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PHYTOCHEMICAL SCREENING OF *RICINODENDRON HEUDELOTII* (EUPHORBIACEAE) FOR INSECTICIDAL ACTIVITY IN THE CONTROL OF TWO STORAGE INSECT PESTS

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ABSTRACT
Experiments were conducted in the laboratory to screen the leaf extract of *Ricinodendron heudelotii* (Baill) Pierre ex Pax for the presence of chemical groups with insecticidal activity against *Sitophilus zeamais* (Mots.) and *Callosobruchus maculatus* (F.). The plant materials were collected, dried and ground to fine powder while extraction was carried out using 90% ethanol. Concentration of the extract was carried out using rotary evaporator while phytochemical screening involved the use of TLC analysis for chemical compounds such as tannins, alkaloids, saponins, terpenes, flavonoids and anthraquinones. Partitioning of the extract was done by dissolving the extract in water to obtain the aqueous phase which was partitioned between equal volumes of chloroform, *n*-hexane, *n*-butanol and ethyl acetate. The different fractions were screened for insecticidal activity and were found to exhibit significant ($P < 0.01$) mortality against the two insect species. Complete protection to grains was obtained with reduced progeny development as well as significant repellency of insect pests compared with the control. The result from the study confirmed the presence of insecticidal compounds in the candidate plant which could be explored as a safe alternative to synthetic insecticides and thus averting the unpleasant consequences of the use of synthetic insecticides.

KEYWORDS: Ricinodendron; natural insecticide; screening; butanol; toxicity; progeny.

INTRODUCTION

The problems caused by synthetic pesticides and their residues have increased the need for effective, biodegradable pesticides with greater selectivity. Alternative strategies have included the search for new types of insecticides and the re-evaluation as well as the use of traditional botanical pest control agents (Obeng-Ofori and Akuamoah, 1998). Common insecticides of plant origin are pyrethrum from *Chrysanthemum cinerareaefolium* which is the best known natural insecticide containing six active components in varying proportions with the most active being pyrethrins (Escoubas *et al*., 1994). Pyrethrum breaks down rapidly when exposed to sunlight or strong artificial light and is therefore not suitable for residual surface treatments. However, toxicity of pyrethrum is increased by the addition of a synergist such as piperonyl butoxide. Azadirachtin obtained from the neem tree, *Azadirachta indica* A. Juss and *Melia azadirach* is a tetranortri terpenoid and effective against a wide range of insect pests by acting as a growth regulator and feeding deterrent (Schmutterer, 1995; Addae-Mensah, 1998). Quizolizidine alkaloids have insecticidal and insect deterrent properties and are found in many leguminosae (Wink, 1993). Others are quassins, ryanodine, vratum alkaloids, phototoxins, anonains and acetogenins, N-Alkylamides. The plant kingdom is a vast storehouse of chemical substances manufactured and used by plants for defense against attack by insects. These substances may elicit strong physiological responses in various stages of an insect's life. Since these naturally occurring phytochemicals are usually biodegradable and non-toxic to plants and animals, they offer the potential for safe and effective control of stored product pests (Rembold, 1994). More than 30,000 secondary metabolites have been reported from plants (Wink, 1988) with major group of compounds with insecticidal activity being alkaloids, amines, non-protein amino acids, cyanogenic glycosides, glucosinolates, lectins, protease inhibitors; all of which are nitrogen containing allelochemicals. Other allolochemicals are monoterpenes, sesquiterpenes, diterpenes, triterpenes/ steroids, tetraterpenes, polyketides, polyacetylenes, flavonoids and phenylpropanoids (Nakanishi and Suzuki, 1998). The use of various products derived from certain plants is known to be an age-old practice for the protection of field crops and stored grains against damage by pests (Biller *et al*., 1994). Other products obtained from plants include nicotine from tobacco and rotenone from *Derris elliptica*. These plants have been used by the native farmers in many parts of the world and usually great results are always obtained. Phenolic compounds, often tannins and non-tannins in cereal grains which are found primarily in the pericarp and pigmented inner integument layer of kernels are associated with resistance to insect attack on grain crops in the field (Wongo, 1998, Bottenberg and Sing, 1996). The present study therefore aimed at investigating the different chemical

