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EVALUATION OF THE ANTIMICROBIAL AND LARVICIDAL POTENTIALS OF SEED EXTRACTS OF *PICRALIMA NITIDA.*

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ABSTRACT: *The increasing rate of resistance to antibiotics and insecticides by microorganisms and various insect pests have become an issue of Public Health concern. In this research, various concentrations of extracts were tried against three Gram negative isolates, zones of inhibitions were recorded in (mm) after 24hrs. Fourth (4th) instar larvae of A. gambiae were tested against various extract concentrations, and mortality recorded over 72hours. Results obtained showed that both methanolic and aqueous extract of seed samples showed excellent antimicrobial activity against the isolates tested. The aqueous extract showed more activity against both E. coli and E. aerogenes, however Klebsiella spp were more susceptible to the methanolic extract. The Minimum Inhibitory Concentration (MIC) values of isolates determined using broth dilution method reveals that the MIC of the methanolic Extracts were ≤20mg/ml, ≤30mg/ml and ≤10mg/ml for E. coli, E. aerogenes and K. pneumonia respectively. MIC values of the aqueous extracts were found to be ≤30mg/ml, ≤20mg/ml and ≤30mg/ml for E. coli, E. aerogenes and K. pneumonia respectively. Result of larvicidal assay revealed that the methanolic seed extract of P. nitida showed a remarkable activity against the insect larva with a mean mortality of 18.7 ± 1.5, 19.3 ± 1.2 and 19.7 ± 0.6 for extract concentrations of 0.5mg/ml, 1.0mg/ml and 2.0mg/ml respectively after 24hours exposure. However, aqueous extract had mean mortalities of 4.7 ± 1.2 and 6.3 ± 1.2 at concentrations of 4.0mg/ml and 5.0 mg/ml after 72hours exposure time. LT_{95} values of methanolic seed extract at ($P \leq 0.05$) was found to be 29 hrs and 16 hrs at concentrations of 0.5mg/ml and 1.0mg/ml, while LT_{95} value for aqueous extract at ($P \leq 0.05$) was 489 hrs at 1.0mg/ml. This research shows that seed extracts of P. nitida are both efficient antimicrobials and larvicidal agents and its potential can be harnessed for future use.*

KEYWORDS: Antimicrobial, Larvicidal, Seed Extracts, Picralima Nitida

INTRODUCTION

Research has shown that various plants are potential source of bioactive agents which can be used in chemotherapy and pest control. *Picralima nitida* is an entirely glabrous shrub of 3-10 m high. Its fruits are ovoid and yellowish at maturity (Adjanohoun *et al.,* 1996). This plant is widely distributed throughout Africa forest regions. Seeds bark and roots of *P. nitida* have a reputation as a febrifuge and remedy for malaria (Kouitcheu *et al.,* 2008). Ubulom *et al.,* (2011), evaluated the antifungal activity of aqueous and ethanolic extracts of this plant against *Aspergillus flavus, Candida albicans* and *Microsporum canis.* Dibua *et al.,* (2013) and Ubulom *et al.,* (2012) also evaluated the larvicidal efficacy of this plant against *Anopheles gambiae* larvae. The plant, *Picralima nitida* has provided drugs used in the treatment of many diseases. Extracts of the plant have been used in the treatment of pathogenic diseases (Ubulom *et al.,*

