed lower ochratoxin A values when compared with groups 5 and 6 in set 2. After administering ochratoxin A at 1 mg per 250 g bw to animals in the two sets, set 1 had average value 0.62 ng/mL ochratoxin A in serum, while animals in set 2 averagely obtained 0.38 ng/mL ochratoxin A.

3.2 Ochratoxin A Levels in Rat Livers Seven Days after Intoxication

Ochratoxin A level among the treatment groups varied slightly from each other among the 2 sets except for animals in Groups 1 and 2 (negative and positive controls) across the 2 sets which were not administered the toxin. Table 2 showed animals administered 2 mg OTA/250 g bw had values ranging between 2.22 ± 0.24 ng/g and 2.30 ± 0.50 ng/g liver tissue. Fifty three percent reduction in the values on average was recorded when the concentration of OTA reduced by 50 %. Animals in set 1, group 6 had the least concentration of ochratoxin A 0.38 ± 0.05 ng/g. Drastic reductions were noticed with the extract treatment after intoxication with OTA as presented in Table 2. Result showed the values were significantly different from the control at $p<0.05$.

3.3 Ochratoxin A Concentration in Rat Kidney Seven Days after Intoxication

Table 3 showed that ochratoxin A levels in the kidney were lower when compared with values in the liver (Table 2) and serum (Table 1). The highest ochratoxin A value was obtained in the set 1 (animals administered 2 mg ochratoxin A per 250 g bw) with 0.88 ± 0.06 ng/g. The Table presented ochratoxin A concentrations in kidney of animals in group 5 (Set 1) showing drastic reduction to 0.23 ± 0.03 ng/g, but the reduction to 0.17±0.01 ng/g in group 6 of the same set was not significant. It goes to show that at 1 mg ochratoxin A per 250 g bw, pretreatment had little

or no effect compared to treating after intoxication.

4. DISCUSSION

Toxicity enhances liver injury which is brought about by series of metabolic process dysfunction to include DNA damage, disrupted protein synthesis, increased catabolism involving phenylalanine, leakage of damaged tissues, lipid peroxidation, and mitochondrial respiration interference [41,42]. Administration of *Lippia alba* to animals at concentrations between 100 and 800 mg/kg body weight from earlier work [43] (Orole, 2017 – unpublished thesis) presented no abnormal physiology in the kidney, liver, nor the heart lending support to the safety of the plant extracts when administration is through the oral route. Extracts of the plants not only confers health advantages, but presented no toxic effects at the administered concentrations, thus ascertaining the safety of the extracts for further experimental study. Sacrificing of animals on day 7 was due to the short half-life of ochratoxin A which is about 100 h [44].

The result of residual ochratoxin A concentrations as reported in this study agreed with Haack-Micheal [45], Hagelberg et al. [46], and Heussner et al. [14] that ochratoxin A has half-life ranging between 55 - 120 h. The administration of a single dose of ochratoxin A

Table 1. Ochratoxin A concentration in rat serum seven days after intoxication

Treatment	Set 1	Set 2
	Animals pre-treated with *L. alba* before intoxication (ng/mL)	**Animals treated with *L. alba* after intoxication with OTA (ng/mL)**
Group 1	0.00	0.00
Group 2	0.00	0.00
Group 3	1.12 ± 0.12^{c}	1.02 ± 0.09^{c}
Group 4	0.62 ± 0.12^{b}	0.38 ± 0.09^{ab}
Group 5	0.12 ± 0.06^{a}	0.58 ± 0.01^{b}
Group 6	0.08 ± 0.02^{a}	0.10 ± 0.06^{ab}

Values are mean ± SEM; Values with different superscripts within the column are significantly different at p<0.05 by Tukey HSD test