groups or compounds present in *Ricinodendron heudelotii* (Baill) Pierre ex Pax (Euphorbiaceae) that could be utilized for insecticidal activity against *S. zeamais* (Mots.) (Coleoptera: Curculionidae) and *C. maculatus* (F.) (Coleoptera: Bruchidae) being serious primary insect pests attacking stored maize and cowpea, respectively. *R. heudelotii* commonly called wood-oil-nut tree, is a tropical plant with leaves being digitately alternate and composed of long elliptic leaflets from 10 – 15 cm in length and 3 – 10 cm in width. The tree is dioecious and deciduous, and begins fruiting after 7 – 10 years (Richter and Dalwitz, 2000). The tree is endemic to tropical Africa with distribution spanning from Senegal to East Africa and Madagascar. The economic, industrial and medicinal uses of *R. heudelotii* have been variously reported (Etukudo, 2003; ICUC, 2004; Momeni *et al*., 2005).

MATERIALS & METHODS

Standard methods as outlined by Sofowora (1993) were employed in the phytochemical screening tests for various chemical groups including alkaloids, saponins, anthraquinones, terpenes and flavonoids.

Preparation of plant material and ethanol extract

The leaves of *R. heudelotii* were collected in the wild from Uyo metropolis, Akwa Ibom State, Nigeria and left to dry at room temperature in a shade at the Pharmacognosy Laboratory, University of Uyo, Nigeria. The air dried materials were powdered using laboratory mortar and pestle, and extracted at room temperature with 90% ethanol by maceration. The ethanol extract was concentrated to dryness in a vacuum using rotary evaporator.

Fractionation of the dried extract

The solvent free extract was dissolved in 200ml of distilled water and transferred to a separating funnel and partitioned between ethyl acetate, chloroform, *n*-Hexane and *n*-Butanol. Each fraction was concentrated to dryness using a rotary evaporator at 40^{o}C for 8 hours (Udo *et al*., 2004).

Chromatographic techniques

Spotting and development of the chromatogram

One gram of each extract was dissolved in 5 ml of methanol in a small beaker. The solution was applied to each TLC plate as a single small spot of about 2 mm on the origin (1 cm from bottom of plate) using a capillary tube. The spots were then allowed to dry in air at ambient temperature for about 30 minutes. The dry plates were placed in the developing chambers and covered, and left to stand undisturbed. The plates were developed in an ascending manner as the solvent travelled by capillary action to the solvent front (1 cm from top of the plate). The plate was then removed and the solvent front marked with a pencil. Drying in air later took place for about 15 minutes before detection of spots.

Detection of spots

Detection of spots was done by the use of ultraviolet (UV) lamp (λ366nm). The UV lamp was held over each plate to visualize the spots, which were circled lightly with a pencil and the retention factor (Rf) was calculated for each spot thus:

Rf = distance from center of spot to origin / distance of solvent front from origin.

Contact toxicity of extract fractions by topical application

Ten adult insects each of *S. zeamais* and *C. maculatus* respectively were placed in petri dishes lined with moist filter paper (Obeng-Ofori *et al*., 1998). Insects were picked individually and with the aid of micropipette, 20 µl/ml of the various extract fractions were applied to the dorsal surface of the thorax of each insect. Distilled water was used in the controls and each treatment replicated four times. Insects were examined daily for mortality with 96 hours. Any insect that did not move or responded to three probing with a blunt probe was considered dead.

Contact toxicity of the extract fractions by grain treatment

Toxicity of the different extract fractions on maize and cowpea grains was tested in the laboratory by applying dosages of 200 mg/kg and 400 mg/kg to 50 g of grains in a 200 ml plastic cup (Epidi *et al*., 2009). The extracts were allowed to dry for 30 minutes before introducing 10 pairs of each insect into the plastic cups and covered with white muslin cloth held in place with rubber bands. The control was treated with distilled water only and mortality was recorded after 24 hours and up to 96 hours. Insects were presumed dead on failure to respond to three probing with a blunt probe after 5-minute recovery time.

