

Research Article

Fractional Extraction, Isolation and Identification of Biologically Active Compounds from *Anamirta cocculus* (L.) Wight & Arn to Control *Culex quinquefasciatus* Say and *Aedes albopictus* Skuse

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DOI: <https://doi.org/10.24321/0019.5138.202031>

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How to cite this article:

Anju Viswan K, Pushapalatha E, Arivudainambi S, Selvamuthukumaran T. Fractional Extraction, Isolation and Identification of Biologically Active Compounds from *Anamirta cocculus* (L.) Wight & Arn to Control *Culex quinquefasciatus* Say and *Aedes albopictus* Skuse. *J Commun Dis* 2020; 52(3): 53-57.

Date of Submission: 2020-03-12

Date of Acceptance: 2020-05-16

A B S T R A C T

Background & Objectives: Development of resistance towards synthetic insecticides is one of the major problems in vector control programmes and it can be reduced to an extent by the alternate use of botanical or bio insecticides. The present study aims to find out the larvicidal activities of *Anamirta cocculus* (L.) Wight and Arn against *Culex quinquefasciatus* Say and *Aedes albopictus* Skuse and to isolate the most active compounds present in the active fraction of the plant extract.

Methods: Cold extracts of *A. cocculus* seeds were taken using methanol as solvent and fractionation was done using the solvents n-hexane, ethyl acetate and acetone by column chromatographic method. Bioassays were conducted using all these extracts and LC₅₀ were calculated using the probit analysis developed by Finney. The structural elucidation of the compounds of most active fraction isolated from chromatographic studies after bioassay was done by Liquid chromatography–mass spectrometry (LCMS) and Nuclear magnetic resonance (NMR) spectroscopy.

Result: *A. cocculus* treated larvae exhibited more restlessness, convulsions and sluggishness, and the restless activity is more in *Ae. albopictus* than in *Cx. quinquefasciatus*. The activity of column fractions of *A. cocculus* was in the order Hexane (H) > Acetone (A) > H:EA (3:1) > H:EA (2:1) > H:EA (1:1): Ethyl acetate (EA): H:EA (1:2) > H:EA (1:3). The compounds present in the most active fractions were Picrotin and Menispermene respectively.

Interpretation & Conclusion: From the results it has been proved that the plant is having potential larvicidal activity.

Keywords: *Anamirta Cocculus*, *Aedes albopictus*, *Culex quinquefasciatus*, Larvicidal Activity, NMR, LCMS

Introduction

The pharmacological and insecticidal properties of herbs have been well established by researchers all around the world, especially in India where the climate is highly conducive for such pesticidal plants. Utilization of plant derived products in the management of insect pests/vectors are not a new concept but renewed recent past due to the development of wide resistance of pests against chemical/ synthetic insecticides. The ill effects of synthetic insecticides could be reduced to an extent by the use of botanical insecticides.¹

Plants are the reservoirs of complex phyto-chemicals like alkaloids, flavonoid, saponins and tannins, which are known to possess medicinal and pesticide properties.² The plant derived metabolites, such as saponine, steroids, isoflavonoids, essential oils, alkaloids and tannins were proved as potent mosquito larvicidal compounds by many researchers in their earlier studies. In addition to natural secondary metabolites, synthetic derivatives also provide alternative source for the management of mosquitoes.³ Bioactive organic compounds exerted by plants have a complex mode of action such as growth and oviposition inhibitory, repellent activity, toxic and deterrence.^{4,5}

In this study, *Anamirta cocculus* (L.) Wight and Arn., was tested for its efficacy against *Aedes albopictus* Skuse and *Culex quinquefasciatus* Say. *A. cocculus* is large woody climbing shrub with vertically furrowed ash coloured bark and glabrous young parts. Leaves large, simple, alternate, long petiole, petioles thickened at the base and apex broadly ovate, subcoriaceous, cordate or truncate at the base, tufts of hairs in the axils of the nerves except the basal ones, flowers greenish in long panicles, drooping from the nodes of the old wood, fruits druped kidney shaped turning red on ripening.

Materials and Methods

Test Organism

The mosquito species *Aedes albopictus* Skuse and *Culex quinquefasciatus* Say were used for the study.

Plant Used in the Study

Anamirta cocculus (L.) Wight and Arn. (Family: Menispermaceae).

