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Antioxidant and Antimicrobial Potentials of Alkaloid-Rich Fraction of *Zanthoxylum Zanthoxyloides*' Leaf

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ABSTRACT

The need to scientifically prove the claims of the trado-medical uses of plants in combating diseases has led to research into the medicinal potencies of some plants. *Zanthoxylum zanthoxyloides* stem has been traditionally used to treat dental and malarial infections. This research was carried out to investigate the medicinal potentials in the leaf of this plant to seek its effectiveness against some diseases. In vitro antioxidant assays were carried out using 1,1-diphenyl-2-picrylhydrazyl radical (DPPH), ferric reducing antioxidant power (FRAP), superoxide radical scavenging activity (SRSA) and hydrogen peroxide (H2O2) scavenging activity. Ascorbic acid was used as standard antioxidant. *In vitro* antimicrobial sensitivity testing was also done using oral microbes obtained from human sputum. Ciprofloxacin was used as positive control, while methanol was the negative control. The minimum inhibitory concentrations for the microbes were determined for K. pneumonia at 100 mg/ml, *S. aureus* at 50 mg/ml and *M. catarrahlis* at 50 mg/ml. The EC_{50} values, the concentration that gives half maximal response of the extract to scavenge the reactive oxygen species, were obtained for DPPH as 0.6043±0.0014 mg\ml, H_2O_2 as 0.7277±0.0339 mg\ml and SRSA as 0.0770±0.0075 mg\ml. Antimicrobial testing also showed the extract was sensitive to *Staphylococcus aureus, Moraxella catarrhalis* and *Klebsiella pneumoniae* at concentrations of 500 mg/ml, 250 mg/ml, 125 mg/ml and 100 mg/ml. Evidently, the alkaloid-rich extract of *Z. zanthoxyloides* possesses potent antioxidant and antimicrobial principles, which lay credence to the traditional use of the plant in the treatment of dental and malarial infections. Consequently, the use of the plant in traditional medicine should therefore be encouraged.

Keywords: *Z. Zanthoxyloides*; Alkaloid-Rich Fraction; Antioxidant; Antimicrobial

Abbreviations: FRAP: Ferric Reducing Antioxidant Power; SRSA: Superoxide Radical Scavenging Activity; As: Absorbance value of Test Samples; Ac: Absorbance of Hydrogen Peroxide; FUTA: Federal University of Technology Akure; S: Sensitive; I: Intermediate; R: Resistant

Introduction

Medicinal herbs have been used for healing as an alternative to medicine by all cultures for several thousands of years. About 80% of the world's population does not have access to conventional drugs and therefore rely on medicinal herbs (Abugassa, et al. [1]). In Nigeria, *Zanthoxylum zanthoxyloides* is used as a chewing stick. Water extracts from the plant showed activities against bacteria significant to periodontal disease (Taiwo, et al. [2]). The anthelmintic activity of the methanolic extract of the root-bark of *Z. zanthoxyloides* was also reported (Ogwal-Okeng, 1999), and it is a very popular anthelmintic amongst the various tribes in Uganda. It has also been found that the alcoholic extracts of the root-bark possesses considerable antibacterial activity (El-Said et al. [3]). Several studies on the various effects of its extracts have been reported. For example, Kassim, et al. [4] reported the anti-malarial activity attributed to benzophenanthridine alkaloid, fagaronine from *Z. zanthoxyloides* root extracts. Anti-malarial activity was also reported in a study using extracts from trunk barks of *Z. zanthoxyloides* (Gansane et al. [5]). On the other hand, Patel

et al. [6] named the compound nitidine as the agent in *Z. zanthoxyloides*' anticancer capabilities while an anti-inflammatory property due to ortho-hydroxymethyl benzoic acid made *Z. zanthoxyloides* useful in the management of pain in sickle cell crisis (Oyedapo, et al. [7,8]). More recently, the potential of *Z. zanthoxyloides* leaf, bark and root extracts as a biopesticide for stored food protection has been reported (Udo [9]).

