Asian Journal of Research in Biochemistry

6(3): 32-40, 2020; Article no.AJRB.57527
ISSN: 2582-0516

Effect of Aqueous Stem Extract of *Loranthus micranthus linn* on Anti-microbial Sensitivity, Cytotoxicity, and *In-vitro* Anti-inflammatory Indices on Human Red Blood Cells

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Authors' contributions

This work was carried out in collaboration among all authors. Author CEU designed the study, wrote the protocol and the first draft of the manuscript. Author UDN performed the statistical analysis. Author SE carried out the collection of materials used in the work. Authors HNO and RCI managed the literature searches and review. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/AJRB/2020/v6i330119
Editor(s):
(1) Dr. Fabio Altieri, Sapienza University of Rome, Italy.
Reviewers:
(1) Kishore Janardhan Patil, Kavayitri Bahinabai Chaudhari North Maharashtra University Jalgaon, India.
(2) Umesh S. Khandekar, Smt. Narsamma Arts, Commerce and Science College, India.
(3) A. Doss, National College (Autonomous), Tiruchirappalli, India.
Complete Peer review History: http://www.sdiarticle4.com/review-history/57527

Original Research Article

Received 15 March 2020
Accepted 21 May 2020
Published 29 May 2020

ABSTRACT

Aim: The present study was carried out to investigate the antimicrobial, cytotoxic, and anti-inflammatory activities of aqueous stem extract of *Loranthus micranthus (*African mistletoe) plant.
Methods: The Disc agar diffusion method was used for the antimicrobial susceptibility test of test organisms to determine the minimum zone of inhibition. The brine shrimp lethality test method was used in determining cytotoxicity, and the heat-induced membrane diffusion method was used for anti-inflammatory indices.
Results: In this study, the antimicrobial activity was evaluated using *Escherichia Coli., Salmonella typhi, Pseudomonas aeruginosa*, and *Staphylococcus aureus* as test organisms which showed

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significant zones of inhibition at increasing concentrations (25, 50 and 100 mg/ml) of the plant extract. The result of the cytotoxic investigation after the use of brine shrimps as test organisms revealed that the plant was not toxic as the LC_{50} did not fall within the concentrations used in this study. Also, the aqueous stem extract of *L. micranthus* showed significantly lower optical densities of hemoglobin compared to the corresponding standard (Aspirin) concentrations of 25, 50, 100, 200 and 300 µg/ml. This result was in agreement with the % protection of the experimental plant, which showed a significant increase with an increase in concentration, which implies that the aqueous stem extract of *L.micranthus* has anti-inflammatory effects.

Conclusion: It can, therefore, be concluded that the usage of the aqueous stem extract of *Loranthusmicranthus* as a therapeutic drug would exert health benefits by virtue of its antimicrobial, anti-inflammatory and less toxicity, proved in this study.

Keywords: Loranthus micranthus;anti-inflammatory; antimicrobial; brine shrimp; cytotoxicity.

1. INTRODUCTION

Ever before the advent of orthodox medication, plant extracts have served as dependable sources of medicine in traditional African societies and beyond [1]. Research by scientists all over the world suggests that plant-based drugs and additives are better and safer in addressing man's ever-growing health challenges and that no plant is entirely useless to man [2]. However, most of these plants have been ingested indiscriminately without minding if they could have any side effects.

The increasing prevalence of antimicrobial resistance (AMR) coupled with the dry antimicrobial development pipeline threatens the success and continuation of clinical medicine as we know it [3]. The increased public health threats caused the World Health Organization (WHO) to declare AMR to be one of the three most significant threats to human health, as reported for World Health Day 2011 [4]. In 2004, when the Priority Medicines for Europe and the World report was published, AMR was given considerable attention [5]. Surveillance programs have been initiated at local, national, and international levels. Successful programs have led to better interventions aimed at assessing AMR and ensuring more appropriate antibiotic prescribing.

