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MOSQUITO-LARVICIDAL ACTIVITY AND ACTIVE CONSTITUENTS OF *ACORUS CALAMUS* L. ESSENTIAL OIL

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Abstract

Fractions of *Acorus calamus* oil obtained by vacuum liquid chromatography showed increased mosquito-larvicidal activity against III instar larvae of *Culex quinquefasciatus* from LC_{50}, 39ppm in unfractionated oil to LC_{50}, 27ppm. The unidentified constituent of oil (GLC retention time, 37.65 min) which had the highest negative correlation (r = -0.954) between the LC_{50} values for the VLC fractions, can be suggested as the major active principle responsible for mosquito-larvicidal activity. It is possible to increase the mosquito-larvicidal activity of calamus oil by removing its major constituent, β-asarone (84%) which is known to be neurotoxic.

Key words. *Acorus calamus* L., Essential oil, mosquito-larvicidal ac tivity, *Culex quinquefasciatus*, LC_{50}, β-asarone, Vacuum liquid chromatography.

1. Introduction

Plants are known to contain compounds of insecticidal activity (Dixit et al., 1955; Somerville & Pockett, 1974; Bandara *et al.*, 1989). Most of these compounds are biodgradable and less harmful to mammals than synthetic insecticides. Therefore, it appears preferable to replace synthetic insecticides which are: (i) toxic to mammals and (ii) responsible for the development of resistant insect strains, with natural insecticides of plant origin (Marini-Bettolo, 1976).

Insecticidal activity of *Acorus calamus* oil against some insects (Dixit *et al.*, 1955; Bhaskar & Srivastava, 1972) and adult *Culex* mosquitoes (Dixit *et al.*, 1955) have been previously reported. Our previous studies have shown that the steam distillate of *Acorus calamus* has significant mosquito-larvicidal activity (Ranaweera, 1996).

The present study describes the larvicidal activity of different fractions of *Acorus calamus* essential oil obtained by Vacuum Liquid Chromatography (VLC) against *Culex quinquefasciatus*.

2. Materials and methods

Plant material:

Specimens of *Acorus calamus* L (rhizome) were washed in running water and were cut into pieces and immediately air-dried.

Test organisms:

Late III instar larvae of *Culex quinquefsciatus,* supplied by. late Professor W. E. Ratnayake, Department of Zoology, University of Sri Jayweardenepura, Sri Lanka, were used for bioassay.

Extraction of *Acorus calamus* oil and fractionation:

For the extraction of *Acorus calamus* oil, air dried rhizomes (250g) of *A. calamus* were cut into small pieces and were steam distilled. Fractionation of this steam volatile oil was carried out by Vacuum Liquid Chromatography (VLC) using different solvent systems. The concentrations of constituents present in each of these oil fractions obtained by VLC were determined by GLC analysis. Each VLC fraction was also tested for its larvicidal activity (LC_{50}) against III instar larvae (*C. quinquefasciatus)* as described below.

Vacuum liquid chromatography (Pelletier *et al.*, 1986 ; Coll & Bowden, 1986) was carried out in a sintered-glass Buchner filter funnel (height, 8cm; diameter, 2.5cm) packed with alumina with binder. Sample (1.0g) was eluted sequentially with hexane, hexane : toluene 1:1, toluene, toluene:ethyl acetate 20:1 and acetone. Solvents in fractions were evaporated to dryness under reduced pressure and for the purpose of bioassay, residues were redissolved in 5% aqueous ethanol. Tween 80 (0.1%)was used as a surfactant.

Fractions obtained by VLC were subjected to further separation by TLC. Samples (5µl) were spotted on pre-coated TLC plates (silica gel $60F_{254}$) and the plates were developed in toluene: ethyl acetate 93:7 and the detection was made by vanillin sulphuric acid (Wagner *et al.*, 1984).

