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# Peptide-rich extracts from leaves of *Newbouldia laevis* (P. Beauv.) Seem. ex. Bureau (Bignoniaceae) with antimicrobial and brine shrimp lethality activities

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**Abstract**
Global concerns over antibiotic-resistant microbial pathogens have spurred a quest for innovative, stable, and target-specific molecules like bioactive peptides. This study evaluates the antimicrobial properties and toxicity of peptide-enriched extracts from *Newbouldia laevis* leaves. Aqueous extraction, thin layer chromatography (TLC), and Phenomenex's Stratum Giant Tube C18-E were used to obtain partly purified fraction (PPF) and crude peptide extracts (CPE). Antimicrobial inhibitory capacity was determined using p-INT dye, with gentamicin and fluconazole as standards. Cytotoxicity was assessed with *Artemia salina* nauplii. Microsoft Excel and GraphPad Prism 5 was used in analysing the data. Peptides were detected in CPE and PPF via TLC and modified G-250**.** PPF demonstrated more efficacy ($IC_{50}$: 5.89µg/mL to 33.94µg/mL) against bacteria and fungi, with low toxicity ($LC_{50}$: 5964.87µg/mL for PPF and 1094.20µg/mL for CPE) in the Brine shrimp lethality assay. The study presents the discovery of bioactive peptides from the leaves of *Newbouldia laevis*. Consequently, bioactive peptides extracted from this plant hold potential as foundational compounds for the development of novel broad-spectrum antibiotics aimed at combating microbial infections.

*Keywords*: Antimicrobial peptides; *Newbouldia laevis;* Brine shrimp lethality assay

## INTRODUCTION

There is an increased research interest on the exploitation of natural and endogenous antimicrobial peptides (AMPs), also referred to as host defence peptides [1] for drug discovery. Antimicrobial peptides (AMPs), which have been known since the 1980s and consist of cationic and hydrophobic amino acids with direct antibacterial activities, are naturally occurring polypeptide sequences of 12 to 50 residues [1]. These classes of peptides have long been considered potential candidates for novel antimicrobial drug development. They are also a component of the innate immune system that all kinds of life possess [2]. Most of the research on AMPs has been focused on finding and describing peptides with strong, broad-spectrum antimicrobial

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characteristics. These peptides have the potential to serve as innovative therapeutic agents and are strong, broad-spectrum antibiotics. It has been demonstrated that AMPs may eliminate or alter cancerous cells, Gram-negative and Gram-positive bacteria, enveloped viruses, and fungi [3].

Many different types of plants have had antimicrobial peptides extracted from their roots, seeds, flowers, stems, and leaves. These peptides have demonstrated efficacy against both human infections and phytopathogens [4, 5]**.** Many other AMPs, including, thionins, cyclotides, defensins, snakins, and in-like proteins based on amino acid sequence and lipid transfer proteins are produced in some plant species[6]

*Newbouldia laevis* is a fast-growing evergreen shrub. It is among the most useful indigenous plants in African ethnomedicine and grows up to 10 metres in height with a cauliflorous habit. It is typically,-referred to as the African border tree. It is a Bignoniaceae-family member angiosperm that is medium-sized, sun-loving, quickly growing, and drought-tolerant [7, 8]. The Bignoniaceae family of plants are widely used in traditional medicinal systems of many countries [9]. In rats, prolonged pentobarbitone-induced hypnosis, spontaneous motor activity, and exploratory activity were all significantly reduced by the methanolic extract of *Newbouldia laevis*. Additionally, it was discovered to lessen the apomorphine climbing response in rats, indicating sedative effects [10]. A previous study [11] documented the potency of ethanolic extracts of *Newbouldia laevis* leaf and root against bacteria often linked to wounds, nosocomial infections, gastroenteritis, septicaemia, and urinary tract infections. In light of this, it has been reported that in traditional medicine, the stem bark of *Newbouldia laevis* has antibacterial properties against bacterial isolates from infected wounds and eyes [12]. However, little is known about the presence of plant-derived peptides from this family and most especially from *Newbouldia laevis*. This study presents the detection of bioactive peptides from the leaves of *Newbouldia laevis* and investigates the antimicrobial activity of its crude peptide extract.

