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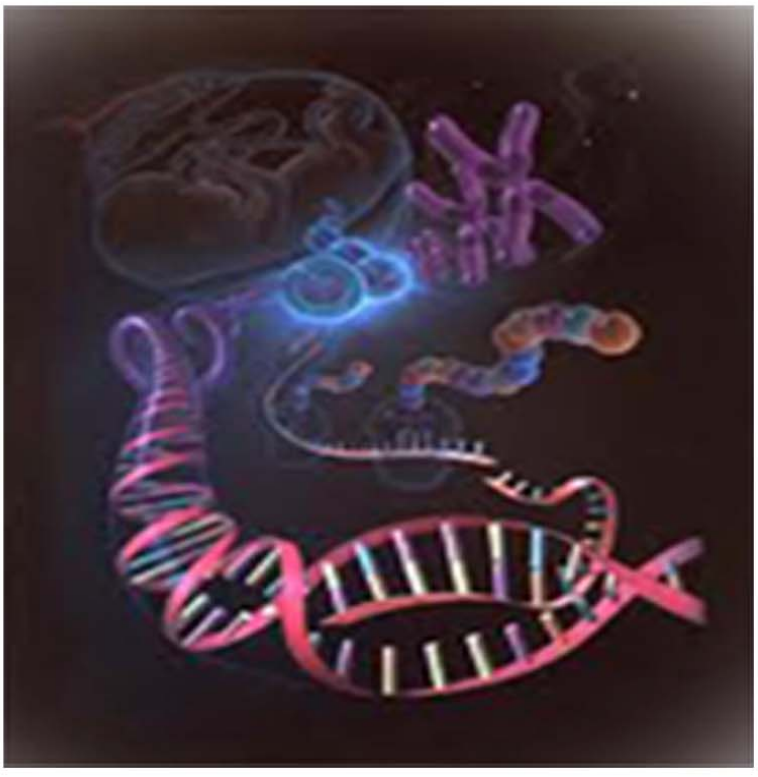

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Research Paper

EVOLUTION OF MOSQUITO LARVICIDAL ACTIVITY OF NEEM SEED KERNEL ALKALOID AGAINST *ANOPHELES STEPHENSI*

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Azadirachta indica (Neem) is commonly used in Indian traditional medicine for the treatments of variety of diseases. Over phytochemical studies of the Neem seed kernel showed the presence of alkaloid. Percentage of yield for methanol extract was 13.0%. The results of spectrum showed presence of sesquiterpene lactone group. The mosquito larvicidal activity of bioactive alkaloid isolated from Neem seed kernel have been tested against *Anopheles stephensi.* Three trials were performed for each concentration along with control and untreated. 24 h LC50 and LC90 values were determined using probit analysis method. The compound gave LC50 value to be 156.19 ppm for second instar larvae and 122.74 ppm for fourth instar larvae. It was noticed that fourth instar larvae are more susceptible than second instar larvae. The results obtained suggest that the bioactive compound of *Azadirachta indica* could be useful in the search for new larvicidal compound of plant origin.

Keywords: Larvicidal, Phytochemical, Alkaloid, Bioactive, *Anopheles stephensi, Azadirachta indica*

INTRODUCTION

Mosquito constitute a major public health problem as vectors of serious human diseases like malaria, filariasis, Japanese encephalitis, dengue fever, chikungunya and yellow fever (Jang *et al.,* 2002) cause substantial mortality and morbidity among people living in tropical and sub tropical zones. Synthetic pesticides have been extensively used for mosquito control by either killing preventing adult mosquitoes to bite human beings or by killing mosquito larvae at the breeding sites of the vectors (Joshep *et al*., 2004). Development of insect resistance to synthetic pesticides such as malathion, DDT, deltamethrin and even bio-pesticides such as *Bacillus thuringiensis* (Yang *et al*., 2005, Tabashnik *et al*., 1994), high operational cost and environmental pollution have created the need for developing alternative approaches to control vector-borne disease (Mittal and Subbarao, 2003). Plants products are emerging as a potential oils have special interest due to their insecticidal properties (Cheng *et al*., 2003, Sukumar *et al*., 1991).

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MATERIALS AND METHODS

Plant Material

The seed kernel of *Azadirachta indica* of family Meleaceae was collected during pre-monsoon month from the campus of SSL Jain College Vidisha (MP) India. The collected plant seeds were shade dried at room temperature in laboratory keeping for one day at 100°C in the oven. A voucher specimen of the plant has been preserved in our herbarium record in Pest control and Ayurvedic Drug Research Laboratory Vidisha for future reference.

Extraction Method

The extraction of seed kernel of *Azadirachta indica* shade dried powder material (40-60) mesh size was carried out separately by soxhlet apparatus in the laboratory using different solvents in increasing order of polarity. The extraction procedure adopted as given by Harborne (1984). The plant material was extracted in n-hexane, benzene, chloroform, acetone and methanol.