Ability of extract fractions to protect grains from damage

Maize and cowpea grains were treated with 200 mg/kg and 400 mg/kg of the different extract fractions and ten pairs of each insect species were introduced into treated and untreated grains as the control. Each treatment was covered with white muslin cloth and held in place with rubber bands and left to stand undisturbed for four weeks. Samples of 100 grains were taken from each cup and the number of damaged grains (grains with characteristic holes) and undamaged grains were counted and weighed. Percent weight loss was calculated following the method of FAO (1985) as:

$$\%\ \text{weight loss} = \frac{[\text{UaN} - (\text{U} + \text{D})]}{\text{UaN}} x100$$

Where:

U = weight of undamaged fraction in the sample
N = total number of grains in the sample
Ua = average weight of one undamaged grain
D = weight of damaged fraction in the sample

Effect of extracts on progeny production

One hundred grams of maize and cowpea grains respectively were measured into 200-ml plastic cups and 200 mg/kg as well as 400 mg/kg of the various extract fractions added. Controls were treated with distilled water and each treatment was replicated four times. The experiment was left to run undisturbed for five weeks (Epidi *et al.*, 2009). The number of insects that emerged was counted after 24 hours up to the 96 hours of the sixth week.

Repellency bioassay

This was one using a choice bioassay system where baked wheat flour cakes were used according to the method described by Udo (2000). Wheat flour was purchased from the Uyo main market, Akwa Ibom State, Nigeria and 100 g

of flour was mixed with one litre of water and the resultant dough made into small round balls of about 10 g each and a total of one hundred and sixty (160) cakes were baked in the oven at 40°C for six hours. Two baked cakes were treated with each extract fraction at the rate of 100 mg/kg while two baked cakes were treated with distilled water to serve as the control. Treated and control cakes were air dried for one hour before introducing 10 adults of each insect species into the center of petri dishes containing the cakes. The treated and control cakes were separated by a space in the centre of the petri dish and each treatment was replicated four times. The number of insects present on the control (N_c) and treated (N_t) cakes was recorded after one hour and up to six hours. Percent repellency (PR) was computed using the formula:

$$PR = \frac{Nc - Nt}{Nc + Nt} x100$$

Where:
PR = Percent repellency
N_c = Insect number present on control cakes
N_t = Insect number present on treated cakes
Negative PR values were treated as zero.

Data analysis
All data generated were analyzed using analysis of variance (ANOVA). Data involving counts were transformed using square root while those involving percentages were transformed using arc-sine before analysis (Wahua, 1999). Correction for natural mortality in control treatment was carried out using the standard formula described by Abbott (1925):

$$AM = \frac{\%T - \%C}{100 - \%C} x100$$

Where:
AM = Adjusted mortality
%T = Percent treated mortality
%C = Percent control mortality