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2011), protozoan infections (Okokon *et al.*, 2007) and non pathogenic diseases. (Kouitcheu *et al.*, 2006) Diabetes mellitus is a major endocrine disease that is treated with the extracts of the plant (Inya-Agha *et al.*, 2006). Extracts from various parts of the plant including seed, stem bark, and rind have been used for the purpose of therapy. Bosede and Oyelola, (2013) evaluated the antihyperglycaemic and antihyperproteinaemic activity of extracts of *Picralima nitida* Seed and *Tapinanthus bangwensis* Leaf on Alloxan-Induced Diabetic Rabbits. In addition to this, Meyer *et al*., (2006) assessed the medicinal composition of *P.nitida* from laboratory analysis, and found that the active component of *P. nitida* is formed by more than 10 alkaloids present in different tree parts (from bark, leaves, roots). Their names derive from the local name "Akuamma" (Okunji *et al*., 2006). According to Meyer *et al*., (2006), these alkaloids play several biological roles such as: anti-inflammatory (pseudo-akuammigine), anti-fever (erythrocytic phase inhibition of *P. falciparum* with use of roots, stem bark and fruit skin), antimicrobial (against Gram bacteria and fungi with use of root bark), Hypoglycemic control (with use of roots and fruits) and, anti-malaria (with fruits), and anti-leishmaniasis (with roots).

The continuous and inappropriate use of various chemical based agent used in the control of insect vector and infectious microorganisms has resulted in the development of resistance both in insect population and in the population of various microorganisms. There is therefore an urgent need to identify new control strategies that will remain effective, even in the face of growing insecticide and drug resistance (Achs and Malaney, 2002). The search for herbal preparations that do not produce any adverse effects in the non-target organisms and are easily biodegradable remains a top research issue for scientists associated with alternative vector control (Redwane *et al.*, 2002). *Escherichia coli*, *Enterobacter aerogenes* and *Klebsiella pnuemoniae* are causative agents of a wide range of diseases and are often implicated in hospital acquired infections. This research hence evaluates the antimicrobial potential of *P. nitida* against three organisms as well as its larvicidal efficacy against larvae of the vector *A. gambiae*

METHODOLOGY

Collection and Sample Preparation

Picralima nitida samples were sourced from Umuagwu, Akabor in Oguta Local Government Area of Imo State, Nigeria. The plant was classified and identified at the herbarium section of the International Center for Ethno–Medicine and Drug Development, Nsukka by a professional plant taxonomist. The seeds were extracted from the fruit, and then washed and air dried under shady condition at room temperature ($28\pm2^{O}C$). 1g of each dried samples was then pulverized and extracted using a soxhlet extractor. Extracts were then evapourized using a rotary evaporator until solvents were completely evaporated to get the solidified crude extracts. The crude extracts thus obtained was stored in sterilized amber coloured bottles and maintained at $4^{o}C$ in a refrigerator.

Phytochemical Screening

Screening was conducted in accordance with Evans, (2002). The phytochemical tested for includes; saponin, steroid, tannins, terpenes, alkaloids, glycosides and flavonoids.

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Stock Preparation

2.5g of the extracts was solubilized in 5ml dimethylsulphuroxide (DMSO), the mixture was then made up with 495ml of water to get a stock solution of 5mg/ml. volume per volume dilution was then carried out to obtain graded concentrations of 4mg/ml, 3mg/ml, 2mg/ml, 1mg/ml and 0.5mg/ml.

Insect Rearing

About 100 healthy larvae of the insect were collected from flooded grass field. The larvae were identified by a parasitologist as Anopheles by a parasitologist at the department of Zoology University of Nigeria, Nsukka. The larvae were fed with quaker oat and biscuit crumbs in the ratio of 3:1. Emerged adult mosquitoes were transferred to an oviposition cage with potted plants. Adult mosquitoes were fed with 10% glucose solution, laboratory reared albino rats were also provided as source of blood meal for the adults. Ovitraps were placed within the cage for laying of eggs. Eggs laid were collected on daily basis and were recycled to the 4^{th} instar before they were used for the bioassay.

Larvicidal Bioassay

Larvicidal bioassay of individual plant extracts was tested against 4th instar larvae of *Anopheles spp.* The tests were conducted, as described by WHO (2005). Trials were conducted in triplicate with a control. Twenty healthy larvae were introduced into each glass beaker and mortality was observed at 24, 48 and 72 hrs. Larvicidal activity of each extract was determined, by counting the number of dead larvae on daily basis (24hrs interval). The dead larvae in the three replicates were combined and expressed as percentage mortality for each concentration. Larvae were recorded dead when they failed to move after probing with a needle.