Inexpensive and readily available diagnostic tools are now available for a variety of infectious diseases. Some of these tools are able to distinguish between viral and bacterial infections, but the pressing need to find a cure for these infections still persists [6].

This study also investigates the cytotoxic activity of the folklore claimed plant *Loranthusmicranthus* using brine shrimp lethality bioassay, which is based on the ability of the plant extract to kill the laboratory cultured brine shrimp *(Artemia nauplii*).

Artemia salina, the brine shrimp, is an invertebrate component of the fauna of saline aquatic and marine ecosystems. It plays an essential role in the energy flow of the food chain [7]. They can be used in a laboratory bioassay in order to determine the toxicity by the estimation of the medium lethality concentration LC_{50} [8].

Standard known non-steroidal anti-inflammatory drugs (NSAIDs) such as Aspirin, ibuprofen, and naproxen are drugs that inhibit the enzyme cyclooxygenase, which synthesizes prostaglandins that cause inflammation and as such, alleviate pain. However, prostaglandins protect the stomach and support platelets and blood clotting; these NSAIDs could cause stomach ulcers and severe hemorrhage of bleeding, resulting in death [9].

However, owing to these threatening challenges in the biochemical research world, this study was carried out to mitigate them as well as proffering other therapeutics (stem extract) to AMR, anti-inflammation, and toxicity as against the conventional leaf and root extracts.

2. MATERIALS AND METHODS

2.1 Plant Material

The plant was collected from Ugba tree *(Pentaclethra macrophylla, Benth*), within the localities of Amaeke Item, Bende Local Government Area, Abia State Nigeria and was identified by Dr. GarubaOmosun of the Department of Plant Science and Biotechnology, Michael Okpara University of Agriculture, Umudike, Abia State.

2.2 The Preparation of Plant Stem Extract

The stem of *Loranthusmicranthus* was collected, rinsed, cut into smaller pieces, and was air-dried for three weeks. The dried stem was pulverized into a fine powder and stored in an air-tight bottle. 400 grams of the fine powder was weighed using a beam balance and was dissolved in two glass jars containing 600ml of boiled distilled water. The mixture was allowed to stand for five days, after which it was filtered using Whatman No.1 filter paper. This process was repeated two more times to obtain a clear filtrate and to ensure the absence of stem particles in the filtrate.

The filtrate was then concentrated to near dryness in a digital electrical oven at 40^0C and kept in an air-tight bottle.

2.3 Method for Anti-Microbial: Disc Preparation

Whatman No.1 filter paper discs of (6 mm in diameter) were punched out with the aid of paper punch and placed in Bijour bottles, which were sterilized by autoclaving at 121°C for 15 min and kept in a refrigerator until required for use. Disc antimicrobial activity testing Agar diffusion method as modified and adapted from the European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2012) was employed. The freshly prepared Mueller-Hinton agar plates were dried in a dryer for about 15 min to remove surface moisture at 40 to 45°C using (GenlabWidness England, Model DC 125) glassware dryer. The discs were prepared using serial double dilution by dissolving 6.25 g, 12.5 g, 25.0 g, 50.0 g, and 100.0 g of the extract in 100 ml of the solvent (water). Half (50 ml) of the extract was introduced into five (5) sterile discs in the Bijou bottle to make 6.25, 12.5, 25, 50, and 100 mg/disc concentrations. 50 ml of the solvent was added into the remaining stock solution making it 100 ml. Each disc was capable of adsorbing 0.01 ml of the solution; the procedure was employed to prepare 6.25, 12.5, 25, 50, and 100 mg/disc concentrations. The plates were aseptically inoculated uniformly with test organisms (*Escherechia Coli, Salmonella typhi, Pseudomonas aeruginosa*, and *Staphylococcus aureus*) by streaking methods. With the aid of sterile forceps, impregnated paper discs (Whatman No. 1 filter paper) containing the extract at different concentrations (6.25, 12.5, 25, 50, and 10) were pressed firmly onto the inoculated agar surface to ensure even contact. Each disc was sufficiently spaced out and kept at least 15 mm from the edge of the plate and 25 mm from disc to disc to prevent overlapping of zones. The plates are incubated at 37°C for 24 hours. The zone diameters of the semi-confluent growths were measured with the aid of a meter rule to the nearest millimeter.

