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Original Research Article

In-Vitro Antioxidant Activity and Acute Toxicity of the Alkaloidal Constituents of *Zanthoxylum zanthoxyloides* Leaves

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ABSTRACT

The roles of plant-derived products in the management of diseases and their safety cannot be overemphasized. In several cases, these plants are used in ethnomedicine without regard to their toxicity. The study evaluated the antioxidant activities of both methanol (MeOH) extract and the bulk alkaloidal fraction of *Zanthoxylum zanthoxyloides* leaves and determined their median lethal dose in mice. The extract was subjected to solvent-solvent partitioning to obtain the bulk alkaloids from the methanol extract. The antioxidant activity of both the methanol extract and the bulk alkaloid fraction was measured *in-vitro* using DPPH radical scavenging; FRAP assays, as well as total antioxidant capacity (TAC) quantitation models. The MeOH extract (IC_{50} 40.22 mg/L; EC_{50} 32.08 µg/mL) and bulk alkaloid fraction (IC_{50} 19.46 mg/L; EC_{50} 30.27 µg/mL) showed significant ($p < 0.05$) antioxidant activities comparable with ascorbic acid (IC_{50} 20.62 mg/L; EC_{50} 32.51 µg/mL). The methanol extract and alkaloid fraction are relatively safe. No death was recorded on the mice upon oral administration of the extract/fraction at maximum dose of 5000 mg/kg. In conclusion, alkaloids constituents of *Z. zanthoxyloides* leaves have the potential to mop up free radicals *in-vitro* suggesting its high antioxidant potentials.

***Keywords*:** *Zanthoxylum zanthoxyloides*, Antioxidants, Free radicals, Alkaloids.

Introduction

Free radicals are unstable biomolecules that have one or more unpaired electrons and play an important role in the pathophysiology of a variety of disorders.[1,2] This explains the renewed interest in drug research and discovery for this class of substances.[3] Oxidative stress is caused by a disparity in the generation of free radicals and the effect of the antioxidants. Medicinal plants can maintain the balance because they contain phenolic and alkaloidal antioxidants mainly from fruits and vegetables.[4,5] Antioxidants serve as guards against oxidative damage.[6] They hold sufficient immunity, repair or maintain homeostasis, and are also involved in stimulating the immune system. The toxicological profile of herbal remedies is an important factor to be considered in drug discovery.[7,8] The safety of herbal remedies is usually ascertained by carrying out toxicity studies on animal models such as mice. However, the toxicities of many medicinal plants used in African folk medicines have not been validated despite claims of safety by the users.

Zanthoxylum zanthoxyloides, Lam. (Family**:** Rutaceae) is used extensively in traditional medicines for the treatment of trypanosomiasis, aches, conjunctivitis and degenerative disorders. [1,3-5,7,9] The plant has gained interest recently because of the presence of bioactive constituents including alkaloids.[14] Its antioxidant activity has been demonstrated by several researches.[11] Its antioxidant property has been attributed to its capacity to mop up free radicals and reduce oxidative stress.[10]

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Phytochemical investigations on *Z. zanthoxyloides* have identified numerous phytochemicals namely alkaloids, flavonoids, tannins and saponins.[11,14] It is therefore important to investigate the relationship between the plants' alkaloidal content and its *in-vitro* antioxidant activity, to validate scientifically the potential values of the plant which are already in use in traditional medicine.

Therefore, in our continued search for antioxidant alkaloids from *Z. zanthoxyloides*, we have previously probed by HPLC-MS dereplication the potential alkaloid constituents of the plants with antioxidant activity.[14] Since the study identified twelve hypothetical alkaloids with antioxidant potential, we report here the extraction, *in-vitro* antioxidant activity and *in-vivo* acute toxicity of the bulk alkaloids. This represents the validation of our initial proposition on the role of alkaloids of *Z. zanthoxyloides* in radical scavenging cascades and the first step in the systematic antioxidant activity-guided isolation of the alkaloidal constituents to validate our earlier hypothesis.