RESULTS

Phytochemical screening of the plant revealed the presence of chemical groups such as saponins, terpenes, flavonoids, tannins, alkaloids and anthraquinones. Lieberman's test showed the absence of cardiac glycosides (Table 1). The TLC result as shown in Table 2 showed the movement of the spots well above the center of origin which is useful in determining the nature of secondary chemicals present in the plant. Insect mortality recorded by topical application of the extract fractions of *R. heudelotii* differed significantly ($P<0.05$) for both *S. zeamais* and *C. maculatus* (Table 3). The hexane fraction produced 100% mortality in both insect species 72 hours after treatment while the aqueous fraction did not affect S. zeamais significantly after 96 hours of treatment. Chloroform and *n*-hexane fractions showed significant mortality ($P<0.05$) over the control with 100 percent mortality after 96 hours of treatment. However, insect mortality observed on grains treated with extract fractions of *R. heudelotii* showed different levels of bioactivity against the two insect species (Table 4). In all the treatments, *S. zeamais* was less affected compared to *C. maculatus*. After 96 hours of exposure to treated grains, the aqueous and ethyl acetate fractions produced 78% mortality against *C. maculatus*. The extract fractions of *R. heudelotii* tested at 400 mg/kg significantly ($P<0.01$) reduced damaged caused by *S. zeamais* and *C. maculatus* to stored maize and cowpea, respectively. Minimal weight loss of 0.08% and 0.43% was observed for maize and cowpea, respectively from the *n*-hexane and ethyl acetate fractions against *S. zeamais* and *C. maculatus* (Table 5). Progeny produced by both insect species was significantly ($P<0.01$) affected by the various extract fractions applied at 200 mg/kg to 100 g of maize and cowpea grains, respectively. The butanol fraction inhibited the number of F_1 progeny produced in the two insects comparative to the other fractions. Altogether, the extracts reduced the number of F_1 progeny of both insect species compared with the control treatments (Table 6). Repellency bioassay using the choice test showed extract fractions of *D. arborea* significantly ($P<0.01$) repelling *S. zeamais* with an overall repellency of 63% (Table 7). *C. maculatus* was also significantly repelled with an overall repellency of 45%. The butanol fraction produced the highest repellent effect of 79% against *S. zeamais* while chloroform repelled *C. maculatus* by 68%.

TABLE 1. Phytochemical screening of crude extract of *R. heudelotii*

Experiment		Inference
Tannins		+++
Flavonoids		++
Saponin		
A.	Ferric chloride test	+++
B.	Frothing test	+++
Terpenes		-
Anthraquinones		+++
Alkaloids		
	Dragendorff's Reagent	++
Cardiac glycoside		
	Lieberman's test	-
	Salkowski test	+++
	Keller-kiliani test	+

Key:
- Negative, + Traces, ++ Positive, +++ Strongly positive

TABLE 2: TLC result of bulked column fractions of *R. heudelotii* showing retention factor values

Bulked fraction	Solvent system	No. of spots	Colour	Retention factor (Rf)
A	Methanol	3	Light blue	0.94
(F_{1-22})	Chloroform		Light blue	0.83
	(3:7)		Yellow	0.48
B	Chloroform	3	Light blue	0.93
(F_{23-34})	Ethyl acetate		Blue	0.87
	(9.5:0.5)		Blue	0.95
C	Chloroform	6	Red	0.78
(F_{35-61})	Ethyl acetate		Light blue`	0.65
	(9:1)		Light blue	0.40
			Light blue	0.31
			Light blue	0.49
			Light blue	0.28
	Chloroform	5	Light blue	0.78
	Methanol		Light blue	0.67
	(9.5:0.5)		Light blue	0.53
			Light blue	0.48
			Blue	0.97
D	Chloroform			
(F_{62-90})	Ethyl acetate	-	No	
	(9:1)		Resolution	

TABLE 3: Toxicity of extract fractions of *R. heudelotii* applied topically against *S. zeamais* and *C. maculatus*

Extract fractions treatment 20 µl/ml	Mean % mortality at different hours after					
	24	48	72	96	Control	LSD
S. zeamais						
Ethyl acetate	65 ± 0.50	80 ± 1.15	80 ± 1.15	85 ± 0.96	0 ± 0.00	27.2
Chloroform	75 ± 0.50	85 ± 0.50	90 ± 0.50	95 ± 0.50	0 ± 0.00	14.6
n-Hexane	95 ± 0.50	95 ± 0.50	100 ± 0.00	100 ± 0.00	0 ± 0.00	9.8
Butanol	95 ± 0.50	100 ± 0.00	100 ± 0.00	100 ± 0.00	0 ± 0.00	7.0
Aqueous	0 ± 0.00	0 ± 0.00	10 ± 1.00	15 ± 0.96	0 ± 0.00	NS
LSD	**12.58**	**7.25**	**7.00**	**7.50**	**NS**	
C. maculatus						
Ethyl acetate	90 ± 1.00	100 ± 0.00	100 ± 0.00	100 ± 0.00	0 ± 0.00	13.8
Chloroform	85 ± 0.96	100 ± 0.00	100 ± 0.00	100 ± 0.00	0 ± 0.00	13.2
n-Hexane	95 ± 0.50	100 ± 0.00	100 ± 0.00	100 ± 0.00	0 ± 0.00	7.0
Butanol	95 ± 0.50	100 ± 0.00	100 ± 0.00	100 ± 0.00	0 ± 0.00	7.0
Aqueous	15 ± 0.50	25 ± 0.50	30 ± 0.58	30 ± 0.00	0 ± 0.00	15.0
LSD	**14.65**	**9.50**	**9.53**	**9.33**	**NS**	