Table 2. Ochratoxin A concentration in rat liver seven days after intoxication

Treatment	Set 1	Set 2
	Animals pretreated with *L. alba* before intoxication (ng/g)	**Animals treated with *L. alba* after intoxication with OTA (ng/g)**
Group 1	0.00	0.00
Group 2	0.00	0.00
Group 3	2.22 ± 0.24^{d}	2.30 ± 0.50^{b}
Group 4	1.08 ± 0.13^{c}	0.99 ± 0.08^{a}
Group 5	0.55 ± 0.06^{b}	0.93 ± 0.09^{a}
Group 6	0.38 ± 0.05^{ab}	0.75 ± 0.08^{a}

Values are mean ± SEM; Values with different superscripts within the column are significantly different at p<0.05 by Tukey HSD test

Table 3. Ochratoxin A concentrations in rat kidneys seven days after intoxication

Treatment	Set 1	Set 2
	Animals pretreated with *L. alba* before intoxication (ng/g)	**Animals treated with *L. alba* after intoxication with OTA (ng/g)**
Group 1	0.00	0.00
Group 2	0.00	0.00
Group 3	0.88 ± 0.06^{c}	0.84 ± 0.07^{c}
Group 4	0.50 ± 0.03^{b}	0.47 ± 0.04^{b}
Group 5	0.23 ± 0.03^{a}	0.33 ± 0.03^{ab}
Group 6	0.17 ± 0.01^{a}	0.17 ± 0.03^{a}

Values are mean ± SEM; Values with different superscripts within the column are significantly different at p<0.05 by Tukey HSD test

for toxicity study was supported by earlier work of Sorrenti et al. [42] who observed that single dose of ochratoxin A caused substantial oxidative damage in rat kidneys.

On the concentrations of ochratoxin A in organs, findings presented in this study contradicted the report of Sorrenti et al. [42] whose study gave higher concentrations of ochratoxin A in albino kidneys when the values were compared to values obtained in the liver. Pfohl-Leszkowicz and Manderville [15] reported that concentration of ochratoxin A in animal tissues and plasma is dose, species, and form of toxin administered dependent. The result presented on ochratoxin A concentrations in body organs disagrees with the report of Haack-Micheal [45], that as a result of higher bonding with serum proteins, concentration in serum is higher than in other body tissues. It should be noted that values obtained after each treatment were still significant because, it is reported that ochratoxin A at low concentrations impact more negatively the cells of kidney and liver. Ochratoxin A concentration of 1 ng/g had teratogenic effects in male albino rat [36].

Reductions in ochratoxin A levels in group 5 and 6 (*Lippia alba* and *Ganoderma lucidum* treated animals) resulted from the scavenging activities of *L. alba* and *G. lucidum* though the former presented a better result by being more effective in scavenging OTA radicals within the cell as seen in the result [47]. When the ochratoxin A values in groups 5 and 6 animals were compared in the serum, liver, and kidney after 7 days treatment with the different extract, it is observed that higher ochratoxin A concentrations were obtained by the serum samples which resulted from OTA binding to serum protein, and so forming adducts which retards ochratoxin A elimination from the serum. The administration of ochratoxin A using the intraperitoneal route was necessitated by the fact that it is easy, faster, and an acceptable delivery method, while administration through the oral route will result in absorption of only 56% of the toxin to the blood stream through the duodenal wall [45,48,49].

5. CONCLUSION

It is essential to isolate active principles of the *Lippia alba*, turn them into pharmaceutical products that can utilize to reduce ochratoxin A concentration in the body, more so that plant is eco-friendly. In conclusion, it was observed that *Lippia alba* presented activity and showed significant abilities to bring about reductions in ochratoxin A levels. Further research work is needed to elucidate the mechanisms employed to bring about reduction and elimination noted animal organs.

CONSENT

It is not applicable.

ETHICAL APPROVAL

All authors hereby declare that "principles of laboratory animal care" (NIH publication no. 85-23, revised 1985) were followed, as well as specific national laws where applicable. All experiments have been examined and approved by the appropriate ethics committee

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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