Isolation and Identification of test Organisms

The test organisms were Gram negative isolates collected from Bishop Shanahan Hospital, Nsukka. The organisms were sub-cultured on a nutrient agar slant and stored at a temperature of 4^oC. Isolates were identified using the conventional biochemical tests as described by Ezeonu *et al.,* (2010). Tests carried out includes: indole, MRVP test, sugar fermentation, Lysine decarboxylase, Urea hydrolysis test, Gram staining, motility test, Oxidase and Citrate test.

Antimicrobial assays

The antimicrobial activity of each crude extract was measured *in vitro* against the test organisms. This was investigated using the well in agar dilution methods, as recommended by the Clinical and Laboratory Standards Institute Anon (1997). Zones of inhibition of extract concentrations ranging from 50mg/ml to 3.13mg/ml against the organisms were measured and recorded as the mean zone of inhibition (mm).

Determination of minimal inhibitory concentrations

The minimal inhibitory concentrations (MIC) of the extracts were determined using the broth dillution method. Each isolate was cultured overnight on a nutrient broth and then serial diluted using the 10 fold serial dilution method to give 10^4 cfu ml^{-1}. Each dried plant extract was dissolved in 50% DMSO to obtain a stock concentration of 50mg/ml. Graded concentrations of 40, 30, 20, 10 and 5mg/ml was then obtained. 0.1ml of each test organism was suspended in Molten Mueller–Hinton agar containing varying concentrations of the extracts and then pour plated. The test set up was then incubated at a temperature of 37 ± 2^oC for 24 hours and the presence or absence of microbial growth checked. The lowest concentration of an extract at which there was no visible growth was taken as the MIC for the extract–microbe combination. As controls, the MIC of gentamicin against each bacterial species was similarly determined.

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Gentamicin was serially diluted with sterile distilled water to give final concentrations between 5 to 20mg/ml.

Statistical analysis
Results obtained from research were analysed using PROBIT.

RESULT
Phytochemical screening of crude methanolic and aqueous seed extracts revealed the presence of bioactive plant metabolites. Phytochemical tested includes: alkaloids, flavonoids, saponins, tannins, terpenes and glycosides.

Bioassay
Larvicidal bioassay results shown in tables 1 and 2 reveals mean mortality values of the two extracts. Results obtained reveals that the aqueous seed extract had a mean mortality of 4.7±1.2 and 6.3±1.2 at concentrations of 4.0mg/ml and 5.0mg/ml after 72 hours exposure. Methanolic extract had a mean mortality of 18.7±1.5 and 19.7±0.6 at concentrations of 0.5mg/ml and 2.0mg/ml respectively after 24 hours exposure.

Table 1: Mean Mortality of Larva to Aqueous Seed Extract

Exract Conc.(mg/ml)	No of Larva Exposed	24 hrs Mean Mortality	48 hrs Mean Mortality	72 hrs Mean Mortality
0.0	20	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
0.5	20	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
1.0	20	1.3 ± 1.2	1.3 ± 1.2	1.7 ± 1.5
2.0	20	1.7 ± 1.2	2.0 ± 1.0	3.3 ± 0.6
3.0	20	1.7 ± 0.6	2.7 ± 0.6	4.0 ± 1.0
4.0	20	3.3 ± 0.6	4.3 ± 1.5	4.7 ± 1.2
5.0	20	4.0 ± 2.0	5.7 ± 1.5	6.3 ± 1.2

Table 2: Mean Mortality of Larva to Methanolic Seed Extract

Exract Conc.(mg/ml)	No of Larva Exposed	24 hrs Mean Mortality	48 hrs Mean Mortality	72 hrs Mean Mortality
0.0	20	0.0±0.0	0.0±0.0	0.0±0.0
0.5	20	18.7±1.5	19.7±0.6	20.0±0.0
1.0	20	19.3±1.2	20.0±0.0	20.0±0.0
2.0	20	19.7±0.6	20.0±0.0	20.0±0.0
3.0	20	20.0±0.0	20.0±0.0	20.0±0.0
4.0	20	20.0±0.0	20.0±0.0	20.0±0.0
5.0	20	20.0±0.0	20.0±0.0	20.0±0.0

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Biochemical Screening

The biochemical profile of the isolates used in this research is shown in table 3 below.