Means are percentages of four replicates of 10 insects each. LSD test (P<0.05). NS = Non Significant

TABLE 4: Contact toxicity of extract fractions of *R. heudelotii* applied on grains against *S. zeamais* and *C. maculatus*

Extract fractions	Mean % mortality at different hours after treatment					
400 mg/kg	24	48	72	96	Control	LSD
S. zeamais						
Ethyl acetate	3 ± 0.50	5 ± 1.00	8 ± 0.96	10 ± 0.82	0 ± 0.00	**NS**
Chloroform	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00	**NS**
n-Hexane	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00	**NS**
Butanol	0 ± 0.00	1 ± 0.25	4 ± 1.50	5 ± 1.83	0 ± 0.00	NS
Aqueous	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00	**NS**
LSD	**NS**	**NS**	**NS**	**NS**	**NS**	
C. maculatus						
Ethyl acetate	20 ± 0.82	60 ± 0.82	73 ± 0.96	78 ± 1.50	0 ± 0.00	14.30
Chloroform	23 ± 0.96	43 ± 1.26	50 ± 1.15	58 ± 1.50	0 ± 0.00	17.20
n-Hexane	23 ± 1.26	33 ± 1.71	35 ± 0.58	43 ± 1.71	0 ± 0.00	23.80
Butanol	10 ± 2.06	10 ± 2.16	10 ± 2.16	30 ± 0.57	0 ± 0.00	**NS**
Aqueous	6 ± 0.68	35 ± 1.32	61 ± 1.50	78 ± 0.86	0 ± 0.00	19.20
LSD	**7.58**	**14.50**	**22.68**	**18.73**	**NS**	

Means are percentages of four replicates of 20 insects each. LSD test (P<0.05). NS = Non Significant

TABLE 5: Effect of extract fractions of *R. heudelotii* on damage caused by *S. zeamais* and *C. maculatus*

Extract fraction	Mean percent weight loss	
400 mg/kg	*S. zeamais*	*C. maculatus*
Ethyl acetate	0.27 ± 0.28	0.15 ± 0.18
Chloroform	0.14 ± 0.17	0.39 ± 0.41
n-Hexane	0.33 ± 0.46	0.49 ± 0.33
Butanol	0.73 ± 0.39	0.79 ± 0.61
Aqueous	0.49 ± 0.20	0.99 ± 0.51
Control	4.79 ± 1.38	9.46 ± 4.08
LSD	**0.95**	**2.54**

LSD test at (P<0.01)

TABLE 6: Effect of extract fractions of *R. heudelotii* on progeny produced by *S. zeamais* and *C. maculatus*

Extract fraction	Mean number of F_1 progeny	
200 mg/kg	*S. zeamais*	*C. maculatus*
Ethyl acetate	36.00 ± 2.16	37.75 ± 26.39
Chloroform	29.25 ± 8.96	23.50 ± 13.99
n-Hexane	34.50 ± 3.11	21.25 ± 5.62
Butanol	19.50 ± 9.33	25.00 ± 5.48
Aqueous	28.00 ± 6.98	23.50 ± 8.81
Control	46.00 ± 10.80	62.50 ± 12.39
LSD	**11.29**	**20.88**