Materials and Methods

Plant material and extraction of crude extract

Healthy *Z. zanthoxyloides* leaves were harvested from gardens in Mbaitolu, Imo State, Nigeria, in November, 2019, while authentication was done by Prof. Charles N. Mba of the Department of Soil Science, Federal University of Technology, Owerri. The sample was then preserved as a voucher specimen (#INTERCEDD/901) in the herbarium of the Bioresources Development and Conservation Program (BDCP) research centre in Nsukka, Enugu state, Nigeria. The extraction of crude methanol extract was done following the previously described method.[15]

Isolation of bulk alkaloids

A 50 g of the dried methanol extract was dissolved in 500 mL of a 10% MeOH, and then partitioned between equal volume of dichloromethane and acidified water (0.1 M HCl) in a separatory funnel. Dichloromethane fractions (containing the neutral and acidic

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alkaloid compounds) were collected and evaporated *in vacuuo* at 40ºC to isolate the bulk alkaloids. Thereafter, the aqueous layer was made basic by treating with 0.1 M aqueous ammonia and re-extracted with 500 mL of dichloromethane. Again, the bulk alkaloids were collected in the dichloromethane layer as free-base. Two drops of Dragendorff's reagent were added to the fractions above and the presence of alkaloids was revealed by the production of an orange-red precipitate.[16]

In-vitro antioxidant studies
The DPPH radical scavenging activity, TAC and FRAP of the methanol extract and the bulk alkaloid fractions were determined using ascorbic acid as standard following the previously described standard method.[16]

Acute toxicity test
Thirty (30) mice (weighing 20.99 ± 7.24 g) obtained from the Faculty of Veterinary Medicine, University of Nigeria, Nsukka animal house, were used for the toxicity test. They were placed in clean aluminium cages under suitable conditions (well ventilated and temperature of 25ºC). The use of mice in this research was reviewed and approved by the University of Nigeria Ethics Committee (FEC/2020/TOA/ZZ/000011) and was conducted in accordance with the guidelines on the handling of laboratory animals. Appropriate concentrations of the methanol extract and bulk alkaloid of the plant were dissolved in 3% aqueous tween 80. Three groups of mice (1, 2, 3) were administered 10, 100, and 1000 mg/kg body weight of methanol extract intraperitoneally, each containing five mice (i.p.). Three other groups of mice (A, B, and C) were administered 10, 100, and 1000 mg/kg body weight of bulk alkaloid intraperitoneally, each containing five mice (i.p.). They were examined for acute toxicity symptoms and behavioural responses for 24 hours. Based on the previous result, further increased doses of 1900, 2600 and 5000 mg/kg were administered i.p. to the six groups above respectively. Individually, they were examined for acute toxicity symptoms and behavioural abnormalities 1 hour after dosage and at least once a day for the next 14 days. The maximum test dose was 5000 mg/kg, as recommended by the Organization for Economic Cooperation and Development (OECD).[17]

Statistical analysis
This was done with IBM SPSS version 25.0 Windows and GraphPad Prism v 8.02. All the values were presented as mean ± standard deviation (SD) and analyzed with one-way ANOVA and post hoc Turkey test. The difference between extract and bulk alkaloid was considered significant at $p < 0.05$ levels compared with the standard, ascorbic acid.[18] The antioxidant activity was expressed as inhibition (%). The geometric mean of the maximum dose of the extract/fraction that produced 0% lethality and the maximum dose that caused 100% death was used to compute the median lethal dose, or LD50.

Results and Discussion

Antioxidant activity of the alkaloid constituents
Traditionally, several plants are used against oxidative diseases;[3-5,19] and several mechanisms of action have been proposed to explain the antioxidant actions of phytochemicals.[20-22] This study identified the potential of the alkaloids constituents of *Z. zanthoxyloides* in the management of oxidative diseases and further confirmed by DPPH, TAC and FRAP assay models that the leaves extract used by traditional healers possess antioxidant properties thus complementing its use in the treatment of trypanosomiasis, aches, conjunctivitis and degenerative disorders.[1,3-5,7,9-14,19-22]

DPPH radical scavenging assay
The methanol extract and the alkaloid fraction of *Z. zanthoxyloides* showed positive qualitative DPPH antioxidant assay test. Both the extract and the alkaloid fraction of *Z. zanthoxyloides* showed concentration-dependent inhibition (Figure 1). At all the concentration tested (0-50 mg/L), the bulk alkaloid caused a significantly ($p < 0.05$) increased inhibition compared with both methanol extract and the standard. At 50 mg/L, the bulk alkaloid elicited 93.64 % inhibition compared with ascorbic acid (53.64 %) and methanol extract 41.10 %. Similarly, the inhibitory effects of bulk alkaloids and extract at 40 mg/L were 85.04 % and 36.52 % respectively.

