BRINE SHRIMPS LETHALITY ASSAY OF THE EXTRACT PRODUCED BY ENDOPHYTIC FUNGI ISOLATED FROM THE ROOT OF Lowsonia inermis

BY

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Abstract

Endophytic fungi produced a secondary metabolites similar to their host plant; therefore it can be used as a source of producing active metabolites that can be used in drug discoveries. This work was aimed to isolate cytotoxic compounds from Endophytic fungi hosted in the root (tissue) of lawsonia inermis plant. The root of this plant was collected, prepared and screened for endophytic fungi. Aspergillus Niger was isolated, fermented on malt broth, extracted with ethyl acetate and partionioned using separating funnel. The crude ethyl acetate extract was tested for in vivo Brine shrimp lethality Assay of the extract and pure isolates. Cytotoxicity of the extract was evaluated in term of Lethal Concentation at 50% confident level (LC_{50}). Ten shrimps were added into three replicates of each concentration 1000, 100, 10µg/ml respectively. After 24hours the number surviving shrimp were counted and LC_{50} was determine. The bioassay from the ethyl acecate fraction (HR-ASN) of the metabolite was obtained using column chromatography, which lead to the isolation of three pure compounds $HR_{66,}$ $HR_{94,}$ HR_{102}. The result showed that the crude extract HR-ASN have LC_{50} of 3.15µg/ml, and that of the isolated compounds $HR_{66,}$ $HR_{94,}$ HR_{102} have the LC_{50} values of 4.094 µg/ml, 5.27 µg/ml, 2.297 µg/ml. HR_{102} revealed more potent activity oftoxicity with LC_{50} value of 2.295µg/ml. Therefore all the tested sample are toxic. It indicated that bioactive components are present and could be the source of pharmacological effect.

Keywords: Endophytic fungi, Cytotoxicity, Brineshrimps, Lowsoniainterims, metabolites.HR-ASN-Aspergillus niger

Introduction

Endophytes have been extensively studied for their potential as novel sources of effective new drugs. Microbes both fungi and bacteria have provided modern medicine or drugs with valuable effective treatments, including penicillium from *fungus penicillium notatum*, and bacitracin from Bacillus subtilis, a common bacterium. Additionally, taxol a potent chemotherapeutic agent is synthesized by an endophyte of the pacific Yew tree. Their diversity and specialized habitation makes them an exciting field of study in search of new drugs like molecule. The hunt for new drugs is particularly important in view of the fact that so many microorganisms are developing resistance to some of the current drugs (Aguero *et al*., (2011); Kumar *et al*., (2014); Stancy & Pradeep, (2013); Samuel *et al*., (2014).These led to the discovery of many other bioactive compounds with antimicrobial, insecticidal, cytotoxic and anticancer activities. Endophytic fungi are group of fungi with very specific ecosystem inside plant tissues and produces varieties of secondary metabolites (2009). The brine shrimp

assay is very useful tool for the isolation of bioactive compounds from plant extract (Sam, 1993). The present study present evaluated the ability of a fungi produced from the root of *Lowsonia Innermis* to produce isolate of active pure compound

Materials and Method

Plant sample: The root of *Lowsonia Inermis* was obtained from Botanical Garden of Botany Department of Bayero University, Kano. Healthy mature plant was carefully chosen for this purpose.

Preparation for isolation of Endophytic fungi: The Isolation of the fungi was carried out based on the processes outlined by (Khan *et al* (2011), Jagaonwala *et al*., (2011) & Strobe (2013), with a slight modification, the root of the plant was rinsed gently with running tap water to remove any adhesives, dust and debris. Thereafter, sections of the back of the root were gently scraped and re-rinsed with running tap water.The scraped root sample were then cut into smaller pieces in highly sterile condition required for isolation of endophytes (Bharathidasan & Panneerselvam 2011). All work including all materials such as glass wares, knife, forcep, blade were carried out in a sterile fume cupboard. Root sample were cut into about 0.5cm*0.5cm.The surface sterilization was done using sodium hypochlorite (5% NAOCl) and 75% ethanol. Each set of the plant materials after the preliminary treatment were further treated with 75% ethanol for three minute followed by immersion in 1% sodium hypochlorite for five minutes and again in 75% ethanol for two minutes. The plant material were then rinsed three times with deionised water and then dried in tissue paper (Khan et al., 2011; Jalgaonwala et al., 2011; Strobel & Daisy, 2003).