The percentage yield of crude extract of neem seed kernel obtained 8.66 for n-hexane, 4.15 for benzene, 6.8 for chloroform, 9.1 for acetone, 13.0 for methanol.

Isolation and Purification of Compound

There are various active compounds present in the plant extract ,so further isolation and purification was carried out, to find out the pure active compound. The crude extract obtained from vacuum evaporator of plant was subjected for isolation, purification, chemical examination, spectral analysis and characterization of the compound.

Phytochemical Screening of Alkaloids

About 0.5 g of each extract separately with a few drops of dilute hydrochloric acid and filter. The filtrates were tested with various alkaloidal reagent such as Mayer's reagent (cream precipitate): Dragendorff's reagents (orange brown precipitate) and Wagner's reagent (reddish brown precipitate).

Mayer's Reagent: Few drops of reagent were added in each extract and observed formation of the white or cream colored precipitates.

Dragendorff's Reagent: Few drops of Dragendorff's reagents were added in each extract and observed formation of the orange yellow or brown colored precipitates.

Wagner's Reagent: Few drops of Wagner's reagent were added in each extract and observed formation of the reddish brown precipitates.

THIN LAYER CHROMATOGRAPHY (TLC)

Separation was carried out on TLC plates to see the presence of different compound in a extract and their purity for the terpenoids and saponins. Different solvent system were used according to the method of Harborne (1984) and measured with the cm scale for determining the Rf value defined by Brimley and Barrett (1953).

Column Chromatography

The small quantity of crude chloroform extract was followed by observation on silica gel column with ethyl acetate solution. Four fractions (AIB1 to AIB4) were collected by using solvent system $CHCl_3$ - MeOH . All fraction were monitored by TLC until single spot was obtained as per phytochemical methods Harborne (1984).

Acid Hydrolysis

10 mL of the fraction collected from column chromatography was mixed with 2 mL of methanolic HCl (10%) and was reflex for 4 h in evaporator at the 4 h the mixture was again diluted with 3 mL of distilled water and then evaporated to remove methanol, it performed 2 times then the aqueous layer was neutralized with 10% sodium hydroxide and concentrated under reduced pressure.

Methylation

Purified fraction were separately dissolved in MeOH. In this process compound was washed by MeOH until it got converted into crystals form. After methylation, solvents were evaporated and residues were controlled by TLC.

Structure Elucidation

Chloroform extract of *Azadirachta indica,* which yielded active fraction AIB2 was further analyzed for spectrum to get IR, UV, CNMR, HNMR and mass spectrum for knowing the probable functional groups present. The result of the spectrum showed molecular weight 177.13, molecular formula $C_8H_9N_4O$ and showing the presence of sesquiterpene lactone group.

Experimental Bioassay

Laboratory colonized *Anopheles stephensi* second and fourth instar larvae were used for the experimental bioassay. Larval study was conducted according to standard of procedure WHO (1981).

For experimental bioassay, 25 s and fourth instar larvae of *Anopheles stephensi* were kept in 500 mL of the test compound. Acetone was used as solvent to dilute the compound to an appropriate test concentration. The treatments were replicated three times. Each replicate set contains one control, which received 1 ml of 50% acetone and 249 mL of distilled water and one untreated, which contained only 250 mL of distilled water. The number of dead larvae, pupae and adults were recorded. Mortality was corrected according to Abbott formula (1925).

Statistical evaluation of data was carried out by probit analysis of Finney (1971) and level of significance Duncan's (1963) multiple range test.

RESULTS AND DISCUSSION

After shade drying the powdered material of neem seed kernel, when soxhleted in different solvent of increasing order of polarity gave maximum yield in methanol extract, which accounted 13.0% and 8.66% in n-hexane, 4.15% in benzene, 6.8% in chloroform, 9.1% in acetone as shown in (Table 1).

Chromatographic separation of crude chloroform extract of *Azadirachta indica* was carried out using solvent system $CHCl_3$:MeOH (13:7) which gave 4 fractions AIB1, AIB2, AIB3 and AIB4 as shown in (Table 2). The biologically active fraction AIB2 was further purified to acid hydrolysis, methylation and re-crystallization.

TLC of the crude extract of *Azadirachta indica* chloroform extract was performed using solvent system $CHCl_3$:MeOH (13:7) which gave 4 spots and Rf value of each spot 0.57, 0.60, 0.63 and 0.72 as shown in (Table 3).