## EXPERIMENTAL METHODS

**Preparation of the extracts.** *Newbouldia laevis* fresh leaves were gathered from a field near the University of Ibadan. Dr Osiyemi of the Herbarium section of the Forestry Research Institute of Nigeria (FRIN), Ibadan, identified and verified the plant. The plant specimen was deposited at the Forest Herbarium Ibadan (FHI), under voucher number 11070**.**

**Fig 1:** *Newbouldia laevis* (P. Beauv) Seem. ex. Bureau (leaves and flowers)

The leaves of the *Newbouldia laevis* were air-dried for two weeks under shade, pulverized and extracted using equal volumes of methanol: dichloromethane under continuous agitation for 18–24 hours at room temperature. Then distilled water was added to the extraction system, vortexed, and left for 24 hours. The supernatant aqueous amino acid/peptide-rich extracts (CPE) were decanted and concentrated using the rotary evaporator to remove methanol before C18

flash pre-purification. The aqueous extracts were pre-purified on RP-C18 solid-phase with C18-parked Cartridges (Strata Gigatubes C18-E; 5 g, 20 mL, Phenomenex, Germany). Following initial preconditioning, equilibration and sample application, bound peptides were washed with 10% buffer B (90%v/v acetonitrile, 0.08% v/v trifluoroacetic acid) and finally eluted with 50 mL of 80% buffer B to obtain the pre-purified peptide fractions (PPF). Cryodesiccation of eluted fractions was achieved with freeze-drying equipment.

**Modified G-250 dye and ninhydrin reagent preparation.** The G-250 dye was prepared by modification of procedures in published reports [13-15]. In the procedure, two hundred milligrams (200 mg) of G-250 was dissolved in forty millilitres (40mL) of orthophosphoric acid and then added to 320 mL of 50% ethanol in distilled water One hundred millilitres (100 mL) of ethanol in which 0.2 g of ninhydrin has been dissolved served as the ninhydrin reagent.

**Chemical detection by Thin Layer Chromatography (TLC), modified G-250 and ninhydrin.** Thin Layer Chromatography (TLC) was used to chemically identify the CPE and PPF extracts. The TLC chemical detection was implemented using a modified version of the approach previously reported [15] and employed pre-coated TLC plates (F254 MERCK, Germany). The solvent mixture was n-butanol: acetic: acid: water (3:1:1). The aqueous peptide fraction from the plant sample that had been solvent reconstituted was spotted on a TLC plate and developed using the solvent system. To find the presence of circular and linear peptides, respectively, newly made modified G-250 stains and Ninhydrin were sprayed over the dried-out plates. The sprayed plates were allowed to dry before being examined in the daylight.

**Lethality test for brine shrimp (*Artemia salin*a (BSLA).** The procedure previously reported [16] was followed for the experiment. Nauplii were harvested after hatching by pipette dropping. Each CPE sample was dissolved in seawater to make a working concentration of 20 mg/mL from which a stock solution of 1000 µg/mL was prepared. The CPE stock solution (1000 µg/mL) was serially diluted and its serial dilutions to concentrations ranging from 1000 to 1 µg/mL were then used. Vials containing various amounts of the CPE's diluted test solutions were introduced. Using a Pasteur pipette, ten active brine shrimp nauplii were then added to each of these vials Seawater served as the control, and each serial dilution was prepared in triplicate. After 24 hours, the number of survivors and mortalities was noted.

**Preparation of stock solution for antimicrobial tests.** The stock solutions of CPE and PPF from *Newbouldia laevis* leaves were prepared by dissolving 20 mg in 2 mL of 1% dimethylsulphoxide (DMSO) which were then kept at 4°C until required for use.

**Microbial cultures.** Gram-positive strains of Methicillin-Resistant *Staphylococcus aureus* (MRSA), *Candida albican,* and Gram-negative strain of *Escherichia coli* (ATCC 25922) were each maintained on Nutrient agar, Mannitol salt agar (MSA), and Sabouraud dextrose agar (SDA), respectively. To produce the bacterial and fungal cultures, a single colony of each organism were inoculated into 5 mL of tryptic soy broth (TSB) for bacteria and 5 mL of Sabouraud dextrose broth (SDB) for fungi. The original culture was sub-cultured for all microorganisms, and they were all incubated at 37°C overnight. The bacteria and fungus were collected from the Pharmaceutical Microbiology Department, University of Ibadan, Ibadan, Nigeria.

