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R.O. Onzago

Department of Public Health
Pharmacology and Toxicology,
University of Nairobi, P.O. Box
29053-00625, Nairobi, Kenya

S.G. Kiama

Department of Veterinary Anatomy
and Physiology, University of
Nairobi, P.O. Box 30197-00100,
Nairobi, Kenya

J.M. Mbaria

Department of Public Health
Pharmacology and Toxicology,
University of Nairobi, P.O. Box
29053-00625, Nairobi, Kenya

D.W. Gakuya

Department of Clinical Studies,
University of Nairobi, P.O. Box
29053-00625, Nairobi, Kenya

J. G. Nduhiu

Department of Public Health
Pharmacology and Toxicology,
University of Nairobi, P.O. Box
29053-00625, Nairobi, Kenya

Correspondence:

R.O. Onzago

Department of Public Health
Pharmacology and Toxicology,
University of Nairobi, P.O. Box
29053-00625, Nairobi, Kenya

E-mail: rokindoronald@gmail.com

Evaluation of antimicrobial activity and toxicity of *Vernonia hymenolepis* (A. Rich) traditionally used for toothache in Kenya

R.O. Onzago*, S.G. Kiama, J.M. Mbaria, D.W. Gakuya, J. G. Nduhiu

Abstract

Aim: The main aim of the study was to ascertain the antimicrobial properties and safety of *Vernonia hymenolepis* leaves to validate its use in treatment of toothache. **Materials and Methods:** The Leaves were collected from Trans Nzoia County, shade dried, ground and both organic and water extraction done. Minimum inhibitory concentration against *Staphylococcus aureus* ATCC 25923, *Bacillus cereus* ATCC 11778, *Pseudomonas aeruginosa* ATCC 27853, *Escherichia coli* ATCC 25922 and *Candida albicans* was done. Cytotoxicity was done using Brine Shrimp lethality test and lethal concentration (LC₅₀) determined using Finney computer program. The Oral Acute Toxicity Testing (ATC method) was performed as per Organization for economic co-operation and development (OECD) guideline. **Result and Discussion:** The results showed that the aqueous extract had an inhibitory activity against *Staphylococcus aureus* and had no significant effect on *Pseudomonas aeruginosa*, *Escherichia coli*, *Bacillus cereus* and *Candida albicans* at concentration of 400 mg/ml. The organic extract had inhibitory effect against *Staphylococcus aureus* at a dose of 100 mg/ml and against *Pseudomonas aeruginosa* and *Escherichia coli* both at a dose of 400 mg/ml, *Bacillus cereus* at a dose of 200 mg/ml and *Candida albicans* at 50 mg/ml. This study has shown that the plant extracts has a moderate Cytotoxicity with the LC₅₀ (µg/ml) of 491.8 (µg/ml) and 481.7 (µg/ml) for water and organic extract respectively. Acute oral toxicity ATC method showed that the plant extracts in both preparations were not toxic even at a high dose of 2000 mg/kg. **Conclusion:** It's concluded that *Vernonia hymenolepis* possesses antimicrobial activity and is not toxic.

Keywords: *Vernonia hymenolepis*, Antimicrobial activity, Cytotoxicity, *In-vitro*.

Introduction

Oral health problems affect all age groups and more especially in rural communities this is because of lack of availability of good oral hygiene due to high cost of tooth paste and other commercial preparations that are used for oral health care and also due to resistance of conventional antimicrobials that are available for treatment of oral conditions. This implies that there is a high demand of oral health care, hence resulting to alternative use of herbal medicine. Many people in various communities in Kenya have used plants and other natural products for treatment of various ailments including oral diseases but this sometimes result to death of individuals due to overdose, this may be attributed to lack of sufficient and scientific knowledge about the dose and toxicity of the herbal preparation.

Vernonia is a genus in the family Asteraceae and has very many species of forbs and

shrubs with numerous subgenera and subsections.¹ *Vernonia hymenolepis* is an ever green shrub, the stem has spines and it's green in color, the leaves are leathery pubescent and tomentose beneath. The flowers are mauve white. The leaves are indigenous vegetable that is commonly cultivated by farmers in Nigeria and Cameroon.² *Vernonia hymenolepis* occurs in montane forest, along rivers, old cultivation areas, roadsides, in forest margins and also in bushed grassland. It has been documented to be used for various treatments which include treating of pneumonia, hypertension and also treating diarrhea in babies and jaundice.³ It has also been validated to treat Amoebiasis, malaria, typhoid, and constipation by communities in Kenya.⁴ It's widely used traditionally by Herbalist and communities in Trans Nzoia County, Kenya in treatment of oral conditions especially toothache. However, its pharmacological properties like antimicrobial and toxicology has not been adequately established on its use in oral health care. The aim of this study was therefore to validate the use of *Vernonia hymenolepis* extracts as an antimicrobial agent and also to ascertain its toxicity.