Total antioxidant capacity (TAC) assay
The reducing effect of the alkaloids fraction concentrations was significantly higher compared with the control. The bulk alkaloids significantly ($p < 0.05$) produced a higher reducing capacity (2.57) at 100µg/mL when compared with the reducing capacity of ascorbic acid (0.30). The MeOH extract caused a reducing capacity of 0.20 slightly lower than the control. At 25µg/mL, bulk alkaloids still produced the highest reducing capacity of 1.43 and the MeOH extract 0.01. (Figure 2).

Ferric ion reducing antioxidant power (FRAP) assay
The FRAP assay model followed similar trends with the DPPH and TAC assay models (Figure 3).

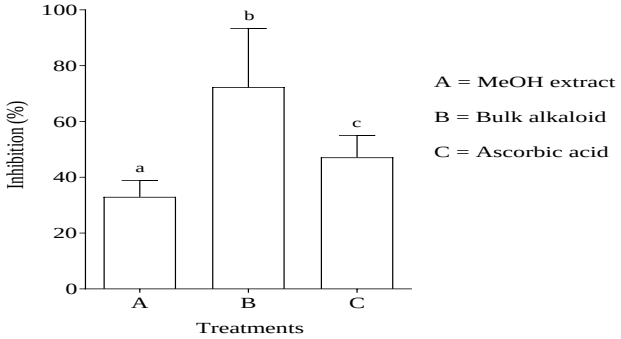

Figure 1: DPPH scavenging activity of extract and bulk alkaloid fraction of *Z. zanthoxyloides*. Bars with different alphabets are significantly different at $p < 0.05$. (Data are mean ± SD, $n = 3$).

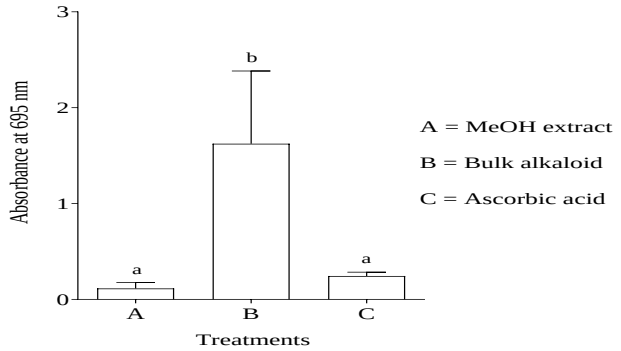

Figure 2: TAC of extract and bulk alkaloid fraction of *Z. zanthoxyloides*. Bars with different alphabets are significantly different at $p < 0.05$. (Data are mean ± SD, $n = 3$).

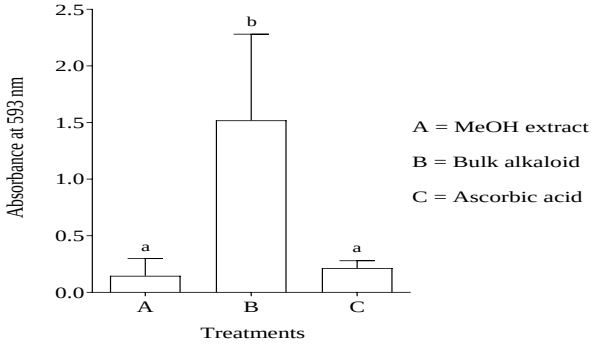

Figure 3: FRAP of extract and alkaloid fraction of *Z. zanthoxyloides*. Bars with different alphabets are significantly different at $p < 0.05$. (Data are mean ± SD, $n = 3$).

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The alkaloids fraction elicited significantly ($p < 0.05$) elevated reducing power at all concentrations tested compared with both MeOH extract and the control. At 100.0 µg/mL, the alkaloids fraction, MeOH extract and the control caused 2.42, 0.43 and 0.27 % inhibition respectively. At 25.0 µg/mL, however, the MeOH extract caused non-significantly ($p > 0.05$) reduced inhibition with the control.

IC_{50} and EC_{50} values of the extract and alkaloids fractions are shown in the table below. Extract and alkaloids fraction with reduced IC_{50} and EC_{50} values show higher potency (Table 1). The alkaloids maintained reduced IC_{50} and EC_{50} values than both the extract and control in both DPPH and TAC assays respectively, an indication of higher antioxidant activity than the MeOH extract and ascorbic acid.