Preparation of Media: Potato Dextrose Agar (PDA) 25g was dissolved in 1litre of distilled water, based on the manufacturer`s instruction. The conical flask containing the PDA solution was sterilized in an autoclave for 15 minutes at 121^{o}C,the sterile PDA(20-25ml) was poured into petri-dishes under sterile environment. The petri-dishes were allowed to cool and solidify.

Isolation of Endophytic Fungi: Four pieces of plant segments were placed on petri-dish containing PDA supplemented with chloremphenicol 280mg-^{1}. The petri-dishes were sealed with cello-tape and incubated at room temperature in fume cupboard for one week (Gove *et al* 2011).

Isolation of Pure Colony: Isolation from the master petri-dishes was done by the transfer of hyphae tips of the colony using a sterile syringe needle tips on a fresh PDA plates suplemented with chloremphenicol 280mg-^{1} and incubated at room temperature and were observed daily to obtain pure cultures while those with contamination were discarded (Hallman *et al*.,2007).

Cultivation of Fungi for the Production of Secondary Metabolites

Fermentation in liquid Media

The screening of fungal for biological active secondary metabolites was first carried out on Malt-broth prepared by dissolving 20ml of the malt in 1000ml of deionised water and autoclave at 121^{o}C for 15 minutes (Goves et al., 2011).

Media Dispensing
200ml of the prepared media dispensed in 250ml conical flask each and covered with bunk (cotton wool) and autoclave at 121^{o}C for 15 minutes. After autoclaving, they were allowed to cool andabout 2cm*2cm of the pure sub-culture fungi was transferred onto prepared media and was allowed to ferment for thirty days at room temperature on a rotator incubator.

Extraction of the secondary Metabolites from Aspergillus species
The mycelium from the bunk flask for 30 days were separated from the liquid broth by sanctionfiltration using vacuum pump. After filtration, the fungal mycelium was soaked in methanol thrice for 1 week each, while the broth filtrate was extracted with ethyl acetate three times. The broth ethyl acetate was partitioned using separating funnel and brine solution (solution of concentration of sodium chloride) was added to remove adhesives. The broth ethyl acetate was then separated from the brine solution and dried with anhydrous magnesium sulphate ($MgSO_4$), and finally concentrated at reduced pressure. The separated mycelia soaked in methanol were filtered by sunction to obtain the methanol extracts which was concentrated at reduced pressure to yield brown oil residue. The methanol crude extract was further partitioned between water and ethyl acetate 1:1 to yield the ethyl acetate fraction which was washed with brine solution and dried with anhydrous magnesium sulphate ($MgSO_4$), and finally concentrated at reduced pressure to afford a dark brown oily solid of 10.98g and labelled as HR (Raviraja *et al*., 2007)

Column Chromatography Analysis: 7g of the ethyl acetate crude extract was powdered with silica gel until homogenous solid mixture was obtained, silica gel was made into slurry with hexane and loaded into sintered-base glass column (121*2.5cm capacity). The powdered mixture of the extract was then loaded onto bed of the silica gel into the column. Small quantity of silica gel was added on top to protect the adsorbent. The loaded column was run by eluting with a total volume of 500ml (per solvent system) n-hexane:chloroform gradient in order of increasing polarity (100% hexane, 4:I, 3:2, 2.5:2.5, 2:3, 1:4, 100% chloroform) and then a mixture of chloroform:ethylacetate in a ratio (4:1, 3;2, 2.5:2.5, 2:3, 1:4, 100% ethylacetate) and finally washed with acetone, 50ml of eluent was collected at time interval (Braithwite & Smith, 1999).

Purification of Column Fraction
Fraction obtained from the first column was further purified on silica gel column chromatography eluting total volume of 200ml Hexane(100%).Hexane : chloroform (4:1 ,3:2 ,2.5:2.5 ,2:3 ,1:4, 100% chloroform) .Chloroform: ethylacetate (4:1,3:2, 2.5:2.5, 2:3, 1:4, 100% ethylacetate) and finally washed with 100% acetone.20ml eluent was collected at an interval.