Materials and Methods

Zanthoxylum zanthoxyloides leaves were obtained from a farm in Ikare Akoko area of Ondo state, Nigeria. The leaves were identified at the Department of Plant science and Biotechnology, Adekunle Ajasin University, Akungba-Akoko, Ondo state, Nigeria. The leaves were washed and air-dried for 8weeks, then it was blended into fine powder. The powdered leaves were soaked with methanol at ratio 5:1(500 ml of methanol to 100 g of the sample) for 72 hours and a clean muslin cloth, was used to filter the soaked sample, the filtrate was then evaporated using the rotary evaporator at 40°C. The resulting solution was then air dried and kept in the refrigerator at -4°C until use.

Extraction of the Alkaloids-Rich Fraction

To 800 g of the plant sample, 4 litres of methanol was added and macerated for 72 hrs and then sieved with a clean muslin cloth. After filtration, the solvent was removed under reduced pressure using a rotary evaporator at 40°C, to minimize any thermal degradation of the alkaloids. Alkaloid was extracted using the procedure described by Hadi and Bremner [10]. The crude alkaloid mixture was then separated from neutral and acidic materials and water solubles, by initial extraction with aqueous acetic acid, followed by dichloromethane extraction and then alkanation of the aqueous solution and further dichloromethane extraction.

Determination of DPPH Radical Scavenging Activity

The DPPH (1, 1-diphenyl-2-picrylhydrazyl) radical scavenging activity of Alkaloid-rich leaf extract of *Zanthoxylum zanthoxyloides* was measured by using assay method described by Shimada et al. [11] with slight modifications. 1 mL each of plant extract at different concentrations (0.2 – 0.8 mg/ml) was added to 1 mL 0.1 mM DPPH dissolved in 95 % ethanol. The mixture was shaken vigorously and incubated in the dark and at room temperature for 30 min. The absorbance was read at 517 nm. Ethanol (95 %) was used as a blank. The control solution consisted of 0.1 mL of 95 % ethanol and 2.9 mL of DPPH solution. Analyses were carried out in triplicates. Percentage inhibition of DDPH radical was calculated.

$$\%\ DPPH\ inhibition\ =\ \left(Abs_{control} - Abs_{sample}\right)\ x\ 100\ /\ \left(Abs_{control}\right)$$

IC_{50} values were estimated from percentage inhibition plot, using a non-linear regression plot.

Determination of Hydrogen Peroxide Scavenging Activity

The ability of the Alkaloid-rich leaf extract of *Zanthoxylum zanthoxyloides* to scavenge hydrogen peroxide was determined according to the method of Ruch et al. [12] as described by Keser et al. [13] with slight modifications. A solution of hydrogen peroxide (4 mM) was prepared in 0.2 M phosphate buffer (pH 7.4). Varying concentrations of the extract (0.2 – 0.8 mg/mL) in distilled water were added to 0.6 ml of 4mM hydrogen peroxide solution. Absorbance value of test samples (As) were read at 230 nm after 10 minutes against a blank solution containing the phosphate buffer without hydrogen peroxide. Absorbance of hydrogen peroxide (Ac) was taken as the control. Ascorbic acid was used as a standard antioxidant. The percentage of scavenging effect was calculated by comparing the absorbance values of the control and test samples using:

$$\%\ Scavenging\ Capacity\ (\%\ Scavenged\ \left[H_2O_2\right]) = \left[\left(AC - AS\right)/AC\right]\times 100$$

IC_{50} values were estimated from the % inhibition versus concentration plot, using a non-linear regression plot.