Materials and Methods

Antimicrobial activity

Preparation of plant extracts

The plant sample (Leaves) were collected from Trans Nzoia county, delivered to the Department of Public Health, Pharmacology and Toxicology, University of Nairobi, and then shade dried and milled into powder. The powder was packed in a clean air tight polythene papers.

Dichloromethane/Methanol extract

100 grams of the plant material were dissolved in Methanol: Dichloromethane (1:1) i.e. 500 mls of dichloromethane and 500 mls of methanol then placed in a soxhlet evaporator and extracted at 60°C for 8 hours. The resulting extract was then evaporated to dryness in a rotary evaporator (Ugo Basile, Italy) at 40°C and a pressure of 376 Pascals, the extract was weighed and stored for analysis. The percentage yield was 21.5%.

Water extracts

100 grams of the dried plant material (Leaves) was soaked in one liter of distilled water for 72 hours in a conical

flask. Filtration was done using Whatman No.1 filter paper and the filtrate collected in sterile beaker which was then secured. The filtrate was then freeze dried and 32% (w/w) was yielded. The freeze dried preparation were placed in airtight amber colored sample bottle and stored in a refrigerator at 4°C for further tests.

Test organisms

Strains of bacteria and a fungus were obtained from stock cultures at the Department of Public Health, Pharmacology and Toxicology, University of Nairobi for antimicrobial assays. The gram positive bacterial strains used were *Staphylococcus aureus* (ATCC 25923) and *Bacillus cereus* (ATCC 11778) while gram negative bacteria strains used were *Escherichia coli* (ATCC 25922) and *Pseudomonas aeruginosa* (ATCC 27853). *Candida albicans* was also used.

Broth dilution method

Standard microbials: *Pseudomonas aeruginosa*, *Escherichia coli*, *Bacillus cereus*, *Staphylococcus aureus* and *Candida albicans* strains were cultured overnight at 37°C in blood agar for 18 hours. The test organisms were suspended in physiological saline to equal that of 0.5 McFarland standards.

The Minimum Inhibitory Concentration (MIC) test was conducted as per the Clinical Laboratory Standards institute.⁵ The plant extracts were weighed and mixed in 4ml of sterile Mueller Hinton broth to make a master dilution of 400 mg/ml for both gram positive and gram negative. Eight culture tubes containing 2 ml Mueller Hinton broth were arranged in duplicate, and then two fold serial dilutions were made from the master dilution. The concentrations made were 400 mg/ml, 200 mg/ml, 100 mg/ml, 50 mg/ml, 25 mg/ml, 12.5 mg/ml, 6.25 mg/ml, and 3.125 mg/ml. The plant extract was inoculated with 0.1 ml of individual microorganism into the tubes. All inoculated dilutions were incubated at 37°C for 24 hours. The lowest concentration of the plant extract that retains its inhibitory effect resulting in no growth (absence of turbidity) of a micro-organism was recorded as the MIC value of the extract. A control experiment was run in parallel to study the impact of the solvent itself (without plant components) on growth of the micro-organisms.⁶ The positive controls were Amoxicillin and Benzyl penicillin antibiotics while 2% DMSO was used as negative control.⁷

For determination of MBC, 100 µl of broth from all tubes that showed turbidity was aseptically cultured on TSA plates using pour plate method. The plates were incubated at 37C° for 24 hours. After incubation the lowest concentration of the plant extracts showing no bacteria growth was recorded as MBC. Minimum bactericidal concentration is the lowest concentration at which 99.9% or more of the initial bacteria inoculums were killed. All the experiment was performed in duplicate.