One well from each plate was filled with sterile distilled water as a negative control, and another one was filled with the antibiotic Gentamycin as the positive control, prepared in the concentrations as the extract. The plates were incubated at a temperature of 37°C for 24 hours.

2.4 Brine Shrimp Bioassay:Hatching of Brine Shrimp

Artemia salina leach (brine shrimp eggs) collected from pet shops was used as the test organism. Seawater ($1^1/_2$ teaspoon salt per liter of water) was poured in a v-bottom, semi-translucent bottle/container while the shrimp eggs were added to the container. The pH of the water was maintained at 8.6 using baking soda. The temperature was also maintained at 80-82°F, and two days were allowed to hatch the shrimp as *nauplii*. The constant oxygen supply was ensured through the hatching time to avoid the *nauplii* from settling to the bottom of the container. When the color of the water changed from brown to orange, the oxygen supply was stopped, and light (phototaxis) was channeled to the bottom of the container so the *nauplii* can settle since they are attracted to light. The nauplii were taken from the tank by a pipette and diluted in fresh, clear seawater to increase visibility, and ten (10) *nauplii* were taken carefully by micropipette and placed in another set of beakers containing seawater and the aqueous stem extract of *L.micranthus* at concentrations of 10000, 1000,100, 10 and 1 ppm.

2.5 Counting of *Nauplii*

After 24 hours, the test tubes (triplicate samples) were inspected using a magnifying glass against a black background.And the number of survived *nauplii* in each tube was counted. Potassium dichromate was used as a negative control, while distilled water was used as a positive control.

From this data, the percentage (%) mortality of the brine shrimp *nauplii* was calculated for each concentration, using the formulae;

$$\% \text{ mortality} = 1 - \frac{(\text{numb of survived nauplii})}{(\text{Initial numb of nauplii})} \times \frac{100}{1}$$

2.6 Inflammatory Tests: Preparation of Red Blood Cells (Rbcs) Suspension

Fresh whole human blood (5ml) was collected from human volunteers in the health center of Michael Okpara University of Agriculture, and the administration of non-steroidal anti-inflammatory drugs (NSAIDs) for two weeks before taking the blood was avoided. Collected blood was mixed with equal volume of sterilized Alsever's solution (2% dextrose, 0.8% sodium citrate, 0.5% citric acid and 0.42% sodium chloride*).* The blood was transferred to the centrifuge tubes containing ethylene diamine tetra-acetic acid (EDTA) to prevent clotting. The tubes were centrifuged at 3000 rpm for 10 mins and were washed three times with an equal volume of normal saline. The volume of blood was measured and reconstituted as a 10% v/v suspension with normal saline [10].

2.7 The Heat-Induced Human Red Blood Cell (HRBC) Membrane Stabilization Method

Various concentrations of the extract were prepared in a test tube giving 25, 50, 100, 200, and 300 µg/ml, arranged in triplicate sets (3 sets per dose). 200 µl or 0.2 ml of RBC suspension was added to each.

Aspirin was taken as a standard drug at different concentrations (25, 50, 100, 200, and 300µg/ml) and was compared with respective concentrations of plant extracts. Standard and control were prepared to omit the extracts. These were incubated at 50°C for 30 minutes and centrifuged at 3000rpm for 20 minutes. The hemoglobin content in the supernatant solution was estimated spectrophotometrically at 560nm [11].