Determination of Ferric Reducing Antioxidant Property (FRAP)

The reducing power of alkaloid-rich leaf extract of *Zanthoxylum zanthoxyloides* was measured according to the method of Oyaizu [14] with slight modification. An aliquot of 1 ml of different concentrations (0.2 – 0.8 mg/ml) of alkaloid-rich leaf extract (0.2 M PBS, pH 6.6) was mixed with 1 ml of 1% potassium ferric cyanide solution. The mixture was incubated at 50°C for 30 minutes followed by the addition of 1 ml 10% (w/v) TCA. 1 ml of the incubation mixture was added with 1 ml of distilled water and 0.2 ml of 0.1% (w/v) ferric chloride in test tubes. After a 10 min reaction time, the absorbance of resulting solution was read at 700 nm. Higher absorbance suggested stronger reducing power. Ascorbic acid was used as the reference antioxidant. An aqueous solution of known Fe (II) concentrations ($FeSO_4 \cdot 7H_2O$; 2.0, 1.0, 0.5, 0.25, 0.125, 0.063 mM) was used for calibration. Results were expressed as mM Fe^{2+}/mg extract. All the tests were performed in triplicate. The EC_{50} of extracts were calculated from the graph of A700 versus extracts concentration.

Determination of Superoxide Radical Scavenging Activity

The method described by Xie et al., (2008) was used to determine SRSA. Samples (1 mg/mL final concentration) were each dissolved in 50 mM Tris–HCl buffer, pH 8.3 containing 1 mM EDTA and 80 µL was transferred into a clear bottom microplate well; 80 µL of buffer was added to the blank well. This was followed by addition of 40 µL 1.5 mM pyrogallol (dissolved in 10 mM HCl) into each well in the dark and the change in the rate of reaction was measured immediately at room temperature over a period of 4 min using a spectrophotometer at a wavelength of 420 nm. The superoxide scavenging activity was calculated using the following equation:

$$Superoxide\ scavenging\ activity(\%) = (\Delta Abs / \min_b - \Delta Abs / \min_s) / \Delta Abs / \min_b \times 100$$

where b and s are blank and sample, respectively.

Antibiotics Susceptibility Testing

Susceptibility of bacteria and fungi isolate to plant extract was determined following the BSAC Diffusion Method for Antimicrobial Susceptibility Testing Version 8 (Andrews [15]). This test was carried out to determine the antimicrobial ability of the plant extract to inhibit the growth of the bacteria and fungi isolate that was collected from Microbiology Department of Federal University of Technology Akure (FUTA). The plate diffusion technique of Willey et al. [16] was used for the antibiotic sensitivity test. Overnight cultures of the organisms were swabbed on sterile Muller Hilton solidified Agar plates using sterile swab sticks. 8mm sized cork borer was used to bore hole on the agar surface at equidistance the well was filled with the diluted plant extract, a known antibiotic was used as positive control while distilled water was used as negative control. All the plates were incubated at 37^0C to 24 hours. The zones of inhibition generated by the antibiotics were measured to the nearest millimetres (mm) and interpreted as sensitive (S), Intermediate (I) and resistant (R). The zones of inhibition were measured and interpreted according to (NCCLS, 2000).

Statistical Analysis

Results were expressed as mean of replicates ± standard error of mean. The data were statistically analyzed using t-test and Duncan's multiple range tests. Differences were considered statistically significant at $p<0.05$ using SPSS and GraphPad Prism version 7.0 (GraphPad Software, San Diego, CA, USA).

Results

Antioxidant Results for Alkaloids

DPPH Radical Scavenging Activity (%)

DPPH EC_{50}

Bars are expressed as means ± standard error of mean of triplicate determinations (n=3). Values with the same superscripts do not differ significantly while values with different superscripts are significantly different ($p<0.05$) from one another.

Hydrogen Peroxide Scavenging Activity (%)

H_2O_2 EC_{50} (mg/ml): Bars are expressed as means ± standard error of mean of triplicate determinations (n=3). Values with the same superscripts do not differ significantly while values with different superscripts are significantly different ($p<0.05$) from one another.

Ferric Reducing Antioxidant Power (FRAP) (mmol Fe^{2+}): Figure 1 shows the ferric reducing antioxidant properties of ascorbic acid (control) and the alkaloid rich extract of *Z. zanthoxyloides*. It significantly ($p<0.05$) reduced antioxidant activity which is concentration dependent when compared to ascorbic acid.