**Standard antibiotic.** The positive controls were the normal medications, gentamicin (10 µg/mL), and fluconazole (10 µg/mL).

Dimethylsulphoxide (DMSO), 1% was used as the negative control.

**Determining the minimum inhibitory concentration (MIC) in a quantitative antibacterial test.** The MIC was calculated using the broth micro-dilution technique and a sterile 96-well microtiter plate was used for this procedure [17]. CPE and PPF extract stock solutions (1 mg/mL) were diluted to a working concentration of 200 μg/mL to generate concentrations ranging from 200 to 6.25 μg/mL, serial two-fold dilutions of the working concentrations were made in the microtiter plate using TSB (in triplicate). All of the controls were evenly dispersed throughout the 96-well plates, including the sterile control (broth and plant extract), negative control (culture broth and 1% DMSO without antimicrobial agent), positive control (Gentamicin), and the negative control (broth and organism). Each bacterial suspension was introduced into the test and control wells in 50 L (0.5 MacFarland). The micro-dilution plates were incubated at 36°C for 24 hours while each experiment was run in triplicate. After 24 hours, the plates received 20 mL of an alcoholic solution of 0.5 mg/mL p-iodonitrotetrazolium violet (p-INT), Sigma® Plates were incubated at 36°C for a further 30 minutes. The lowest concentration of each extract at which microbial growth was entirely suppressed was designated as the MIC value. Bacterial growth was formally quantified using optical density measurements (ELISA reader, CLX800-BioTek Instruments).

**Data analysis.** GraphPad Prism 5 software was used to perform non-linear regression analysis and obtain the 50% inhibitory concentration ($IC_{50}$) for each extract using concentration-effect curves.

## RESULTS

**Chemical detection by Thin Layer Chromatography (TLC).** The TLC chemical detection of CPE and PPF of *Newbouldia laevis* leaves produced a violet-pink and a bright blue colouration respectively upon reaction with the sprayed ninhydrin and modified G-250 stain indicating the presence of free amino acids and cysteine argine-rich peptides as shown in (Fig. 2a & 2b).

**Antimicrobial activity.** Table 1 Shows that the partially purified peptide (PPF) has a better activity than the CPE, on all the organisms tested. At 25 μg/mL, PPF showed a better inhibitory activity compared to the CPE with percentage inhibition of 68.47% compared to 48.47% respectively on *E. coli*. The inhibitory effect of PPF on the MSRA was more as compared to CPE with percentage inhibition of 50.91% compared to 36.92% respectively at 12.5 μg/mL. Furthermore, PPF showed a better activity at 25 μg/mL with 67.60% as compared with CPE with 46.18% on *C. albicans*. Table 2 shows that MRSA is more susceptible to PPF and CPE with $IC_{50}$ of 5.89 μg/mL and 33.94 μg/mL respectively than *E. coli* (PPF = 6.48 μg/mL, CPE = 38.62 μg/mL) and for *C. albican*s (PPF = 9.27 μg/mL, CPE = 46.91 μg/mL) as compared to the standards gentamicin ($IC_{50}$ = 4.14 μg/mL) and fluconazole ($IC_{50}$ = 3.51 μg/mL). Table 3 shows the better inhibitory activity of the PPF than the CPE. Therefore, MIC value of the PPF on the *E. coli* and MRSA is 6.25 μg/mL and that of *C. albicans* is 9.25 μg/mL while that of the CPE is 25 μg/mL for the tested organisms compared to the standards which is less than 6.25 μg/mL.

**Lethality test for brine shrimp (*Artemia salina;* (BSLA).** The $LC_{50}$ values obtained for the extracts were 5964.87 μg/mL for PPF and 1094.20 μg/mL for CPE.