For bioassay, VLC fractions were further fractionated on preparative thin-layer chromatographic (PTLC) plates and developed in toluene : ethyl acetate: 93:7. Five fractions were obtained by dividing the developed plate into five equal bands and each of them were tested for their larvicidal activity against *C. quinquefasciatus.*

GLC analysis of essential oils was performed on a Gas chromatograph-Varian 2700 with F. I. D.; Column : Carbowax 20M 15% coated on Gaschrom Q (1.5M x 20mm); Injection block temp., 250°C; Detector oven

temp., 200°C; Carrier gas (Argon) flow rate: 10 psi ; Samples (0.2µl), with programming from 100 to 250°C at 5mm/min ; Recorder, Shimadzu Chromatopack 6A.

Mosquito bioassay:

LC_{50} values of plant extracts for mosquito larvae were determined by the procedure followed by World Health Organization (Busvine, 1971) with slight modifications. For bioassay, healthy late III instar mosquito larvae were distributed in batches of 20 in small beakers containing 25ml water. Test dispersions (25ml) were prepared in separate beakers by adding different amounts of extract to give a series of ten final concentrations ranging from 10 to 100ppm, when contents with larvae in small beakers were added to the latter. Mortality counts were taken after 24 hours and the bioassay was carried out at 29°C in 4 replicates. LC_{50} values were estimated with sufficient accuracy from a probit/log concentration graph.

3. Results:

A yield of 2.6 per cent essential oil on dry weight basis, was obtained from the rhizome of *A. calamus* by steam distillation. The essential oil obtained was a brown viscous liquid with pleasant spicy odour.

Results of GLC analysis indicate that β-azarone is the major constituent found in *A. calamus* oil which accounts for about 84%. There are twelve other major components present in oil, but in low concentrations, which range from 0.4-2.5% (Figure 1; Table1)

Table 1. Major essential oil constituents of *Acorus calamus* oil.

Essential oil component			
Peak Number	Retention time, min	Compound	Content, %
1.	19. 71	Unidentified	1.35
2.	20.81	Unidentified	0.40
3.	21.84	Unidentified	0.62
4.	24.55	Unidentified	1.28
5.	25.31	Unidentified	0.71
6.	26.84	Unidentified	1.96
7.	31.21	Unidentified	1.39
8.	32.94	Unidentified	2.53
9.	35.41	Unidentified	1.48
10.	35.68	Unidentified	0.68
11.	37.65	Unidentified	1.44
12.	41.13	β-asarone	84.18
13.	46.07	Unidentified	1.23