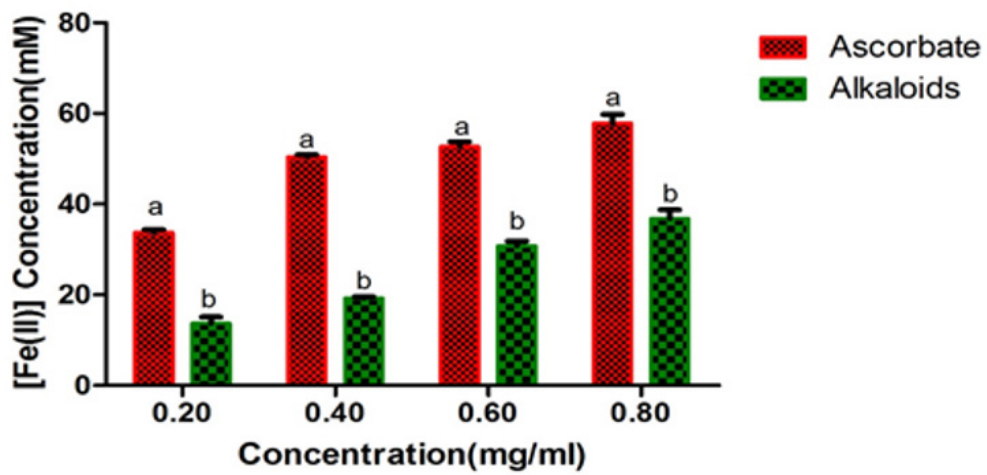

Figure 1.

Note: Bars are expressed as means ± standard error of mean of triplicate determinations (n=3). Values with the same superscripts do not differ significantly while values with different superscripts are significantly different ($p<0.05$) from one another.

Bars are expressed as means ± standard error of mean of triplicate determinations (n=3). Values with the same superscripts do not differ significantly while values with different superscripts are significantly different ($p<0.05$) from one another.

Superoxide Radical Scavenging Activity (%)

Superoxide Radical EC_{50} (mg/ml)

Antimicrobial Assay Results

(Tables 1-3).

Table 1: Result of antibacterial test using alkaloids-rich extracts of Z. zanthoxyloides against some bacteria.

Name of bacteria	Alkaloid (MM)	Distilled water (MM)	Tetracycline (MM)
Klebsiella pnuemoniae	12.0	0.00	35.0
Staphylococcus aureus	17.0	0.00	45.0
Bacillus subtillis	15.0	0.00	40.0
Citrobacter amalonaticus	11.0	0.00	32.0
Escherichia coli	15.0	0.00	38.0

Note: Sensitive (S) ≥ 21, Intermediate (I) 20≤ 15 and resistant (R) ≤ 14

Table 2: Result of antifungal test using alkaloid-rich extracts of Z. zanthoxyloides against some fungi.

Name of fungi	Alkaloid (MM)	Distilled water (MM)	Nystatin (MM)
Aspergillus favus	15.0	0.00	24
Candida tropicalis	12.0	0.00	26
Candida albican	14.0	0.00	24
Rhyzopus stolonifer	16.0	0.00	28
Fusarium solani	150	0.00	23

Note: Sensitive (S) ≥ 21, Intermediate (I) 20≤ 15 and resiq stant (R) ≤ 14

Table 3: Result of minimum inhibitory concentration test using alkaloid-rich extracts of Z. zanthoxyloides against some bacteria.