Table 3: Biochemical Profile of Isolates

	Organisms		
Test	*E. coli*	*E. aerogenes*	*K. pneumoniae*
Indole	+	-	-
MR	+	-	-
VP	-	+	+
Urase	-	-	-
LDC	+	+	+
Glucose	+	+	+
Citrate	-	+	+
Lactose	+	+	+
Mannitol	+	+	+
Gram	-	-	-
Mortility	+	+	-
Oxidase	-	-	-
Sorbitol	+	+	+

Minimum Inhibitory Concentration

The MIC of the extracts against the test isolates showed that the methanolic seed extract had an MIC of ≤20mg/ml, ≤30mg/ml and ≤10mg/ml against *E. coli, E. aerogenes and K. pneumoniae* respectively. However, MIC values for aqueous seed extracts were found to be ≤30mg/ml, ≤20mg/ml and ≤30mg/ml for the same test organisms. Minimum Inhibitory Concentration of the control synthetic antibiotic was found to be ≤5mg/ml for all the test isolates (Figure 1). Mean zones of inhibition of varying concentrations extracts against the isolates tested is shown in table 4

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Table 4: Antimicrobial activities of samples

Organisms	Concentration (mg/ml)	Methanolic Extract Mean ± SD	Aqueous Extract Mean ± SD
E. coli	50.00	18.3 ± 1.5	23.3 ± 1.5
	25.00	16.0 ± 1.0	19.3 ± 1.2
	12.50	12.7 ± 0.5	17.0 ± 1.0
	6.25	8.7 ± 1.2	13.7 ± 1.5
	3.13	2.0 ± 1.0	9.7 ± 1.5
E. aerogenes	50.00	17.1 ± 1.2	26.2 ± 1.3
	25.00	14.7±0.6	21.0 ± 2.0
	12.50	11.0±1.0	17.7 ± 0.6
	6.25	5.3 ± 0.6	12.3 ± 1.5
	3.13	1.7 ± 0.4	7.7 ± 1.2
K. pneumoniae	50.00	24.3 ± 2.1	21.3 ± 1.5
	25.00	18.3 ± 1.5	18.7 ± 1.5
	12.50	14.7 ± 1.5	14.0 ± 1.0
	6.25	6.7 ± 0.3	9.7 ± 0.3
	3.13	4.7 ± 0.6	5.3 ± 0.1