* As per the gas chromatogram in Figure 1

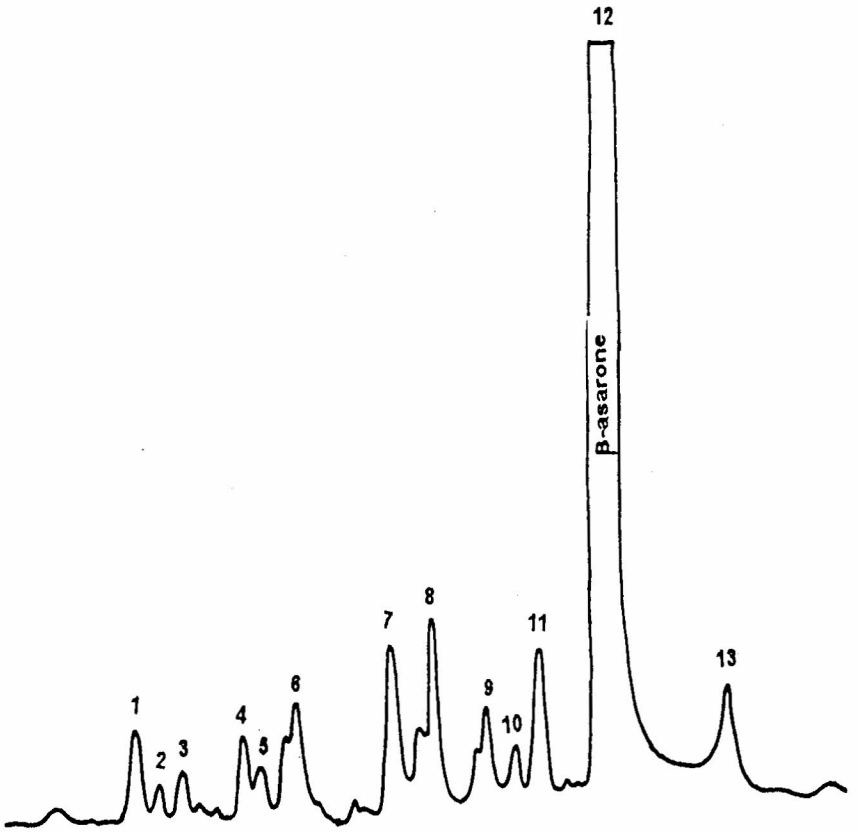

Figure 1. GLC Chromatograma of *Acorus calamus* oil.

Retention times, min: 1 - 19.71 ; 2 - 20.81 ; 3 - 21.84 ; 4 - 24.55 ; 5 -25.31; 6 - 26.84 ; 7 - 31.21 ; 8 - 32.94 ; 9 - 35.41 ; 10 -35.68 ; 11-37.65; 12- 41.13 (β-asarone) ; 13 - 46.07

Oil was fractionated by vacuum liquid chromatography which gave fractions with adequate concentrations of constituents required for the bioassay. As a result, six fractions were obtained and each of them were tested for their larvicidal activity. Results on the larvicidal activity of oil fractions have indicated that two fractions, F5 and F6 had higher activities (Table 2) having LC_{50} values of 27 and 36ppm respectively when compared to unfractionated oil which had LC_{50} value of 39ppm. Other fractions had lower activity and their LC_{50} values were above 125ppm.

Table 2. Mosquito-lcarvicidal activity (LG_{50}, ppm) VLC fractions of *Acorus calamus* oil against III instar larvae of *Culex quinquefasciatus.*

* Fraction No:	F1	F2	F3	F4	F5	F6	Oil (unfractionated)
LC_{50}, ppm:	65.0	127.0	127.5	166.5	27.0	36.0	39.0

* Fraction number is in the order of elution from the VLC column

The highest negative correlation (r= -0.95) was observed between unidentified compound (GLC retention time, 37.65 min) found in oil fractions and the LC_{50} values for fractions (Table 3) Therefore, it is possible to hypothesize that this unidentified compound could be mainly responsible for the larvicidal activity. Another unidentified compound (GLC retention time. 47.07min) too had a negative correlation but with a lower degree (r= -0.316). All the other constituents of oil including β–asarone (r= +0.303) were positively correlated with the LC_{50} values for VLC fractions.

Table 3. Coefficients of correlation observed between the LC_{50} values (ppm) for VLC fractions of *Acorus calamus* oil against III instar larvae of *Culex quinquefasciatus,* and the essential oil constituents in fractions.

	Essential oill component		
Peak number	Retention time, min	Compound	Coefficient of Correlation
1.	19.71	Unidentified	0.453
2.	20.81	Unidentified	0.674
3.	21.84	Unidentified	0.287
4.	24.55	Unidentified	0.845
5.	25.31	Unidentified	0.553
6.	26.84	Unidentified	0.659
7.	31.21	Unidentified	0.451
8.	32.94	Unidentified	0.359
9.	35.41	Unidentified	0.932
10.	35.68	Unidentified	0.900
11.	37.65	Unidentified	- 0.954
12.	41.13	β .-asarone	0.303
13.	46.07	Unidentified	- 0.316

Further separation of VLC fractions of oil by TLC (Figure 2) has shown that the fractions (F5, F6 and F1) which had high activity contained a lower concentration of β-asarone than that in fractions with lower activity (F2, F3 and F4). Results on bioassay of five bands obtained by PTLC, for larvicidal activity has shown that the activity was mainly located in the uppermost (Rf= 0.8-1.0) and lowermost (Rf= 0.0-0.2) bands. The uppermost band always had a higher larvicidal activity than that of the lowermost band. Further separation of compounds in these fractions to isolate active principles was not successful.