Toxicity testing

Preparation of the marine salt solution and Hatching the brine shrimps

Thirty three grams of marine salt was weighed and dissolved in one liter of distilled water in a conical flask to constitute the marine salt solution.^{8, 9} A shallow brine shrimp hatching tank with two chambers and a divider with several holes were filled with the marine salt solution. Sterile spatula was used to scoop one gram of brine shrimp eggs and applied on a large compartment of the improvised tank. Five milligrams of yeast was also added to the chamber to feed the hatched nauplii, and then the large compartment was covered with aluminium foil. The smaller compartment was illuminated by a 40 watt electric bulb for 48 hours for incubation. The hatched nauplii were collected by use of sterile Pasteur pipette to be used in brine shrimp lethality assay.¹⁰

Preparation of stock solution

One hundred milligrams (100 mg) of both extracts (water and dichloromethane/methane) were used. Dichloromethane/methane extract was dissolved in 0.5% DMSO topped up to ten millilitres using marine salt and mixed with vortex to dissolve completely. Water extract was dissolved in 0.5% distilled water in marine salt. This gave a final stock solution of 10,000 µg/ml which were used for serial dilution.^{8,9}

Cytotoxicity test

In-vitro lethality assay of brine shrimp was used to detect cell toxicity of the plant extracts as described by Meyer *et al.* and McLaughlin *et al.*^{11, 12} Ten nauplii were placed in vials containing 5 ml of marine salt solution and increasing concentrations of *Vernonia hymenolepis* leaves extract

(10-1000 ppm).The control was prepared using the same volume of 0.5% DMSO and distilled water. Live nauplii were counted and recorded after 24 hours and the percentage mortality calculated for the plant extracts and controls. The lethal Concentration (LC₅₀) was calculated using the probit analysis at 95% confidence interval.¹³

The Oral Acute Toxicity Testing

Female albino mice were fasted prior to dosing for 3-4 hours but water was given ad libitum. Three concentrations of the Dichloromethane/methanol and aqueous extracts (50 mg/kg, 300 mg/kg, and 2000 mg/kg) were prepared. The test substance was administered in a single dose by gavage using intubation canula. The animals were weighed and the plant extracts administered at a starting dose of 300mg/kg body weight according to animal welfare recommendation. This dose was repeated with three mice.

The same number of mice were dosed at the next higher dose level of 2000mg/kg for both the extracts and dose repeated.

Results

Antimicrobial test (MIC and MBC)

The results showed that the aqueous extract had significant inhibitory activity against *Staphylococcus aureus* and had no significant effect on *Pseudomonas aeruginosa*, *Escherichia coli*, *Bacillus cereus* and *Candida albicans* at concentration of 400 mg/ml of plant extract (Table 1). The DCM/M extract had better inhibitory effect compared to the aqueous against *Staphylococcus aureus* at a dose of 100mg/ml, *Pseudomonas aeruginosa* and *Escherichia coli* both at a dose of 400 mg/ ml, *Bacillus cereus* at a dose of 200 mg/ml and *Candida albicans* at 50 mg/ml (Table 2).

The Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of both the aqueous and Dichloromethane/methanol extracts against the test microorganisms are summarized in table 3.

Table 1: The antimicrobial activity of aqueous extract of *Vernonia hymenolepis* against bacteria standard cultures

Bacteria Species	400 mg/ml	200 mg/ml	100 mg/ml	50 mg/ml	25 mg/ml	12.5 mg/ml	6.25 mg/ml	3.125 mg/ml
<i>Escherichia coli</i>	+	+	+	+	+	+	+	+
<i>Pseudomonas aeruginosa</i>	+	+	+	+	+	+	+	+
<i>Bacillus cereus</i>	+	+	+	+	+	+	+	+
<i>Staphylococcus aureus</i>	-	+	+	+	+	+	+	+
<i>Candida albicans</i>	+	+	+	+	+	+	+	+

Table 2: The antimicrobial activity of dichloromethane/Methanol extracts against bacteria standard cultures

Bacteria Species	400 mg/ml	200 mg/ml	100 mg/ml	50 mg/ml	25 mg/ml	12.5 mg/ml	6.25 mg/ml	3.125 mg/ml
<i>Escherichia coli</i>	-	+	+	+	+	+	+	+
<i>Pseudomonas auriginosa</i>	-	+	+	+	+	+	+	+
<i>Bacillus cereus</i>	-	-	+	+	+	+	+	+
<i>Staphylococcus aureus</i>	-	-	-	+	+	+	+	+
<i>Candida albicans</i>	+	+	+	+	+	+	+	+

Table 3: Shows minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the plant extracts and standard antibiotics

Plant extract,	Bacteria Species	MIC (mg/ml)	MBC (mg/ml)
Standard antibiotics			
	Water extract		
	<i>Pseudomonas aeruginosa</i>	400	400
	<i>Bacillus cereus</i>	400	400
	<i>Staphylococcus aureus</i>	100	100
	<i>Escherichia coli</i>	400	400
Dichloromethane/methanol	<i>Pseudomonas aeruginosa</i>	400	400
	<i>Bacillus cereus</i>	100	200
	<i>Staphylococcus aureus</i>	50	100
	<i>Escherichia coli</i>	200	400

