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Isolation and Characterization of Pyrrolizidine Alkaloids from *Heliotropium indicum* Leaf for Postharvest Management of Stored Grains

Boluwaji M. Adeniyi[1,2], Barnabas A. Kyenge[1], Christiana A. Adah[1],Abolanle O. Ilori[2], and Omoniyi S. Oyewole[2]
[1]Department of Chemistry, Faculty of Sciences, Benue State University. Gboko Road, Makurdi, Benue State,Nigeria.
[2]Nigerian Stored Products Research Institute, Km3, Asa Dam Road, Ilorin, Kwara State, Nigeria

Corresponding Author's email:fowopee@gmail.com

ABSTRACT
The crude methanolic extract of *Heliotropium indicum* leaf was isolated and purified using chromatographic techniques by loading it on a glass column of length 100 cm and diameter 5 mm with 10 g silical gel (60-120 mesh) as the stationary phase. The compound caused approximately 98% mortality for *Sitophilus species* and *Callosobruchus maculatus* in a manner similar to standard insecticide (cypermethrin). Isolation was achieved in gradient elution system that started with 100% hexane followed by gradual increase of 5% ethyl acetate in hexane, then ethyl acetate, and lastly 5% methanol in ethyl acetate and were gradually increased by 5%.The separated fractions were examined using TLC. The pure IPM-65 isolate obtained as pure crystal was washed with methanol prior characterization using nuclear magnetic resonance spectroscopy, and the results were compared with standards in the literature. Liquid Chromatography Mass Spectrometry (LCMS) analysis revealed that the pure IPM-65 isolate was a mixture of pyrrolizidine alkaloids, including intermedine, supinine, lindelofine, and trachelanthine, as shown by their exact masses on the LCMS chromatogram.

KEYWORDS: Spectroscopy, Chromatography; Isolation; Characterization; *Heliotropium indicum.*

1. INTRODUCTION

Plants develop toxins as a type of secondary metabolite mainly for the purpose of self-defense against bacteria, fungi, insects and herb thieving enemies[1]. Well known bioactive compounds such as alkaloids, flavonoids, and terpenoids have major ecological functions and are responsible for preventing herbivores and microbial growth [2]. *Heliotropium indicum* L is a weed from the family Boraginaceae which is found almost everywhere and contains large amounts of secondary metabolites with considerable pharmacological value [3]. This plant grows as an annual or perennial herbaceous plant with a typical height of 15 to 50 cm and is widely distributed in the tropics and subtropics notably in Africa and Asia [4]. Distinct morphological traits like hairy stems and oppositely arranged leaves help this plant survive in a wide range of environments. In folk medicine, *H.indicum* is widely believed to serve many purposes including treatment for inflammation, wounds, and microbial infections because of its antimicrobial and anti-inflammatory properties[5].

Considering its bioactive component, this study aims to: (i) assess the effectiveness of pyrrolizidine compounds (IPM-65) obtained from leaf extracts of *H. indicum* on insect pests that affect stored grains, (ii) determine the structure of active compounds in the leaf isolate using nuclear magnetic resonance (NMR) spectroscopy and liquid chromatography mass spectrometry (LCMS), (iii) and measure the mortality rate of stored grains insect pests after application of pyrrolizidine compounds isolated from *H.indicum* leaf extracts. The pyrrolizidine alkaloid which is 65 mg of pure isolate obtained from methanolic leaf extract of *Heliotropium indicum* (IPM-65). It is a mixture of intermedine, supinine, lindelofine and Trachelanthine as confimed by their exact masses using liquid chromatpgraphy mass spectrometry technique. They are referred to as necine bases and are naturally occurring alkaloids based on the structure of pyrrolizidine. The necic acid moiety is joined to the necine base at position 9 (C_9) of the entire pyrrolizidine compound [6]. This study may help develop botanical insecticides as less harmful to the environment than chemical pesticides.

2. MATERIALS AND METHODS

2.1. Collection of Plant Sample

Fresh leaves of *H. indicum L* were collected from botanical garden of Benue State University, Makurdi, Benue State. The plant was authenticated at the herbarium section of the Forestry Research Institute of Nigeria (FRIN), Ibadan and a specimen copy with herbarium number **FHN/113768** was deposited [7].

2.2. Preparation of Crude Extract

The separated leaves were washed, air-dried for two weeks, and ground into coarse powder, then stored in an airtight bag. For extraction, 416 g of powder was soaked in 1248 mL hexane for 72 h, following [8]. The hexane extract (3.41 g) and subsequent ethyl acetate (2.17 g), methanol (9.98 g), and aqueous (7.72 g) extracts were obtained [7].