The percentage of hemolysis of HRBC membrane was calculated as follows:

$$\% \text{ Inhibition of Hemolysis} = 1 - \frac{(O.D3 - O.D1)}{O.D2 - O.D1} \times 100$$

Where

$O.D_1$ = Absorbance of Control
$O.D_2$ = Absorbance of Standard
$O.D_3$ = Absorbance of Plant Extract at different concentrations

3. RESULTS

The antimicrobial activity of the extract was concentration-dependent, which shows that an increase in the extract concentration will result in a broader zone of inhibition.

4. DISCUSSION

The use of plants (leaves, herbs, and roots) as an alternative to orthodox medicine dates back into history. *Loranthus micranthus*has been studied and confirmed to possess several the rapeutic values, including antidiabetic [12], antioxidant [13] and antimotility [14] activities. Hence, it seems necessary to investigate its antimicrobial and anti-inflammatory properties, as well as establish the safe levels of consumption, therefore its cytotoxic properties.

Inhibition of microbial activity is key to limiting their ability to cause diseases. This inhibition is observed in cultures as gapped zones of the microbial colony. From the antimicrobial screening process conducted in this study using disk diffusion method, it was seen that at concentrations of 6.25 and 12.5 mg/ml, the aqueous stem extract of *L.micranthus* had no significant ($p > 0.05$) antimicrobial effects on *E. Coli*., *Pseudomonas aeruginosa*, *Salmonella typhi,* and *Staphylococcus aureus*, but had significant ($p < 0.05$) antimicrobial effects on all test organisms (except *Pseudomonas aeruginosa* at concentration of 25 mg/ml) in a dose-dependent manner from concentrations of 25, 50 and 100 mg/ml (Table3). This is very significant when compared to the normal control, which showed no zone of inhibition at all in all test samples. At a concentration of 25 ug/mL, the extract had a significantly higher zone of inhibition on *Salmonella typhi* than Gentamycin even though it was significantly lower at all other concentrations and test samples. Despite the variation in concentrations, the results agree with that of Orji et al. [15], which shows the activity of *L. micranthus* extract on the same clinical isolate, such as *S. aureus, E. coli, Pseudomonas aeruginosa* and *Salmonella typhi*. Furthermore, the extract is of concentration-dependent, which shows that an increase in concentration will result in a wider zone of inhibition. However, previous work [16] shows that ethyl acetate and petroleum extracts from the plant did not have activity on *E. coli* at any concentration against the result obtained in this study. This variation might be due to differences in host plant and solvent used for extraction, as suggested by Osadebe [17].

The brine shrimp lethality bioassay is a simple, high throughput cytotoxicity test of bioactive chemicals. It is based on the killing ability of the compound on a simple zoological organism-brine shrimp [18]. It is a handy tool to screen a wide

Table 1. Result showing the antimicrobial effect of a minimal zone of inhibition of aqueous stem extract of *L.micranthus* against test organisms

Test Sample	Concentration (ug/mL)	Average zone of inhibition (mm)		
		Water (normal control)	Plant extract	Gentamycin (positive control)
Escherichia coli.	6.25	0	0	5.2±0.2[b]
	12.5	0	0	9.7±0.5[b]
	25	0	5.0±0.5[a]	13.4±0.04[b]
	50	0	6.3±0.7[a]	19.9±0.8[b]
	100	0	12.0±0.8[a]	24.3±0.8[b]
Salmonella typhi	6.25	0	0	3.1±0.3[b]
	12.5	0	0	6.5±0.5[b]
	25	0	17.0±0.2[b]	12.0±0.8[a]
	50	0	17.0±0.8[a]	19.8±0.8[b]
	100	0	10.3±0.8[a]	21.4±0.2[b]
Pseudomonas aeruginosa	6.25	0	0	2.0±0.2[b]
	12.5	0	0	5.3±0.6[b]
	25	0	0	4.0±0.3[b]
	50	0	5.7±0.3[a]	17.8±0.7[b]
	100	0	10.3±0.7[a]	22.3±0.6[b]
Staphylococcus aureus	6.25	0	0	8.4±0.4[b]
	12.5	0	0	13.7±0.7[b]
	25	0	7.0±0.5[a]	17.2±0.4[b]
	50	0	8.0±0.4[a]	25.3±0.5[b]
	100	0	12.5±0.5[a]	27.8±0.8[b]