Cold Extraction Method

Powder of *A. cocculus* packed as 20 g packets using Whatman No. 40 filter paper and held by stapler pin. The thimbles were placed carefully in to one litre conical flask thus leaving three packets per flask. Five such conical flasks were kept. Then methanol added to conical flask in such a way that the packets were submerged in the solvent. The mouth of the conical flasks was tightly plugged

with non-absorbent cotton, wrapped with aluminium foil and paraffin wax paper and secured using rubber band. Whenever needed methanol was added to the content. After 48 hours, the extracts obtained from the conical flasks were filtered through Whatman No. 40 filter paper. Then the collected extracts were reduced in Vacuum rotary evaporator and collected miscella were transferred to amber coloured bottle, tightly covered, labelled and stored in a deep freezer at -20°C.

Fractionation of *A. cocculus* Extracts

Fractionation of miscella was done by following column chromatography techniques. A glass column of 50cm length was used. Silica gel powder (60/ 120 mesh) was packed in the column and loaded with 5g of miscella. Then the miscella was eluted with different solvents (HPLC grades) and solvent systems such as n-hexane, EA and acetone and combination of solvents at different ratio viz., n-hexane, EA, hexane: EA (1:1), hexane: EA (1:2), hexane: EA (1:3), hexane: EA (3:1), hexane: EA (2:1) and acetone.

Bioassay

Conducted using the protocol of WHO (1981).⁶ LC₅₀ were calculated using a Probit programme developed by Finney (1971).⁷

Identification of Compounds

The structural elucidation of the compounds isolated from chromatographic studies done by LCMS and NMR spectroscopy.

Result

The different column fractions of *A. cocculus* eluted using column chromatographic techniques were tested against III instar larvae of *Cx. quinquefasciatus* and *Ae. albopictus* (tables 1 & 2). The data on 24h LC₅₀ (ppm) of the eight different fractions of *A. cocculus* tested against III instar larvae of *Cx. quinquefasciatus* were provided in the table 2. The 24 h LC₅₀ values ranged from 10.1 ppm to 28.86 ppm for the different column fractions viz; hexane, EA, hexane : EA (3:1), hexane : EA (2:1), hexane : EA (1:1), hexane : EA (1:2), hexane : EA (1:3) and acetone. 24 hr LC₅₀ and LC₉₀ (ppm) values obtained after the treatment of the eight column fractions of *A. cocculus* with III instar of *Ae. albopictus* is provided in table 3. As in the case of *Cx. quinquefasciatus* the activity of *A. cocculus* was same as that for *Ae. albopictus*. The 24 h LC₅₀ values for the different column fractions ranged between 9.81 ppm and 24.22 ppm for all the eight column fractions.

NMR and LCMS Analysis

The hexane fraction of *A. cocculus* shown more activity against III instar larvae of both *Ae. albopictus* and *Cx. quinquefasciatus*. This fraction was subjected to NMR and LCMS analyzes.

LCMS and NMR Data of *A. cocculus*

Fraction I

The fraction I was subjected to ^1H and ^{13}C NMR and LCMS analysis. The ^1H and ^{13}C NMR as well as LCMS data are given in Plate 1A, 1B and 1C.

Compound I

It is interesting to note that the structure isolated resembles the structure of 4, 6a-dihydroxy-1a1-methyl-2-oxo-5-(prop-1-en-2-yl)octahydro-1a1H-oxireno[2',3':1,2]indeno[7,1-bc]furan-6-carboxylic acid. International Union of Pure and Applied Chemistry (IUPAC) Name: 4, 6a-dihydroxy-1a1-methyl-2-oxo-5-(prop-1-en-2-yl) octahydro-1a1H-oxireno[2',3':1,2]indeno[7,1-bc]furan-6-carboxylic acid, Chemical Formula: $\text{C}_{15}\text{H}_{18}\text{O}_7$.

^1H NMR (400 MHz, CDCl_3), δ (ppm): 1.18 (s, 3H, H(31), H(32) and H(33)). 1.54 (d, 1H, H(24) J=1, 7 Hz). 1.95 (d, 1H, H(23), J=1.5Hz). 1.84 (s, 3H, H(35), H(36) and H(37)). 2.23 (d, 1H, H(29) J=2). 2.29 (t, 1H, H(28) J=1.9Hz). 3.59 (s, 1H, H(34)). 5.27 (s, 1H, H(26)). 7.20 (s, 1H, H(39)) (Plate No. 1A).

^{13}C NMR (100 MHz, CDCl_3), δ (ppm): 15.2(C15), 23.0 (C20), 30.9 (C20), 34.06 (C2), 44.4 (C10), 48.6 (C3), 51.5 (C4), 61.58 (C5), 72.3 (C1), 76.7 (C8), 77.1 (C7), 112.9 (C21), 130.0 (C19), 174.5 (C11), 179.5 (C16) (Plate No. 1B).