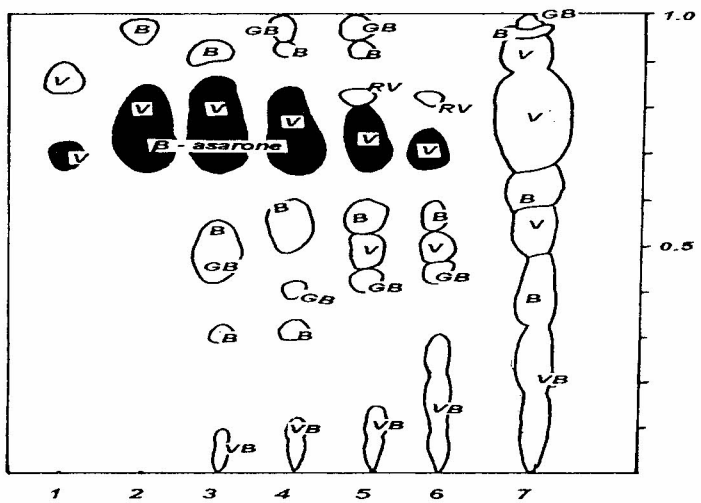

Figure 2. TLC chromatograma of *Acorus calamus* oil and its VLC fractions

Tracks : 1 - fraction *1; 2 - fraction 2 ; 3 fraction 3; 4 - fraction 4 ; 5 - fraction 5 ; 6 - fraction 6 ; 7 - unfractionated oil*
B-blue ; V-violet ; GB - greyish blue ; BV - bluish violet ; RV - reddish violet

4. Discussion

Our studies on an *A. calamus* variety grown in Sri Lanka have shown that its essential oil content ranged from 2.5 to 2.8% This coincides with the results obtained previously (Kelkar and Rao, 1934). It has also been reported that the polyploidism in *A. calamus* affects qualitative and quantitative composition of its essential oil (Mazza, 1985) and the oil content in the Indian hexaploid variety varied from 3 to 8 per cent (Singh et al., 1974). However, no work has been done to determine the ploidy of *A. calamus* varieties grown in Sri Lanka.

Thirteen major and about thirty minor volatile components were detected in *A. calamus* volatile oil, β-asarone being the major component (84%). GC-MS analysis (Mazza, 1985) of Indian calamus (tetraploid) oil has shown that it contained 93 volatile compounds including β-asarone with a high content (77.68%).

As a result of fractionation of oil, the larvicidal activity of fractions was increased when compared to the unfractionated oil and the Fractions (F5 and F6) which had higher larvicidal activity contained lesser content of β-asarone than that in other fractions (Figure 2).

High negative correlation (r= -0.954) observed between the unidentified compound (GLC retention time, 37.65min) and the LC_{50} values of fractions, indicates that this unidentified compound could be the active priciple lethal to mosquito larvae. It was also observed in the chromatogram (Figure 2) that compound (with Rf = 0.85) has appeared in increased amounts in the fraction (F5), which had the highest activity. This could be the same unidentified compound responsible for the activity. Lesser activity located in the lowermost band of TLC (Rf= 0.0 - 0.2) indicates that there could be another compound responsible for lethality but with lesser activity. It was also observed that the increment of the activity as result of fractionation of oil was not as high as expected. This can be due to loss of possible synergistic effects of compounds suggested here and other compounds of fractions on the activity. Studies on synergistic effects of different oil fractions and their compounds on the larvicidal activity has to be conducted.