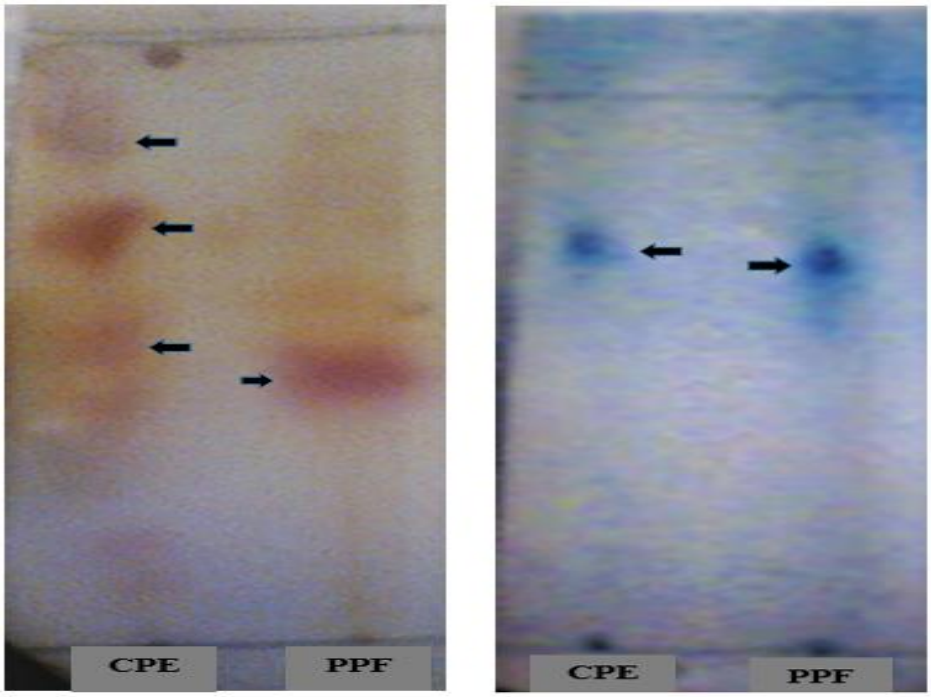


**Figure 2:** 2a & 2b, Chromatogram showing CPE and PPF of *Newbouldia laevis* leaves produced a violet-pink and a bright blue colouration on the TLC plate respectively upon reaction with ninhydrin and modified G-250 stain indicating the presence of free amino acids and cyclotides with potential disulphide linkage.

**Table 1:** Percentage inhibition of PPF and CPE extracts of *Newbouldia laevis* on selected organisms

| Concn. (µg/mL) | Percentage inhibition | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | *E. coli* | | | MRSA | | | *C. albicans* | | |
| | PPF | CPE | Gentamicin | PPF | CPE | Gentamicin | PPF | CPE | Fluconazole |
| 200 | 86.26 | 86.26 | 98.05 | 92.33 | 78.26 | 95.24 | 81.80 | 82.03 | 83.61 |
| 100 | 84.59 | 84.54 | 97.68 | 81.33 | 77.21 | 93.65 | 75.71 | 76.95 | 67.34 |
| 50 | 79.39 | 79.39 | 97.05 | 61.56 | 54.43 | 91.27 | 73.09 | 67.80 | 54.89 |
| 25 | 68.47 | 48.47 | 95.34 | 58.86 | 50.92 | 90.34 | 67.60 | 46.18 | 53.94 |
| 12.5 | 55.47 | 45.04 | 95.20 | 50.91 | 36.90 | 88.20 | 49.55 | 33.24 | 52.22 |
| 6.25 | 33.01 | 33.03 | 94.84 | 48.66 | 29.89 | 84.82 | 46.18 | 27.75 | 51.65 |

*Escherichia coli* (ATCC 25922), Methicillin-Resistant, *Staphylococcus aureus* (MRSA), and *Candida albicans*
Crude peptide extracts (CPE), Partially-purified fraction (PPF)

**Table 2:** $IC_{50}$ of the different samples on the test organisms

| Organism | Concentrations (µg/mL) | | | |
|---|---|---|---|---|
| | CPE | PPF | Gentamicin | Fluconazole |
| MRSA | 46.91 | 5.89 | 4.14 | N/A |
| *E. coli* | 38.62 | 6.48 | 2.64 | N/A |
| *Candida albicans* | 33.94 | 9.27 | N/A | 3.51 |

N/A= Not applicable, Crude peptide extracts (CPE), Partially-purified fraction (PPF)