Data are expressed as mean±SD; n = 3. One-way analysis of variance (ANOVA) followed by POSTHOC test ($p < 0.05$). [a] significantly lower compared to the corresponding in another treatment; [b] significantly higher compared to the corresponding in another treatment group

Table 2. The Result showing the effect of aqueous stem extract of *L. micranthus*on brine shrimps lethality

	Dose level (ppm)	Initial Nauplii added in the 3 test tubes	Number survived after 24hrs			Total No. survived after 24hrs	%Mortality
			T1	T2	T3		
Distilled Water	1	30	10	10	10	30	0
(positive	10	30	10	10	10	30	0
control)	100	30	10	9	10	29	3.3
	1,000	30	10	10	10	30	0
	10,000	30	9	10	10	29	3.3
Plant extract	1	30	5	5	6	16	46.7
	100	30	7	5	6	18	40
	100	30	8	7	6	21	30
	1,000	30	8	8	7	23	23.3
	10,000	30	8	9	9	26	13.3
$K_2Cr_2O_7$	1	30	0	0	0	0	100
(negative	10	30	0	0	0	0	100
control)	100	30	0	0	0	0	100
	1,000	30	0	0	0	0	100
	10,000	30	0	0	0	0	100

Key T= Test tubes. The percentage mortality of the brine shrimps at various concentrations of plant extract indicates that the LC_{50} is less than 1ppm

Table 3. Result of the effect of aqueous stem extract *L. micranthus* on heat-induced HRBC hemolysis test

Concentrationof test/std (µg/ml)	Mean $O.D_3 \pm$ S.D. (Plant extract)	Mean $O.D_2 \pm$S.D. (Standard)	% Inhibition of Haemolysis
25	0.17±0.03[a]	0.21±0.04 [b]	57
50	0.25±0.04 [a]	0.42±0.08 [b]	60
100	0.23±0.03 [a]	0.32±0.05 [b]	50
200	0.35±0.05 [a]	0.60±0.06[b]	54
300	0.30±0.06 [a]	0.66±0.06 [b]	69

Data are expressed as mean±SD; n = 3. One-way analysis of variance (ANOVA) followed by POSTHOC test (p < 0.05). [a] significantly lower than the corresponding standard control; [b] significantly higher than the corresponding test group. OD_1 = 0.14±0.02.