It has been previously suggested that β-asarone was responsible for mosquitocidal (adults) activity (Dixit et al., 1955). Positive correlation observed here for β-asarone (r= +0.3) conclusively shows that β-asarone is not responsible for the mosquito-larvicidal activity. β-asarone was also found to be a depressant of central nervous system resembling meprobamate and chlordiazepoxide (Chak and Sharma, 1965).

Therefore, by removing β-asarone which accounts for 84-90% from the *Acorus calamus* oil, it is possible to increase its mosquito larvicidal activity and as well as to eliminate its neurotoxicity.

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References

1. Bandara, B. M. R., A. A. L. Gunatilake, S. Sotheesswaran and E. M. K. Wijeratne, 1989. Insecticidal Properties and an Active Constituent of *Limonia acidissima. J. Natn. Sci. Coun. Sri Lanka,* **17** (2): 237-239.

2. Bhaskar, P. S. and J. B. Srivastava, 1972. Effects of *Acorus calamus* L. Oil Vapours on *Dysdercus koenigii* F. *Indian J. Experimental Biology,* **10** (50) 391-393.

3. Busvine, J. R., 1971. A Critical Review of the Techniques for Testing Insecticides: *Test Methods with Mosquito Larvae,* pp 182-183, 2nd Edn. London: Commonwealth Agricultural Bureaux.

4. Chak, I. M. and J. N. Sharma, 1965. Effect of Asarone on Experimentally Induced Conflict Neurosis in Rats. *Indian J. Exp. Biol.,* **3**:252-254.

5. Coll, John. C. and Bruce F. Bowden, 1986. The Application of Vacuum Liquid Chromatography to the Separation of Terpene Mixtures. *Journal of Natural Products,* **49** (5): 934-936.

6. Dixit, R.S., S.L. Perti and S.K.Ranganathan, 1956. Evaluation of *Acorus calamus* Linn. - An Insecticidal Plant of India. *J. Scientific & Industrial Research,* **15** (5): 16-22.

7. Kelkar, N. C. and B. Sanjiva Rao, 1934. Essential Oils from the Rhizomes of *Acorus calamus* Linn. *J. Indian Indust. Sci.,* **17**: 25-31.

8. Marini-Bettolo, G. B., 1976. Modern Trends in the Use of Natural Products for Controlling Pests and Plant Diseases: *Proceedings of Natural Products and the Protection of Plants,* 18-23 October 1976, 5-14p, Vatican: Pontificiae Academiae Scientiarum Scripta Varia.

9. Mazza Giacomo, 1985. Gas Chromatographic and Mass Spectrometric Studies of the Constituents of the Rhizomes of *Calamus.* I The Volatile Constituents of the Essential Oil. *Journal of Chromatography,* 328: 179-194.

10. Pelletier, S. William, Chokshi, P. Hiteshi and Huridut K. Desai, 1986. Separaton of Diterpenoid Alkaloid Mixtures Using Vacuum Liquid Chromatography. *Journal of Natural Products,* **49** (5): 892-900.

11. Ranaweera, S. S, 1996. Mosquito - Larvicidal Activity of some Sri Lankan plants. *J. Natn. Sci. Coun. Sri Lanka, **24** (2) : 63-70.*

12. Sing, Tej, K. L. Handa and P. R. Rao, 1974. Improved Method for Maximum Production of Indian *Calamus* Oil. *Sonderdruck aus Parfumerie and Kosmetic,* **55**: 172-175.

13. Somerville, H. J. and H. V. Pockett, 1974. An Insect Toxin from Spores of *Bacillus thuringiensis. Proc. Soc. Gen. Microbiol.,* **1**:145.

14. Wagner, H., S. Bladt and E. M. Zgainski, 1984. Essential Oil Drugs (Aetherolea): *Plant Drug Analysis,* Springer-Verlag, Berlin Heidelberg, New York, Tokyo, pp 5-24.