**Table 3:** Minimum inhibitory concentration (MIC) of PPF and CPE extracts of *Newbouldia laevis*

| Extract | MIC (µg/mL) | | |
|---|---|---|---|
| | *E. coli* | MRSA | *C. albicans* |
| CPE | 25 | 25 | 25 |
| PPF | 6.25 | 6.25 | 12.5 |
| Gentamycin | < 6.25 | < 6.25 | N/A |
| Fluconazole | N/A | N/A | < 6.25 |

*Escherichia coli (*ATCC 25922), Methicillin-Resistant, *Staphylococcus aureus* (MRSA), and *Candida albicans*
Crude peptide extracts (CPE), Partially-purified fraction (PPF)

## DISCUSSION

In this study, the antimicrobial potentials of the peptide-rich extracts of the plant were investigated using Gram-positive and Gram-negative bacterial candidates as well as the drug-resistant *C. albicans* (fungal candidate). To unveil gaps for future research direction, this study aimed to justify and provide new scientific evidence associated with the increasingly wide and extensive use of the plant in African ethnomedicine for disease conditions caused by bacteria and fungi; these pathogenic organisms have been described by the World Health Organisation as posing great threats to global healthcare and food security and there is now a renewed interest in novel therapeutics discovery such as antimicrobial peptides (AMPs) whose structural diversity and specificity have been documented to beat pathogen resistance. [18].

To identify the presence of antibacterial peptides which are rich in basic amino acids such as arginine, lysine and histidine, the modified G-250 dye was applied to isolated peptides on thin layer chromatograms to produce the observed bright blue colouration [14, 15, 19]. The presence of bioactive peptides such as arginine, lysine, and histidine-containing peptides has been positively identified in *Newbouldia laevis*.

The finding confirms the potent antibacterial and antifungal properties previously reported for aqueous extracts. However, in this study, the antimicrobial efficacy was notably enhanced in the peptide fraction, surpassing that of crude extracts from the same plant. This correlation underscores earlier findings emphasizing the antimicrobial potential of low molecular weight secondary compounds present in the plant's root and stem bark, which includes peptides as low molecular weight molecules.[11, 20, 21]. These secondary compounds, which have antimicrobial properties, include ceramide (newbouldiamide), canthic acid, flavonoids (chrysoeriol), quinones (newbouldiaquinone, 2-acetylfuro-1,4-naphathoquinone, 2-hydroxy-3-methoxy-9,10-dihydroanthracene-1-carbaldehyde, and lapachol), sterols [22].

The peptide-rich extracts used in this study were carefully obtained from the polar aqueous extracts after the elimination of polar secondary compounds whose chemical characteristics do not allow such to bind to the C18 bed during fractionation and separation of peptides from other polar secondary compounds. This eliminates the possibility of associating observed antimicrobial activities to phenolic compounds. Evidence-informed antimicrobial activities of peptides and phenolic compounds from *Newbouldia laevis* suggest that more than one class of compounds with different molecular targets may be responsible for the potent antimicrobial potentials of the plant and this provides the basis for its continued use in African Traditional Medicine for generations. In particular, arginine-rich peptides, many of which are cysteine stabilised via disulphide linkages have been considered as emerging antimicrobial peptide-based therapeutics. [23-25].

The demand for antimicrobial peptides (AMPs) has witnessed a surge in recent years

due to the limited number of effective antibiotics and the increased development of resistance to several of the most effective antibacterial agents [26-28]. More so, the potential applications of AMPs in human health, agriculture and food, indicate a promising future for the extensive application of these peptides. Particularly, cysteine-rich peptides containing basic amino acid residues such as arginine and lysine in their peptide sequences, they are also low molecular weight peptides, they have shown to withstand several steps of solvent extraction and purification without any degradation and loss of activity [15, 29]. In addition, Tyrosine, phenylalanine, and tryptophan are a few of these stable peptides' aromatic acid side chains, which both increase their bioactivity and make them easier to identify when they are in pre-purified form [15, 30].