Name of organism	100 mg Alka extract (MM)	80 mg Alka extract (MM)	60 mg Alka extract (MM)	40 mg Alka extract (MM)	20 mg Alka extract (MM)
Klebsiella pnuemoniae	12.0	00.0	00.0	00.0	00.0
Staphylococcus aureus	17.0	11.0	00.0	00.0	00.0
Bacillus subtillis	15.0	11.0	00.0	00.0	00.0
Citrobacter amalonaticus	11.0	00.0	00.0	00.0	00.0
Escherichia coli	15.0	12.0	00.0	00.0	00.0

Note: Sensitive (S) ≥ 21, Intermediate (I) 20≤ 15 and resistant (R) ≤ 14

Discussion

DPPH Radical Scavenging Activity of Alkaloid-Rich Fraction of *Z. Zanthoxyloides*

DPPH (2,2-diphenyl-2-picrylhydrazyl) analysis is one of the best known, accurate, and frequently employed methods for evaluating antioxidant activity. It is a stable free radical which determines the ability of pure substances or crude extracts for trapping the unpaired electron species by donating hydrogen atoms or electrons, and produced in consequence of the radical disappearance and the formation of less reactive species derived from the antioxidant (Parekh et al. [17]). In this study, the effect of the alkaloid-rich extract of *Z. zanthoxyloides* on DPPH scavenging activity increased, with the increase in concentration of the extract from 0.2-0.8 mg\ml and it is thought to be due to their hydrogen donating ability. The DPPH radical scavenging activities of the alkaloid-rich extract as well as their EC_{50} values in comparison to ascorbate (standard antioxidant) are as presented in Figures 2 & 3 respectively. These results appear to be similar to that obtained by Suman et al. [18] for an aporphine alkaloid crebanine derived from *Alphonsea sclerocarpa*. The EC_{50} values show that the extract scavenged the radical to a 50% inhibition at a concentration of 0.6043±0.0286 mg/ml.

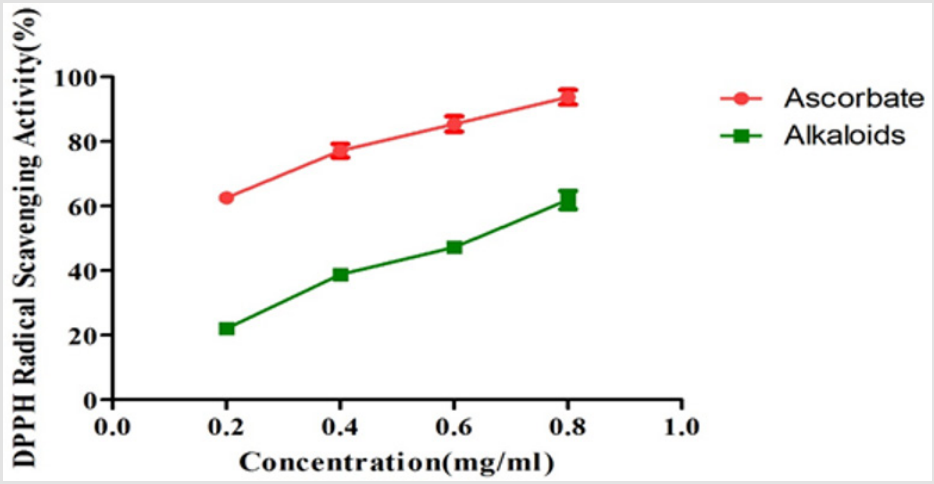

Figure 2: DPPH Scavenging Activities of alkaloid-rich extract of *Z.zanthoxyloides*.

Note: Each point represents an average of triplicate determinations (n=3)