2.3. Isolation and Purification

A 9.0 g methanol leaf extract was adsorbed onto silica gel, dried, and packed into a glass column (100 cm x 5 mm) with 10 g silica gel in hexane. Gradient elution was used with increasing polarity starting with 100% hexane and 5% gradual addition of ethyl acetate in hexane, and methanol in ethyl acetate to yield 87 fractions. Fractions with similar Rf values were combined, washed with acetone, and TLC-tested (hexane: methanol 7:3, Rf 0.42). The compounds' identities were detected by spraying with Dragendorff reagents.Two alkaloid-positive fractions, IPM-85 (85 mg) and IPM-65 (65 mg), were obtained and prepared for NMR analysis [7].

2.4. Spectroscopic Analysis of Pure Isolate

The pure IPM-65 isolate was subjected to ^{1}H, ^{13}C, and 2D NMR (COSY, HSQC, HMBC) using a Bruker Avance III HD spectrometer with acetone as solvent. A 7 mg sample was dissolved in $CDCl_3$, filtered into a clean 5 mm NMR tube, and was analyzed at 400 MHz.TMS was set at 0 ppm which served as the internal reference, and spectra were phase- and baseline-corrected, yielding a complete NMR profile [7] .

2.5. Liquid Chromatography Mass Spectrometer (LCMS)

LCMS of pure IPM-65 was performed on an Agilent 6130 with 1200 series LC. A 1 mg sample in methanol was analyzed with a 1 mL/min flow rate, 3 mL injection volume, and 10-minute runtime. The ionization mode was ESI/APCI in positive/negative polarity, using an Agilent Poroshell 120 C_{18} column [7].

2.6. Insect culture protocols and Insecticidal activity screening of *H.indicum* extracts

Cleaned substrates were placed one-quarter full in kilner jars with added yeast and live adult insects, covered with muslin, and arranged on oil-filled trays to prevent predator access. After 14 days, adult insects were removed to assess emerging age [9]. Separately, 20 g of uninfected rice, sorghum, maize, and cowpea grains were treated with 0.01–0.08 g/mL of *H. indicum* hexane, ethyl acetate, and methanol extracts in jars. Ten pairs of young *S. oryzae*, *S. granarius*, *S. zeamais*, and *C. maculatus* were introduced, and insect mortality was assessed over four days using Abbott's formula [10].

$$\% \text{ Corrected Mortality} = \frac{T-C}{100-C} \times 100 \qquad \text{Eqn (1)}$$

Where T= Treated mortality in %,C = Control mortality in % (Abbott, 1925)

$$\% \text{ Insect mortality} = \frac{\text{No of Dead Insects}}{\text{Total numbers of Insects for Infestation}} \times 100 \qquad \text{Eqn. (2)}$$

3. RESULTS AND DISCUSSION

3.1. Results

3.1.1. NMR Spectroscopy

The ^{1}H and ^{13}C NMR spectra of the pure IPM-65 isolate from methanolic extract of leaf of*H.indicum* L are given in (Figure 1) and (Figure 2) respectively, while important NMR parameters from the 2D-COSY NMR experiment on the isolate are given in (Table 1), which revealed the frequencies for a single isotope, most commonly hydrogen (^{1}H) along both x and y axes. The 2D COSY NMR parameters in Table 1 shows values indicating the chemical shift, and the coupling hydrogen [7].

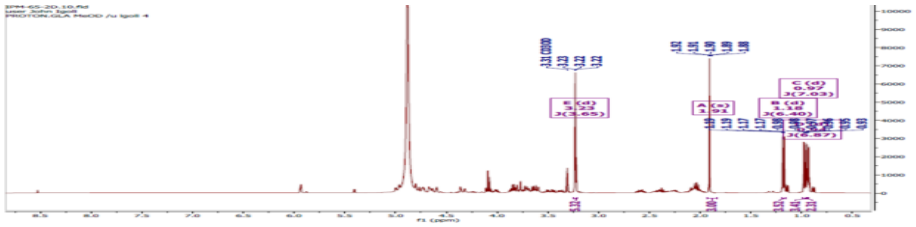

Figure 1: Proton NMR Spectrum of Pure IPM-65 isolate of methanolic extract of leaf of *H. indicum.*