In present study larvicidal activity observed for malaria vector that is *Anopheles stephensi* as indicate in (Table 4). When 5 different concentrations from 100 to 500 ppm were used the LC_{50} and LC_{90} value as 156.19 and 100 ppm for second instar larvae and 122.74 and 499.79 ppm for fourth instar larvae respectively. Ultimately the larvae die either due to failure of ecdysis or by delayed metamorphosis. It was noticed that

Table 1: Percentage Yield of Neem Seed Kernel by Soxhlet Apparatus in Different Solvent

S. No.	Solvent Used	Weight of Plant Material Powder in gram	Temperature	Weight of Extract in gram	Percentage of yield
1	n-haxane	100	40°	8.66	8.66
2	Benzene	100	40°	4.15	4.15
3	Chloroform	100	40°	6.8	6.8
4	Acetone	100	40°	9.1	9.1
5	Methanol	100	40°	13	13

Table 2: Column Chromatography of *Azadirechta indica*

Colour of fraction	Wt. of fraction (g.)	obtained fractions	Solvent system used in column chromatography	Plant extract in solvent
			$CHCl_3$-MeoH (3:17)	Chloroform
		AIB1	$CHCl_3$-MeoH (10:7)	
		AIB2	$CHCl_3$-MeoH-H_2O (13:7)	
		AIB3		
		AIB4		
		AIC1	$CHCl_3$-MeoH (3:7)	Acetone
		AIC2	$CHCl_3$-MeoH (2:8)	
		AIC3		
		AID1	$CHCl_3$-MeoH (3:17)	Methanol
		AID2	$CHCl_3$-MeoH (7:3)	
		AID3		

the 500 ppm concentration caused 100% mortality to both the instar larvae of Anopheles. Mortality of larvae in different concentration during the experiment ranged from 42% to 100% as compared with 4% in the control.

highest % yield 13.0 as indicated in Table 1. (Innocent *et al.*, 2008) have also recorded % yield as 2.1 g in n-hexane, 4.2 in ethyl acetate, 3.1 in acetone, 4.1 in chloroform and 7.2 in methanol from *Lantana camara* plant extracts.

Table 3: TLC of Crude Extract of *Azadirachta indica*

S.No.	Plant Extract	Solvent Used	Number of Spot	Behavioral in			Rf value of each spot from bottom (cm)
				Visible	Iodine Chamber	U.V. Light	
1.	Chloroform	CHCl3:MeOH (13:7)	**4 Spots**				
			Spot 1	Dark Green	Green	Light Green	0.58
			Spot 2	Light Green	Green	Yellow Green	0.6
			Spot 3	Yellowish Green	Light Green	Green	0.63
			Spot 4	Yellow	Light Green	Light Green	0.72
2.	Acetone	CHCl3:MeOH (2:8)	**3 Spots**				
			Spot 1	Light Green	Green	Green	0.65
			Spot 2	Light Yellow	Green	Green	0.71
			Spot 3	Light Yellow	Light Green	Light Green	0.91
3.	Methanol	CHCl3:MeOH (7:3)	**3 Spots**				
			Spot 1	Yellow	Green	Green	0.48
			Spot 2	Light Yellow	Light Green	Light Green	0.58
			Spot 3	Light Yellow	Green	Green	0.61

Table 4: Statistical Data of *Azadiracta indica* Chloroform Extract Treated on Second and Fourth Instar Larvae of *Anopheles stephensi*

Larvalstage	Concn. (ppm)	Larval mortality (%)	Regretion eq.(y=a+bx)	x2=(n-1) Heterogeneity	LC_{50} (ppm)	LC_{90} (ppm)	Log LC_{90} ± S.D.	95%Fiducial limits (ppm)
	100	42						
II^{nd}instar	200	56						M1=139.05
	300	64	1.65+1.51x	2.31(6-1)	156.19	100	2.218+0.08	
	400	76						M2=286.28
	500	100						
	Control	04						
	100	46						
IV^{th}	200	63						M1=145.47
instar	300	78	1.362+ 1.74x	0.36(6-1)	122.74	499.79	2.089+ 0.07	
	400	82						M2=273.65
	500	100						
	Control	04						

In the present study 5 different concentration of *Azadirachta indica* chloroform extract were tested against second and fourth instar larvae of *Anopheles stephensi* a dose dependent larvicidal activity was noticed. Similar, dose dependent larvicidal effect by *Lantana camara* against mosquitoe species *Aedes aegypti* and *Culex quinquefasciatus* was noticed by Kumar and Maneemegalai (Kumar *et al*., 2008) . Earlear studies by (Das *et al.*, 2007) have also reported the similar view regarding mosquito larvicidal activity of methanol and ethanol extract of different parts of 5 indigenous plants. The value was found to be minimum 17.30 ppm for *Aristolochia sacata* extract (Krishan *et al*., 2008 and Saxena *et al.,* 1993) have also reported the larvicidal and chemosterilant activity of *Annona squamosa.*

CONCLUSION

The findings of present studies, therefore suggest the use of *Azadirachta indica* chloroform extract as a local resource in controlling mosquito larvae.

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