Amoxicillin	<i>Pseudomonas aeruginosa</i>	-	-
	<i>Bacillus cereus</i>	-	-
	<i>Staphylococcus aureus</i>	100	200
	<i>Escherichia coli</i>	3.125	6.25
Benzyl penicillin	<i>Pseudomonas aeruginosa</i>	-	-
	<i>Bacillus cereus</i>	0.6	0.6
	<i>Staphylococcus aureus</i>	0.1	0.1
	<i>Escherichia coli</i>	-	-

Brine Shrimp Lethality Test

The LC₅₀ (µg /ml) of plant extracts of various concentrations (10-1000 µg /ml) were 491.8 (µg /ml) and 481.7 (µg/ml) for water extract and

Dichloromethane/Methanol extract respectively (Table 4). There was no mortality to brine shrimp in both marine salt and DMSO at the tested concentration.

Table 4: Lethal concentration LC₅₀ of *Vernonia hymenolepis* extracts

Plant extract	LC ₅₀ (µg /ml)	95% CI
Water extract	491.8	186.3-1501.9
Dichloromethane/Methanol 1:1	481.72	165.44-318350

Acute Oral Toxicity

There was no mortality of the mice died at 300 mg/kg (b.t.w full name) even on repeating the same dose as per OECD guidelines. At a dose of 2000 mg/kg (b.t.w) none of the three mice died at the first set of dosing in both the aqueous and Dichloromethane: Methanol plant extracts but on repeating the dose with the three set of mice one died in DCM: M extract. Hence both the aqueous and organic extract are in category 5 of Global Harmonization System (>2000-5000 mg/kg b.w.t) with LD₅₀ of 2500. Acute oral toxicity ATC showed that the plant extracts are not toxic at a high dose of 2000 mg/kg b.w.t.

Discussion

The water extract of the plant showed significant inhibitory effect on only *Staphylococcus aureus* while the organic extract showed inhibitory effects on all the tested

micro-organisms *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Bacillus cereus* and *Candida albicans*. This was an indication that organic extract (dichloromethane/methane 1:1) is a good solvent to extract active antimicrobial ingredients from the plant leaves compared to aqueous extract.

The plant extracts inhibited the Gram-positive micro-organisms better than the Gram-negative ones. This is in agreement with previous reports that plant extracts are more active against Gram-positive bacteria than Gram negative bacteria.^{14, 15} Some species of *Vernonia* such as *Vernonia cruda*¹⁶, *Vernonia colorata*¹⁷ and *Vernonia amygdalina*¹⁸ have shown to have antimicrobial activity. Even though the aqueous extract preparation was aimed at mimicking the traditional use, very little antimicrobial activity was observed in this study. These findings relates with that found previously where aqueous extracts showed low or no antimicrobial activity^{19, 20}

which suggested that water is not the most effectual solvent for extracting the active compounds from plants.

Studies have shown that brine shrimp lethality assay is an excellent cytotoxicity test. It has been used to detect fungal toxins, pesticide and cytotoxicity of dental materials.²¹ The interpretation of cytotoxicity was done according to Meyer *et al.*¹¹, Santos *et al.*²² such that LC₅₀ of between 500-1000 µg /ml is classified as weak cytotoxicity, 100-500 µg /ml as moderate cytotoxicity, 0-100 µg /ml as strong cytotoxicity while >1000 µg /ml as non toxic. In the present study the plant extracts had moderate cytotoxicity having LC₅₀ 491.8 (µg /ml) and LC₅₀ 481.7 (µg/ml) for water extract and Dichloromethane/Methanol extract respectively.

Acute oral toxicity ATC method showed that the plant extracts in both preparation, the aqueous and Dichloromethane: Methanol 1:1 were not toxic at a high dose of 2000 mg/kg and hence in category 5 of Global Harmonization System (>2000-5000 mg/kg b.w.t) with LD₅₀ of 2500. LD₅₀ of 2500 validates that the plant is not toxic even at high dose of 2000 mg/kg b.w.t. The antimicrobial activity shown by this study suggests that the plant extracts may be incorporated into modern oral health care products as antimicrobial agent after further studies are done.

Conclusion

Antimicrobial activities exhibited by this plant in the current studies validate its use by herbalist to treat toothache and other oral conditions. The studies have proofed that the plant leaf is not toxic though herbalist have been discouraging patients not to swallow its juice as they initially thought it was toxic. The usage of these plants by traditional healers in the treatment of oral conditions especially toothache is therefore supported by these findings.

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