Mean of four replicates of 20 insects each. LSD test at (P<0.01)

TABLE 7: Mean percent repellency (PR) values for extract fractions of *R. heudelotii* on *S. zeamais* and *C. maculatus* in a choice test

Extract fraction	Mean percent repellency (PR)	
	S. zeamais	*C. maculatus*
Ethyl acetate	55	35
Chloroform	62	58
n-Hexane	44	61
Butanol	24	52
Aqueous	53	34
Overall PR	48	48
LSD	**26.42**	**NS**

Mean of four replicates of 10 insects each. LSD test at (P<0.01)

DISCUSSION

Phytochemical screening result revealed the presence of saponins, tannins, anthraquinones and flavonoids as secondary metabolites. Their detection was possible because of the reaction of the functional groups with reagents to produce different colours and changes in physical nature (Adesina, 1986; Trease and Evans, 1989). The reduction of the plant extract fractions by Fehling's solution was an indication of the presence of triterpene saponins and this probably resulted in the oily nature of the isolates obtained (Harborne, 1973). The TLC result using chloroform: ethyl acetate showed a separation of the extract into different components with precoated TLC plates. The different components separated were likely non-polar because they tend to be soluble in non-polar solvents and were solvated by the non-polar mobile phase which moved them rapidly up the plate. The various retention factor (Rf) values obtained showed the differences in the partition coefficients with the component having the highest Rf value indicating the highest partition coefficient (Elujoba and Nagels, 1985). Spectral characterization of the isolates showed various proton shifts indicating a cloud or shift in electron charges between CH_3 and CH_2. The ^{13}C spectral result revealed the presence of between 18 – 20 carbon atoms besides the ester peak noticed. With hydrolysis of the esters and further spectral characterization using GC-MS equipment, structure elucidation of the actual chemical compound would be possible. The result of the spectral characterization was in line with the nature of isolates obtained which were oily in nature (Okunji *et al*., 1996). The result of the contact toxicity of the extract fractions applied on grains was significantly different from the control while topical application was more effective. The insects appeared to avoid the treated grains as some were observed hanging on the inside of the cups and the underside of the muslin cloth, without contact with treated grains. Secondary plant chemicals are known to attract or repel insects and influence their locomotion, oviposition, feeding behaviour, developmental and physiological process as well as behavioural patterns (Obeng-Ofori *et al*., 1998). It is therefore possible that the two insect species were repelled from treated grains. The significant reduction in damage caused to stored grains as well as high insect mortality could be attributable to the presence of toxic secondary metabolites in *R. heudelotii* as previously reported (Okunji *et al*., 1996; Momeni *et al*., 2005). It is known that some secondary metabolites may act both as insecticides and antifeedants as had been observed for rotenone against *T. castaneum* (Nawrot *et al*., 1988) and other rotenoids against lepidopterous pests such as *Spodoptera exempta*, *Eldana saccharina* and *Maruca*

testulalis (Hassanali and Lwande, 1989). The result obtained suggests good potential for the use of *R. heudelotii* in stored product pest management particularly in the tropics.

The extract fractions were effective in reducing the progeny produced in both insect species and this suggest the presence of ovicidal and larvicidal properties in the plant. It would appear that progeny inhibition of *S. zeamais* and *C. maculatus* in treated grains could also arise from the inability of last larval instars of insects to chew through the seed coats of treated grains due to the presence of phenolic compounds (Wongo, 1998).

The significant repellent action observed against S. zeamais further confirms the presence of secondary metabolites in the plant with antifeedant properties (Boeke *et al*., 2004). The degree of repellency however could depend on the habit of the insect species which are often in close association with crops in the field as well as in stores and this tends to expose them to secondary constituents of botanicals, hence the low degree of repellency observed in *C. maculatus*.

Botanical pesticides represent an important component of integrated pest management systems in traditional grain storage as they are broad spectrum in action, based on local materials and potentially less expensive. Many are also safe to the environment and harmless to man and other mammals.

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