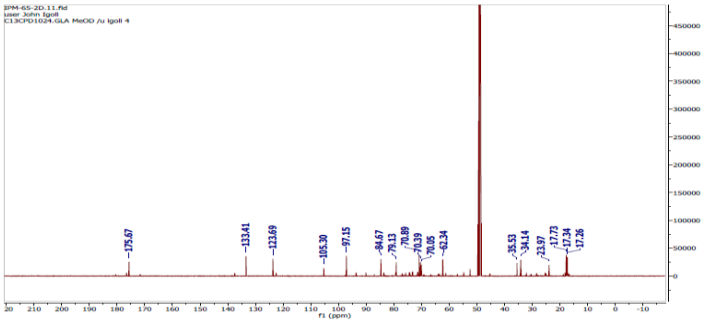

Figure 2:^{13}C-NMR spectrum of IPM-65solate of methanolic extract of leaf of *H. indicum.*

Table 1: NMR including 2D of pure IPM-65 isolate

Position	Atom	^{1}H NMR (ppm)	^{13}C NMR (ppm)	COSY/HSQC/HMBC
1	C=CH	5.90	123.69	2, 4.45, 4.65, 4.90
2	C=CH	5.45	133.14	3.45, 5.45, 5.90
3	CH_2	2.10	34.14	0.97, 2.55, 3.52, 3.58, 4.10
4	N	-	-	-
5	CH	3.45	70.05	5.45
6	CH_2	2.55	35.53	2.10, 3.80
7	CH	3.80	70.89	2.10, 2.55
8	CH	4.10	79.13	2.10, 3.23
9	CH_2	4.40	84.67	4.65
10	C=O	-	175.67	-
11	CH	3.23	62.34	3.60
12	CH_3	1.91 (s)	23.97	1.18, 1.91, 2.10
13	CH_3	0.97 (d)	17.34	0.97,2.10
14	CH_3	1.18 (d)	17.26	1.18, 4.10
15	CH	2.45	-	-
16	C	-	-	-

Based on the analysis of the proton and carbon spectra data and the 2D NMR compared with the reported data in literature, a new structure of pure IPM-65 isolate was proposed (Figure 3).

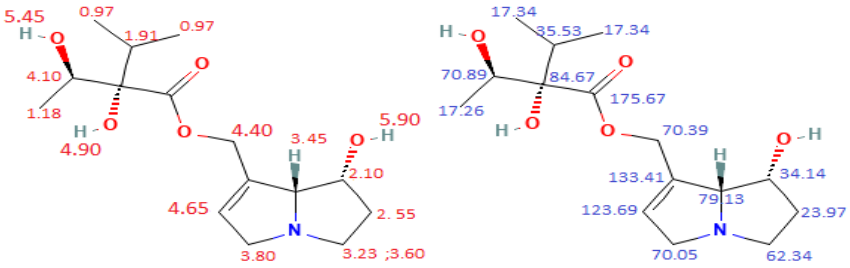

Figure 3: Structure of predicted Compound with assignment of proton and carbon

3.1.2. LCMS

Table 2 shows the result from LCMS chromatogram resulting from analysis of pure IPM-65 isolate run on High Resolution Electron Ionization Mass Spectrometry (HREI-MS) at University of Strathchlyde, Glasgow, Scotland, UK.In line with the proposed compound structure in (Figure 3), the proposed structure of pure IPM-65 isolate having pyrrolizidine skeleton were found to be that of Intermediine, Supinine, Lindelofine, and Trachelanthine based on their exact masses as revealed by LCMS.

Table 2: Exact masses of IPM-65

SN	Compd	E.M (g/mol)	M.F	RT	General name
1	Intermedine	299.1736 g/mol	$C_{15}H_{25}NO_5$	0.200	(7S, 8S)-7-hydroxy-5, 6, 7, 8-tetrahydro-3H-pyrrolizin-1-yl] methyl 2-hydroxy-2-[(1S)-1-hydroxyethyl]-3-methylbutanoate,
2	Supinine	283.1785 g/mol	$C_{15}H_{25}NO_4$	0.202	(8*S*)-5,6,7,8-tetrahydro-3*H*-pyrrolizin-1-yl]methyl (2*S*)-2-hydroxy-2-[(1*R*)-1-hydroxyethyl]-3-methylbutanoate.
3	Lindolefine	285.1923 g/mol	$C_{15}H_{27}NO_4$	0.205	2S,3R)-[(1R,7AR)-hexahydro-1H-pyrrolizin-1-yl]-methyl-2,3-dihydroxy-2-isopropylbutanoate,
4	Trachelanthine	301.1897 g/mol	$C_{15}H_{27}NO_5$	0.208	(1S,8S)-4-oxido-2,3,5,6,7,8-hexahydro-1 H-pyrrolizin-4-ium-1-yl]methyl (2R)-2-hydroxy-2-(1-hydroxyethyl)-3-methylbutanoate