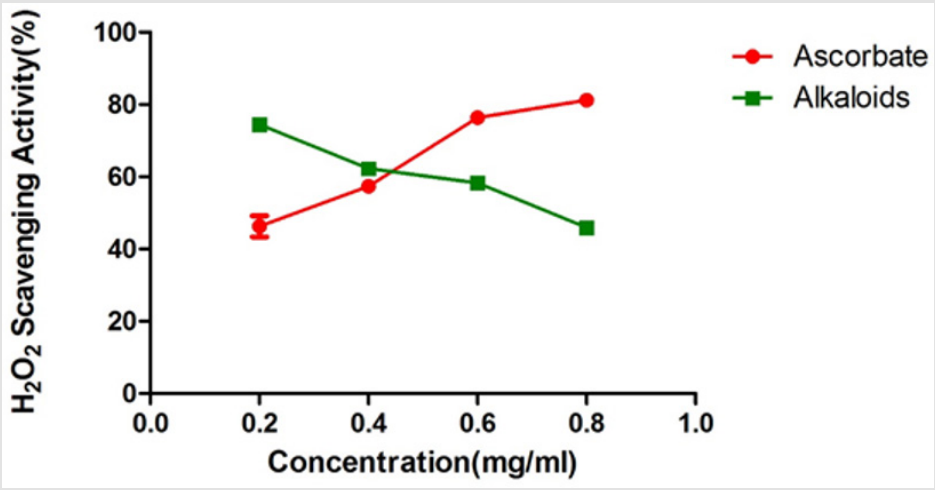

Figure 3: Hydrogen peroxide scavenging activity of alkaloid-rich extract of *Z.zanthoxyloides*.

Note: Each point represents an average of triplicate determinations (n=3)

Hydrogen Peroxide Scavenging Activity of Alkaloid-Rich Fraction of *F. Zanthoxyloides*

Hydrogen peroxide (H_2O_2) is a weak oxidizing agent and crosses cell membranes rapidly to enter the cell. There, H_2O_2 can react with Fe^{+2} ions to generate the hydroxyl radical and this may be the origin of many of its toxic effects (Halliwell, et al. [19]). H_2O_2 is relatively stable in the absence of reducing compounds. Scavenging of H_2O_2 by the extract may be attributed to their electron donating abilities (Wettasinghe, et al. [20]). The H_2O_2 scavenging activity of alkaloid-rich extract of *Z. zanthoxyloides* and their EC_{50} values are presented in Figures 4 & 5 respectively. The result revealed a higher antioxidant activity of the extract at concentrations 0.2 and 0.4 mg\ml greater than the standard and at concentrations 0.6 and 0.8 mg\ml, the scavenging activity was significantly reduced compared to that of the standard. The extract scavenged the radical to a 50% inhibition at a concentration of 0.7277 ±0.0339. The ability of bioactive compounds to reduce ferric ions has a strong correlation with their antioxidative properties. FRAP method is based on the reduction of Fe^{3+} to Fe^{2+} by antioxidants in acidic medium (Benzie, et al. [21]). The FRAP activity of the alkaloid-rich extract is presented in Figure 1. The radical scavenging activity of the extract is concentration dependent, the scavenging activity increased with increase in the extract concentration. This result was found to be similar to that obtained by Suman et al. [18] for a aporphine alkaloid crebanine derived from *Alphonsea sclerocarpa*.

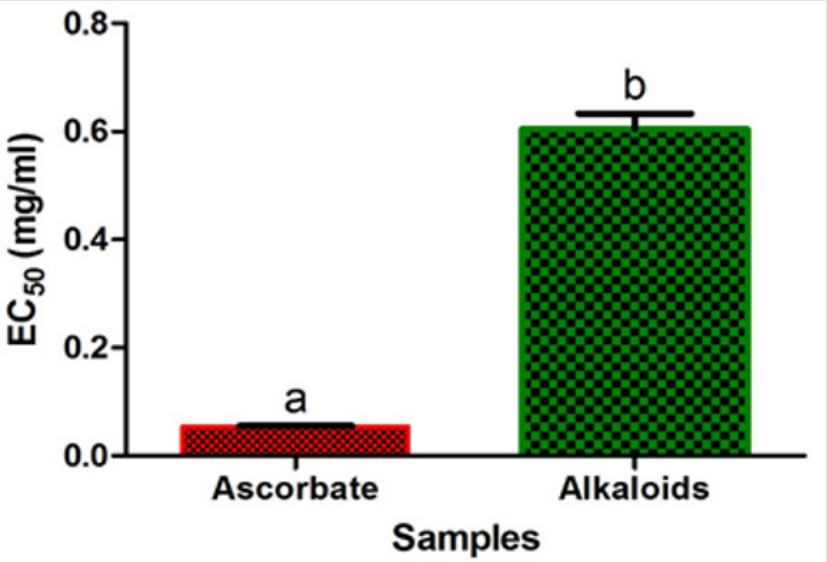

Figure 4: DPPH Radical EC_{50} values alkaloid-rich extract of *Z. zanthoxyloides*.