Mass spectra ESI [M-H] $^-$ 309.13 (Actual Mol. Wt. 310.11) (Plate No. 1C).

Fraction II

The fraction II was subjected to ^1H and ^{13}C NMR and LCMS analysis. The ^1H and ^{13}C NMR as well as LCMS data are given in Plate 2A, 2B and 2C.

Compound 2

It is interesting to note that the structure isolated resembles the structure of 11-hydroxy-1, 2, 10-trimethoxy-6, 6-dimethyl-5, 6, 6a, 7-tetrahydro-4H-dibenzo [de, g] quinolin-6-ium. IUPAC Name: 11-hydroxy-1, 2, 10-trimethoxy-6, 6-dimethyl-5, 6, 6a, 7-tetrahydro-4H-dibenzo [de, g] quinolin-6-ium, Chemical Formula: $\text{C}_{21}\text{H}_{26}\text{NO}_4^+$.

^1H NMR (400 MHz, CDCl_3), δ (ppm): 1.05 (s, 6H, H(27), H(28), H(29), H(50), H(51), H(52)). 1.96 (t, 2H, H(32), H(33) J= 6.7Hz. 3.11 (d, 2H, H(36), H(37) J= 2.3Hz. 3.79 (t, 2H, H(30) and H(31) J=3.5Hz. 1.32 (s, 9H, H(40), H(41), H(42), H(43), H(44), H(45), H(46), H(47), H(48), H(49)). 5.27 (t, 1H, H(35) J=1.6Hz. 4.49 (s, 1H, H(43)). 6.99 -7.325 (Ar. C-H). (Plate No. 2A).

^{13}C NMR (100 MHz, CDCl_3), δ (ppm): 29.80(C20), 29.80(C20 and C25), 31.94 (C15), 34.0(C1 and C6), 46.7 (C12), 43.2 (C4), 50.7 (C3), 61.6 (C11), 113.0-139 (Ar-C) (Plate No. 2B).

Mass spectra ESI [M-H] $^-$ 355.21 (Actual Mol. Wt. 356.19) (Plate No. 2C).

Discussion

The *A. cocculus* treated larvae exhibited more restlessness, convulsions and sluggishness and the restless activity is more in *Ae. albopictus* than in *Cx. quinquefasciatus*. The sluggish and peculiar coiling movement in treated larvae might be due to the neuronal or muscular disturbances caused by active ingredients released by the extracts in to the water. After exposure to *A. cocculus* extracts, the larvae showed abnormal motions, tremors and convulsions followed by paralysis and finally settle at the bottom of the container. The result is in corroboration with the reports of Sagar and Seghal against *Cx. quinquefasciatus*.⁸

The column fractions of methanol extracts were taken using the column chromatography and analyzed their larvicidal activity against 3rd instar larvae of both *Cx. quinquefasciatus* and *Ae. albopictus*. The mortality rates were recorded and the LC_{50} values were calculated. The calculated values show that the hexane fraction of *A. cocculus* shows more activity than the other seven fractions. A slight difference is seen in the activity against *Cx. quinquefasciatus* and *Ae. albopictus*. With all the eight fractions, *Ae. albopictus* showed less LC_{50} values for mortality as compared with *Cx. quinquefasciatus*, which may be due to its breeding habit that they are living in fresh water and *Cx. quinquefasciatus* mostly breeds in the polluted water.

The activity of column fractions of *A. cocculus* was in the order hexane (H) > Acetone (A) > H:EA (3:1) > H:EA (2:1) > H:EA (1:1): EA: H:EA (1:2) > H:EA (1:3). And the LC_{50} values against *Ae. albopictus* and *Cx. quinquefasciatus* were 9.81 > 12.36 > 14.44 > 17.96 > 18.64 > 19.4 > 22.22 > 24.22 and 10.1 > 15.12 > 16.84 > 19.96 > 20.44 > 22.4 > 24.98 > 28.86 respectively.

The most active fraction of *A. cocculus* was further purified using 'Thin layer chromatography' and the fractions were again tested for their larvicidal activity and the most active fractions were analyzed by NMR and LCMS to elucidate the structure of compound present in the fraction.

Chromatographic techniques are used to identify fractions and help to isolate chemical constituents from the selected extract. The fractions isolated from the hexane fraction of *A. cocculus* were I and II. The selected fractions which showed potent activities were further analyzed using Liquid Chromatography-Mass Spectrometry. The fraction I produced the molecular ion signal (m/z) of 309.13 and the identified compound may be 4, 6a-dihydroxy-1a1-methyl-2-oxo-5-(prop-1-en-2-yl)octahydro-1a1Hoxireno[2',3':1,2]indeno[7,1-bc]furan-6-carboxylic acid and its molecular formula is $\text{C}_{15}\text{H}_{18}\text{O}_7$ (Picrotin).