3.1.3. Insect Mortality

Table 3 shows percentage mortality of *Sitophilus* species and *Callosobruchus maculatus* exposed to pyrrolizidine compounds isolated from H.indicum leaf methanolic extract at 0.25 g/kg and 0.5 g/kg. At 0.5 g/kg, the pure isolate caused 98.33% mortality in cowpea weevils, 96.67% in maize weevils, 85% in sorghum weevils, and 81.67% in rice weevils within 96 h, comparable to cypermethrin ($P \leq 0.05$).

Table 3: Mortality of stored grain insect pests postexpoure to pure IPM-65 isolate

IPM-65	Conc. (g/kg)	substrate	Exposure time (Mean ±SEM)		
		Rice	Sorghum	Maize	Cowpea
@24 h	0.25	3.33±1.67^{a}	1.67±1.67^{a}	6.67±1.67^{a}	3.33±1.67^{a}
	0.5	6.67±1.67^{a}	8.33±1.67^{b}	10.00±0.00^{b}	6.67±1.67^{a}
@48 h	0.25	8.33±1.67^{a,b}	15.00±2.89^{c}	46.67±3.33c	51.67±6.00^{b}
	0.5	11.67±1.67^{b}	15.00±0.00^{c}	55.00±5.77c	58.33±9.27^{b}
@72 h	0.25	35.00±2.89^{c}	38.33±4.40^{d}	75.00±2.89^{d}	70.00±5.77^{c}
	0.5	46.67±1.67^{d}	48.33±1.67^{e}	81.67±1.67^{d}	81.67±1.67^{c}
@96 h	0.25	66.67±3.33^{e}	75.00±2.89^{f}	86.67±1.67^{e}	88.33±1.67^{d}
	0.5	81.67±1.67^{g}	85.00±0.00^{g}	96.67±3.33^{f}	98.33±1.67^{e}
Contr.	0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Cyp.	0.5 ml	68.33±4.41^{f}	76.67±6.67^{f}	96.67±3.33^{f}	100.00±0.00^{e}

The values are expressed as the mean ±SE at (P ≤0.05) level of significance
Where Contr = Control, Cyp.= Cypermethri

3.2. Discussion

The characterization of IPM-65 as (1R,7aR)-1-hydroxy-2,3,5,7a-tetrahydro-1-H-pyrrolo[1,2-a]pyrrol-7-yl]methyl(2S,3R)-2,3-dihydroxy-2-isopropylbutanoate (intermedine) was confirmed through NMR and LCMS analyses. NMR spectra showed proton and carbon signals characteristic of a necic acid moiety and pyrrolizidine skeleton [11]. Key proton signals and COSY, HSQC, and HMBC correlations confirmed structural details, and LCMS identified the compound at 299.1736 g/mol [12]. Major PAs in *Heliotropium indicum* included supinidine, retrorsine, and echimidine, which are known for hepatotoxicity [13].

This study revealed the active potentials of this plant product as plant-derived insecticides against stored grain weevils, and provides a scientific rationale for the use of this botanical as alternative to synthetic insecticides in post harvest management of stored grains. The treatment with pure isolate of *H.indicum* had the highest percentage mortality of 98.33% for cowpea weevils. This was supported by the report of Adedire and Lajide [14] on *Piper guineense* belonging to the piperaceae family and stated that the plant possesses some forms of insecticidal properties against the eggs of stored cowpea grains, (bruchid) which are capable of suppressing various developmental instars of *Callosobruchus maculatus,* while Fasakin and Aberejo [15], have also reported that pulverized plant material from *P.guineense* inhibited egg hatchability and adult emergence of *Dernlestes maculatus* during storage.

4. CONCLUSION

The isolation and characterization of pyrrolizidine alkaloids as intermedine, Supinine, Lindelofine and Trachelanthine were successfully carried out from the leaf methanolic extract of *Heliotropium indicum.* The chemical identification of these compounds was validated using NMR and LCMS as pyrrollizidine alkaloids (PAs). It can be concluded that this plant has the potential as biopesticides against the insect pests of stored grains. Further research on the toxicity of this plant is therefore recommended to ensure its safe use as potential biopesticides in the postharvest management of stored grains.

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