Note: Bars are expressed as means ± standard error of mean of triplicate determinations (n=3). Values with the same superscripts do not differ significantly while values with different superscripts are significantly different ($p<0.05$) from one another.

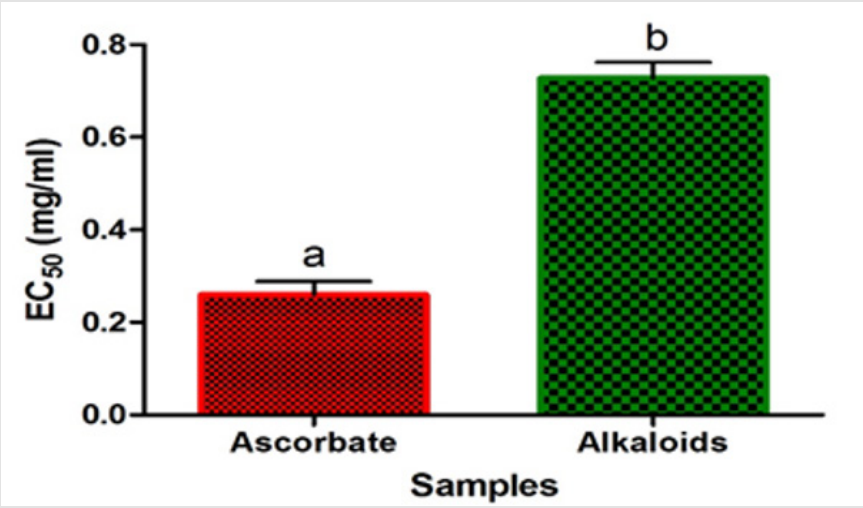

Figure 5: Hydrogen peroxide radical EC_{50} values of alkaloid-rich extract of *Z.zanthoxyloides*.

Note: Bars are expressed as means ± standard error of mean of triplicate determinations (n=3). Values with the same superscripts do not differ significantly while values with different superscripts are significantly different ($p<0.05$) from one another.

Superoxide Radical Scavenging Activity of Alkaloid-Rich Fraction of *Z. Zanthoxyloides*

Superoxide dismutase catalyses the dismutation of the highly reactive superoxide anion to oxygen and hydrogen peroxide. These species are producewd by a number of enzyme systems in auto-oxidation reactions and by non-enzymatic electron transfers that reduces molecular oxygen. It can also decrease certain iron complexes such as cytochromes (Sena et al. [22] Gulein et al. 2011). (Korycka-Dahl, et al. [23]). The superoxide activity of alkaloid-rich extract of *Z. zanyhoxyloides* and their EC_{50} values were presented in Figures 6 and 7 respectively. This study reveals that the alkaloid-rich extract showed a potent superoxide scavenging activity but lower to that of ascorbic

acid. The extract scavenged the radical to a 50% inhibition at a concentration of 0.0770±0.0075 mg\ml. The presence of secondary metabolites like flavonoids, carotenoids and triterpenes in higher plants have excellent antioxidant activity by scavenging reactivity oxygen species which prevent possible damage to cellular components such as DNA, proteins and lipids (Ksouri et al. [24]) [25-35].