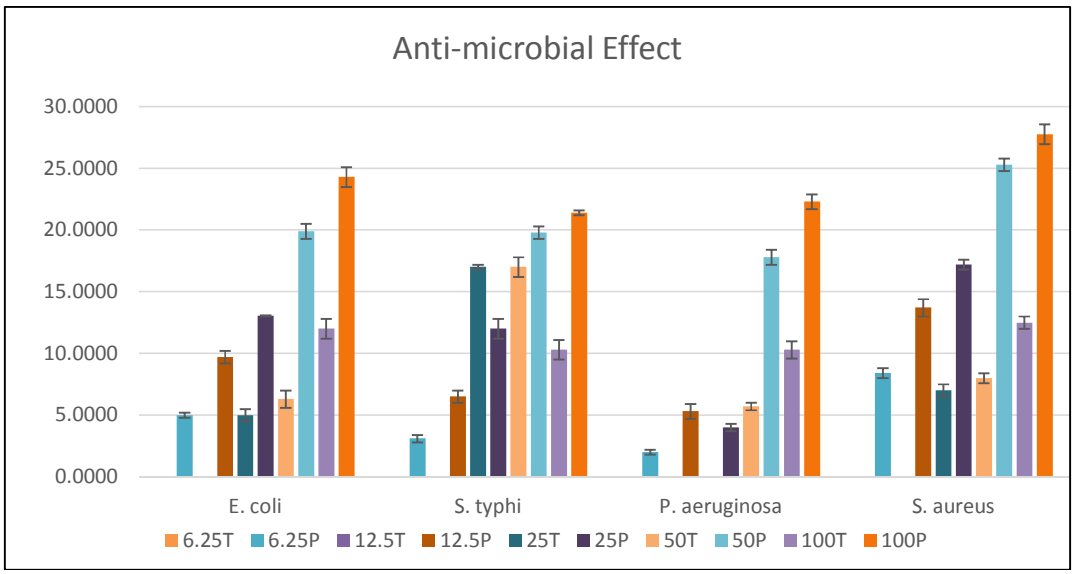

Fig. 1. Result showing the antimicrobial effect of a minimal zone of inhibition of aqueous stem extract of *L. micranthus* against positive control

Key T= Test group; P=Positive control

range of chemical compounds for their various were prepared in parts per million (PPM), and it was observed that the aqueous stem extract of *L.micranthus* did not significantly ($p > 0.05$) induce mortality in the brine shrimp. It was found that increase in the concentration of the extract correlated with a decrease in mortality rate with percentage mortality/lethality of 46.7% and 13.3% at concentrations of 1 and 10,000 ppm, respectively (Table 2), which were the extreme doses. This suggests that contrary to being cytotoxic, the extract could actually be elucidating cytoprotective properties. Also, the $LC_{50,}$ as seen in Table 2, has indicated that the concentration required to kill 50% of the brine shrimp could be less than 1ppm. Comparatively, the positive and negative control has clearly shown the effect of normal and harsh environmental conditions, respectively with respect to the effect of the plant extract.

Stabilization of human red blood cells inhibits lysis and subsequent release of the cytoplasmic contents, which in turn limits the tissue damage and exacerbation of the inflammatory response [19]. The exposure of red blood cells to hypotonic medium, heat and injurious substances such as methyl salicylate or phenylhydrazine, result in the lysis of membranes accompanied by hemolysis and oxidation of hemoglobin [20]. In this study, the aqueous stem extract of *Loranthus micrathus* is observed to have a significant ($p < 0.05$) protective effect against hemolysis with an increase in concentration and as compared to the standard drug used, which is Aspirin. While the mechanism for this response cannot be pinpointed, there have been studies suggesting that NSAIDs, Aspirin inclusive, have a damaging effect on cell membranes, human erythrocyte particularly, by lowering surface tension on such membranes [21].

5. CONCLUSION

The aqueous stem extract of *L.micranthus* was subjected to an antimicrobial, cytotoxic, and anti-inflammatory test. It was assumed to have an LC_{50} greater than 10,000 which did not fall within the concentrations observed in this study and this suggests that the plant extract is not toxic. The result of this study validates earlier reports of the anti-inflammatory and antimicrobial activities of aqueous stem extract of *Loranthus micranthus* by Channabasava and Govindappa [22], which can be used in the production of drugs that treat bacterial infections.

CONSENT AND ETHICAL APPROVAL

Ethical approval was sought from the Health Center of Michael Okpara University of Agriculture, Umudike. Approval of consent was also sought from participants, wherein the following information was given to participants to ensure that they make an informed choice; a complete description of the aims of the study, details of sample collection procedures, potential benefits, and risks of their participation in the research and assurance of confidentiality of any information given as well as of the test results, all these were explained to the subject in English language, and/or their native languages and consent were sought through the signing of an informed consent form. The dignity of the study participants was upheld throughout the study.

ACKNOWLEDGEMENTS

Our gratitude goes to God Almighty for helping us finish this work. We want to appreciate the efforts of our loved ones for their financial and spiritual support.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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Peer-review history:
The peer review history for this paper can be accessed here:
http://www.sdiarticle4.com/review-history/57527