Bioassay
Cytotoxicity Assay against Brine Shrimp

Hatching Shrimp: 1000ml of sea water was poured in a beaker and brine shrimp (Artemia Salina) eggs were added, the beaker was kept under light for 72hours at room temperature to enable the eggs hatch into larvae. Larvae were separated from eggs by aliquoting them three times in small beakers containing sea water (Hamidi et al., 2014).
Brine Shrimp Assay: Toxicity of the extract was monitored by the brine shrimp lethality test. According to the method of (Olowa and Nuneza, .2013), with slight modification. Each of the extracts (20mg/2ml) was dissolved in methanol, from which 500µL, 50µL, 5µL of each solution was transferred into vials corresponding to 1000, 100, 10µg/ml respectively. This was allowed to evaporate to dryness in about 24hours at room temperature. Each dosage was tested in triplicates (9 vials per test sample). 2-3 drops of dimethylsulfoxide (DMSO) was added to each vial to dissolve the fractions ,which was further diluted with 4ml sea water to each vial ten shrimps were added using Pasteur pipette and the contents of the vials were made up to 5ml using sea water. Afeter 24hours the number of surviving shrimp were counted and LC_{50} was determined at 95% confidence interval using regression analysis (Kaniz et al., 2013).

Result and Discussion
Screening for Endophytic fungi in the plant sample of *Lawsonia inermis, Aspergillus nigar* was isolated and identified based on their morphologies. The result displayed in Table 1 showed that *Aspergillus Niger* grows suitably on malt broth.Cytotoxicity test of the crude extract HR-ASNshowed LC_{50} value of 3.165µg/ml and that of isolated pure compounds HR_{66}, HR_{94}, and HR_{102}, has an LC_{50} of 4.094µg/ml, 5.52µg/ml and 2.295µg/ml respectively.

Table 1: Toxicity of the Extract and Isolated Compounds

Fraction after24h (µg/ml)	Concentration (µg/ml)	Replicate	No. of Shrimp	Survivor	Death	LC_{50}	Brine	after 24hr
HR-As	1000	3	10	0, 0, 0	10,		10,	10
	100	3	10	0, 0, 0	10,	10,	10	3.165
	10	3	10	1, 1, 1	9,	9,	9	
HR_{66}	1000	3	10	0, 0, 0	10,	10,	10	
	100	3	10	0, 1, 0	10,	9,	10	4.094
	10	3	10	3, 3, 3		9,	9,	9
HR_{94}	1000	3	10	0, 0, 0	10,	10,	10	
	100	3	10	0, 0, 0	10,	10,	10	5.527
	10	3	10	1, 1, 1	9,	9,	9	
HR_{102}	1000	3	10	0, 0, 0	10,	10,	10	
	100	3	10	0, 0, 1	10,	10,	9	2.295
	10	3	10	1, 0, 1	9,	10,	9	
$K_2Cr_2O_7$	1000	3	10	0, 0, 0	10,	10,	10	
	100	3	10	0, 0, 0	10,	10,	10	4.470
	10	3	10	1, 2, 2	9,	8,	8	

Discussion
From Table 1, Fungal strain HR-ASN with LC_{50} value of 3.165µg/ml and that of isolated pure compounds HR_{66}, HR_{94}, and HR_{102}, has an LC_{50} of 4.094µg/ml, 5.52µg/ml

and 2.295µg/ml were all toxic respectively.Te explanation for this according to Moshi *et al* (2010) is that LC_{50}< 1.0 µg/ml as highly toxic, LC_{50}1.0-10µg/ml is toxic, LC501.0-30µg/ml is moderately toxic, LC_{50}>30<100µg/ml is mildly toxic, LC_{50}>100µg/ml is considered non-toxic.

Conclusion

The cytotoxicity of both the extract and isolated pure compounds produced by Endophytic fungi from the Root of lawsonia inermis are all toxic is an indicative of the presence of potent cytotoxic components suggesting anti-tumur and anti-infammatory which provided a rationale for ethno medicinal use of the plant in traditional medicine.

Recommendation

Further research on the Endophytic fungi of lawsonia inermis plant is required in order to find more potential endophytes and isolation of more potent biological active compound.

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