Plants make many variations of natural products commonly called secondary metabolites, which determined their pharmacological properties.[13] One of the most diverse classes of secondary metabolites with useful pharmacological properties, including their use as antioxidants and free-radical scavengers is alkaloids.[8,23-28] The antioxidant property of the alkaloids of *Z. zanthoxyloides* was evaluated by the DPPH assay, FRAP and TAC models. The alkaloids caused a concentration-dependent inhibition of the oxidation process at the tested concentration, significantly higher than the control. It is plausible, therefore, that alkaloids are the major antioxidant constituent of the plant via intercepting and suppressing free radical production, implying a function in the preventive and complementary treatment of diseases. The insulin level of diabetic rats was improved by the alkaloidal extracts of *Z. zanthoxyloides* leaves, why restoring the histoarchitecture of liver and kidney.[29] They were also found to improve hepatoprotective damage and body weight/liver ratio.[9]

Table 1: IC_{50} and EC_{50} of the extract and alkaloid fraction of *Z. zanthoxyloides*

Treatments	DPPH IC_{50} (mg/L)	TAC EC_{50} (µg/mL)	FRAP EC_{50} (µg/mL)
Extract	40.22 ± 7.80	32.08 ± 0.13	34.14 ± 4.79
Bulk alkaloid	19.46 ± 2.24	30.27 ± 1.72	28.37 ± 1.58
Control	20.62 ± 3.82	32.51 ± 0.25	31.22 ± 0.61

Data are mean ± SD; $n = 3$

Acute toxicity test

During the period of the study, *Z. zanthoxyloides* leaves did not cause mortality (lethality) in the mice treated. No substantial weight loss or changes in feeding habits were noted (Table 2). Furthermore, during the study time, the mice did not exhibit hair loss, skin dryness, or overall weakness.

The safety of the extract and alkaloidal constituents was also supported by this study as the LD_{50} was performed in animals. Both the methanol extracts and the alkaloids fraction found that when extracts/fractions were administered to mice orally, they did not die, even at doses as high as 5000mg/kg. This signifies that the crude methanol extracts and the alkaloids fraction are relatively safe. Previous work has been done on the toxicology of the different parts of *Z. zanthoxyloides*. The methanol and aqueous extracts of its root bark were found to be slightly toxic at higher concentrations with reported LD_{50} of 4148 ± 467 mg/kg and 5500 ± 875 mg/kg body weight respectively.[23,30] Similarly, the acute toxicity of the methanolic extract of the root-bark was demonstrated in mice,[31,32] with LD_{50} measured as 5.0 g kg^{-1} body weight. The mice experience cerebral irritation before death. In a similar work, there was no damage on the liver and kidney function after the administration of the root bark extract of *Z. zanthoxyloides* on albino rats. They maintained that the extracts are relatively safe.[3]

The present work further supported the role of alkaloids as antioxidants.[1,9,20,29] While the antioxidant activity of *Z. zanthoxyloides* was formerly attributed to its flavonoid components by previous reports,[11] this study had empirically demonstrated the role of alkaloids in the antiradical activity of the leaves. This further confirms an earlier hypothesis that alkaloids found in *Z. zanthoxyloides* are thought to have a higher reducing capacity than flavonoids.[14] This is the first communication attributing its antioxidant activity to the alkaloid's constituents.

In this study, therefore, it must be noted that the chemical composition or the concentration of the alkaloids was unknown. However, alkaloidal constituents of plants have been implicated in several biological activities of plants, including *Z. zanthoxyloides*.[1,9,20,29] Interestingly, the consistently higher activities of the alkaloid's constituent suggest this as a potential source of antioxidant lead. To confirm this requires eventual isolation of the alkaloids; which is currently in progress.

Table 2: Acute toxicity of extract and alkaloid fraction of *Z. zanthoxyloides*

Extract				Bulk alkaloid			
Group	**Dose (mg/kg)**	**mortality**	**Weight (g)**	**Group**	**Dose (mg/kg)**	**mortality**	**Weight (g)**
Phase I				Phase I			
1	10	0/5	30.5 – 30.4	A	10	0/5	19.5 - 19.3
2	100	0/5	26.0 - 26.1	B	100	0/5	16.9 - 16.9
3	1000	0/5	31.2 - 31.2	C	1000	0/5	17.6 - 17.6
Phase II				Phase II			
1	1900	0/5	13.9 - 14.0	A	1900	0/5	23.7 - 23.8
2	2600	0/5	13.4 - 13.2	B	2600	0/5	32.1 - 32.1
3	5000	0/5	16.5 - 16.5	C	5000	0/5	17.0 - 17.2

Conclusion

This study has validated the earlier proposed hypothesis that the alkaloidal constituents of *Z. zanthoxyloides* could possess antioxidant properties. Furthermore, the alkaloids of *Z. zanthoxyloides* were discovered to be non-toxic and could serve as a source of antioxidant leads.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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