The fraction II produced the molecular ion signal (m/z) of 355.21 and the identified compound may be 11-hydroxy-1, 2, 10-trimethoxy-6, 6-dimethyl-5, 6, 6a, 7-tetrahydro-4H-

dibenzo [de, g] quinolin-6-ium with molecular formula $C_{21}H_{26}NO_4^+$ (Menispermine).

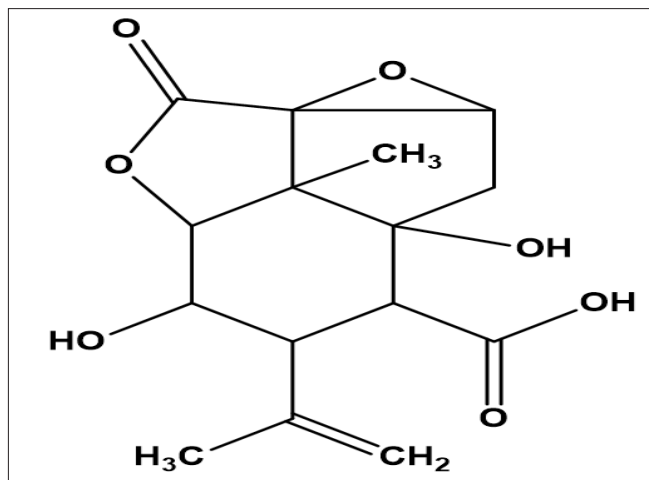


Figure 1. Picrotoxin

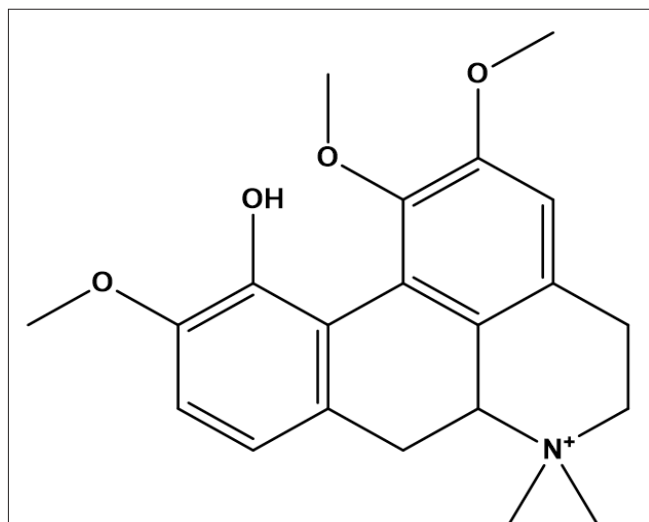


Figure 2. Menispermine

The larvicidal mechanism by the plant extracts is generally due to the presence of alkaloids. It is cytotoxic nature and causes damage of protein kinase which is involved in signal transduction and development process among most of the cells and tissues of mosquitoes.^{9,10} Acetone extracts of *A. cocculus* fruit showed significant reduction in fecundity and hatchability. And it also proved its larvicidal activity against different instars of *Cx. pipiens*.^{11,12}

The rich presence of fats and fatty acids in the seeds of *A. cocculus* might be rendering the extracts a waxy-solid consistency as well as an acidic nature. Due to the presence of various bioactive compounds in *A. cocculus*, antibacterial, antifungal and anti-inflammatory properties have been attributed to it.¹³ *A. cocculus* remains a popular folk remedy in Asia and adjacent regions. Fruits of *A. cocculus* contain picrotoxin, which was also known as cocculin. Picrotoxin was a crystalline equimolar mixture of two sesquiterpene

dilactones, i.e. picrotoxinin and picrotoxin. Furthermore, the fruits contain the isoquinoline alkaloids menispermine, paramenispermine, magnoflorine, stephorine, berberine, palmatine and 1, 8- oxotetrahydropalmatine.¹⁴

From the results it has been proved that the plant is having high larvicidal activity. The insecticidal property provides a safe, easily degradable and suitable alternative to synthetic insecticides. Further purification and field performance of the active compounds are going on to find out the field activity of purified compounds.

Acknowledgement

UGC BSR for providing financial support for carry out this project.

Department of Agricultural Entomology and Department of Chemistry, Annamalai University, Tamil Nadu, for providing facilities to carry out the chromatographic, NMR and LCMS works.

Conflict of Interest: None

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