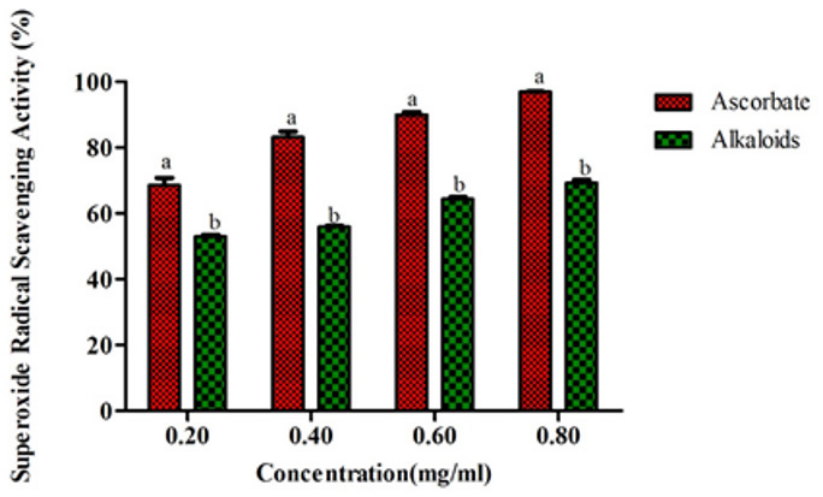

Figure 6: Superoxide scavenging activity of alkaloid-rich extract of *Z.zanthoxyloides.*

Note: Each point represents an average of triplicate determinations (n=3).

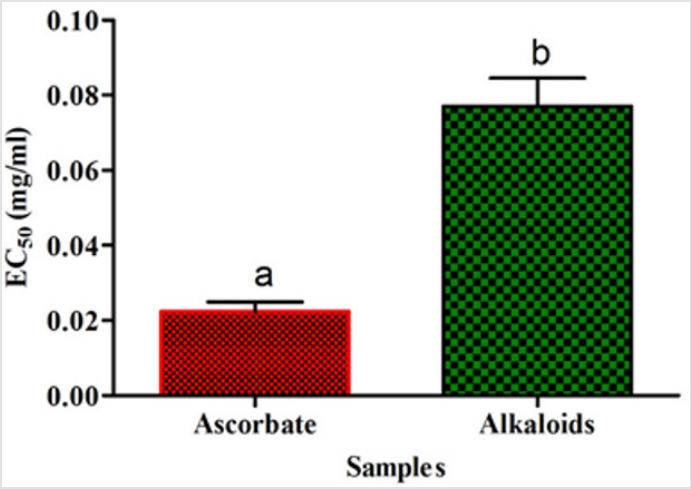

Figure 7: Superoxide radical EC_{50} values of alkaloid-rich extract of *Z.zanthoxyloides*.

Note: Bars are expressed as means ± standard error of mean of triplicate determinations (n=3). Values with the same superscripts do not differ significantly while values with different superscripts are significantly different ($p<0.05$) from one another.

Antimicrobial Assay

Considering the results obtained above, it was confirmed that at 20 mg -40 mg the extract did not show sensitivity to any of the five bacteria on which it was tested. At 60 mg, the alkaloid-rich extract was not sensitive to the bacteria on which it was tested. At 80 mg, the bacteria *Klebsiella pnuemoniae* and *Citrobacter amalonaticus* were not sensitive to the alkaloid-rich extract while the extract was resistant to *Staphylococcus aureus, Bacillus subtillis* and *Escherichia coli*. At 100 mg, the *K. pnuemoniae* and *C. amalonaticus* were resistant to the alkaloid-rich extract while the *S. aureus*, *B. subtillis* and *E. coli* were intermediately sensitive. The minimum inhibitory concentration of the

alkaloid-rich extract for *S. aureus*, *B. subtillis* and *E. coli* is 100 mg. The extract showed intermediate inhibitory activity against *Aspergillus favus, Rhyzopus stolonifer* and *Fusarium solani* while it was resistant against the *Candida tropicalis* and *Candida albican*.

Conclusion

The alkaloid-rich extract showed potent antioxidant properties comparable to those of the synthetic antioxidant. Thus, the fraction can be utilized as a natural source of antioxidant.

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