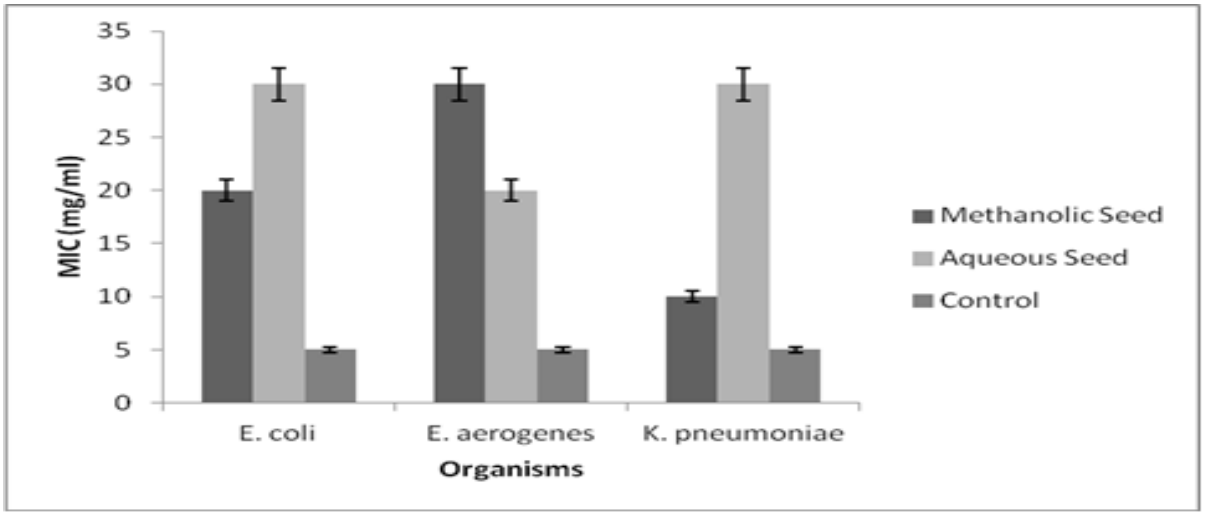

Figure 1: Minimum Inhibitory Concentration of Isolates

DISCUSSION

Insecticide and antimicrobial resistance exhibited by both microorganisms and insect pest demands an urgent alternative strategy in the control of pathogens and vectors of diseases. This research evaluates the antimicrobial as well as larvicidal efficacy of methanolic and aqueous extracts of the seed samples of the

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plant *P. nitida* with a view of exploiting its bioactive potentials as a future remedy in the control of insect vectors and infectious microorganisms. Phytochemicals analysis conducted in this research identified similar phytochemicals like those reported by Dibua *et al.,* (2013), Nkere and Iroegbu, (2005), Ubolum *et al.,* (2012), and Ubolum *et al.,* (2011). This confirms the findings of Wiesman and Chapagain, (2006), Fakeye *et al.,* (2000) and Ramirez *et al.,* (2003) that *P. nitida* contains bioactive components. Larvicidal assay of the extracts revealed a mean mortality of 4.0±2.0, 5.7±1.5 and 6.3±1.2 at 24hrs, 48hrs and 72hrs respectively for the aqueous extract (Table 1). Methanolic extract showed a mean mortality of 18.7±1.5, 19.7±0.6 and 20.0±0.0 with extract concentration 0.5mg/ml at 24, 48 and 72 hrs respectively (Table 2). This is in-line with what Ubolum *et al.,* (2012) reported. Dibua *et al.,* (2013) also reported a similar activity with the methanolic seed extract showing better activity compared to the aqueous.

Antimicrobial properties of seed extracts of *Picralima nitida* evaluated against reported beta-lactam-resistant bacteria revealed that the extracts showed moderate activity on the isolates with the following MIC value for aqueous and methanolic extracts 20mg/ml, 30mg/ml, 10mg/ml and 30mg/ml, 20mg/ml and 30mg/ml against *E. coli, E. aerogenes* and *K. pneumoniae* respectively (Figure 1). This is in-line with the findings of Jacques *et al*., (2011) who reported that glycosides derived from *P. nitida* showed antimicrobial effect against *E. coli, S. aureus*, and *P. vulgaris* at the concentration of 13g/dis. Zones of inhibition of the extracts against the isolates is shown in table 4. None of the isolates tested showed resistance to the extracts, however *E.coli* was more susceptible to the aqueous extract with an inhibition diameter of 23.3mm, were as methanolic extract had an inhibition zone diameter of18.3mm. *E. aerogenes* had an activity similar to that of *E. coli* with inhibition zone diameters of 26.2 and 17.1mm for aqueous and methanolic extracts. This however was not true for *K. pneumonia* which showed more susceptibility to methanolic than to the aqueous extract. This work reveals that extracts of P. nitida can serve both as an agents of insect control and chemotherapy.

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