Thus, the better inhibitory effect of the PPF could be due to the further purification of the CPE which eliminates inactive residues or compounds and optimised the peptides in the concentration used leading to the observed activity against test organisms. The $IC_{50}$ value is the concentration that inhibits the test organisms' growth by 50%. It's possible that the partly purified peptide fraction's (PPF) improved inhibitory efficacy was due to the inclusion of side chains or amino acids like lysine, histidine, or aromatic acids [31]. With regard to a number of pathogens, such as *Mycobacterium tuberculosis, Salmonella Typhimurium, Entrococcus faecalis, and fungus Candida albicans,* these groups of peptides rich in basic and aromatic amino acids have shown greater antibacterial action [32]. Therefore, Both Gram-negative and Gram-positive bacteria and fungi were utilized in this investigation, the $IC_{50}$ and MIC values demonstrate that the PPF has superior activity over the CPE.

Assaying the lethality of brine shrimp is thought of as a rapid initial examination for the presence of biochemical activity and to establish the toxicity of the crude extract [15, 33]. Using brine shrimp extract's classification for toxicity, [34, 35] lethal Concentration > 1000 μg/mL = not toxic, > 500 μg/mL = moderately cytotoxic and < 500 μg/mL = cytotoxic, PPF ($LC_{50}$ =5964.87 μg/mL) and CPE ($LC_{50}$ = 1094.20 μg/mL) could be considered to be non-toxic. In addition, the peptide-rich fraction used in this study has shown even a far lower toxicity ($LC_{50}$ =5964.87μg/mL) to aquatic cellular crustaceans used when compared with the crude extracts (($LC_{50}$ = 1094.20 μg/mL). This emphasizes its non-toxic nature and supports previous reports on low toxicity of several potent antimicrobial peptides in physiological fluids [36, 37] and further raise research interest in their discovery and development to sustainable therapeutics for clinical application. However, a more rigorous, in-depth and extensive study of these suites of promising peptides to reveal their amino acid sequences, primary structure and full characterisation of their 3D configuration represents gaps for further investigations. More importantly, a holistic *in vivo* toxicity assessment of the bioactive peptide rich fraction deserves further scientific attention. Furthermore, extracts of *Newbouldia laevis* have been shown to have a significant inhibitory effect on the CYP1A2, CYP2C9, and CYP2C19 enzymes, which raises concerns about the possibility of herb-drug interactions in patients taking medications with limited therapeutic windows, such as theophylline, caffeine, paracetamol, phenacetin-CYP1A2, warfarin, non-steroidal anti-inflammatory drugs (NSAID) [38]. Thus, the co-administration of extracts of this plant, with orthodox drugs may lead to clinical consequences resulting from herb-drug interaction. Further *in vitro* and preclinical *in vivo* investigation is encouraged in this aspect prior to clinical trials.

*Newbouldia laevis* is until now unsustainably collected from the wild in

addition to few that are used to make fences to demarcate land boundaries. It has been reported that the tropical forests, which are home to up to 50% of the world's blooming plants, are in risk of a continuing decrease at a rate of about 16.8 million ha/annum [39]. The plant and its availability for future generations are threatened by the numerous traditional applications of the herb (both medical and non-medicinal). Consequently, a sustainable usage of the plant should encourage its purposeful development for a variety of purposes. Future research projects might focus on developing the essential plans for preserving *Newbouldia laevis* in Sub-Saharan Africa, where it is indigenous, and using its therapeutic resources. This is owing to the variance in quality and content caused by environmental and genetic variables in plant cultivation versus collecting from the wild. The possibility of variation and the uncertainty of the therapeutic benefit are both reduced by cultivated plants. Since *Newbouldia laevis* is extensively used in African Traditional formulations that are submitted for listing by regulatory bodies, the deliberate and sustainable cultivation of *Newbouldia laevis* has the potential to greatly reduce the possibility of adulteration and wrong identification.

**Conclusion.** While previous studies have implicated other secondary plant metabolites in the studied plant for its antimicrobial activity, this work presents the report on the preliminary detection of bioactive peptides in *Newbouldia laevis* and demonstrated its enhanced antimicrobial activity against the studied organisms. The low toxicity of the bioactive peptide-rich fraction using *Artemia salina* justifies the need for further scientific investigation to isolate, characterize and mechanistically study these suits of peptides for novel antimicrobial drug discovery.

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