FR 1719

Final Report

National Science Foundation Research Project RG/2004/C/01

Insecticidal Compounds from *Gnidia glauca* to develop bio-friendly insecticides

Research Grantees: Principal Investigator: Dr. Vijaya Kumar

Co-Investigator: Dr. Anoma P. Mudalige

Department of Chemistry,
University of Peradeniya,
Peradeniya.

April 17, 2013

Section 1: Information regarding Project/Project Personnel

i. Grant Number – RG/2004/C/01
ii. Title of the Project –
Insecticidal Compounds from Gnidia glauca to develop bio-friendly insecticides
iii. Principal Investigator – Vijaya Kumar
iv. Co-Investigators – Anoma P. Mudalige
v. Institute(s) where research was being carried out
Department of Chemistry, University of Peradeniya
vi. Date of award – August 23, 2004
vii. Date of completion of Project – January 1 2008
viii. Total allocation of funds (Rs) 598,000.00
ix. Total spent (Rs) 575,196.13
x. Number of Research Students employed - One
xi. Post graduate degree completed with dates - None
xii. Number of Technical Assistants and/or labourers employed and period of service – None
xiii. Publications/Communications arising from the project during the reporting period – One Communication

Amarajeewa, B.W.R.C., Mudalige, A.P. & Kumar, V., 2007. Chemistry and mosquito larvicidal activity of *Gnidia glauca*. Proceedings of the Peradeniya University Research Sessions 12(1): 101–102.

Section 2: Executive Summary of the Project

The fresh stem bark and leaves of *G.glauca* is widely used by farmers in some areas in Sri Lanka as a traditional pest control agent in paddy cultivation. Since farmers channel the water entering a paddy field over fresh *G.glauca* leaves and twigs, the project was initiated to test the hypothesis that the activity was due to more polar insecticidal materials extracted by the water. Hexane, dichloromethane and methanol extracts of leaf, bark and stem were subjected to mosquito larvicidal bioassay against *A.egypti* 2nd instar larvae. Plant materials when shade dried were found to be inactive, although plant material when extracted while fresh showed activity. However active extracts and active fractions were found to lose activity gradually with all activity lost in three to five months. Active material was isolated using repeated bioassay guided fractionation. However activity was lost in the process suggesting either easy decomposition of active compounds or a synergetic effect. The study led to the isolation of several compounds, but none with good activity. Two of these compounds were characterized and shown to be a bicoumarin with weak larvicidal activity and an inactive diterpene, although both were isolated through repeated bioassay guided fractionation. ..

Section 3: Report in Detail

i. Introduction/background

Gnidia glauca is a small tree belonging to the Family Thymelaeaceae which has over 1,200 species distributed in 67 genera. The largest genus of this family which includes about 150 species is the genus *Gnidia*. *G. glauca* is found in western part of the Uva province and eastern part of the central province of Sri Lanka and also in South India and tropical Africa.[1] Family Thymelaeaceae is economically important because of the use of many of the plant parts of its members in traditional medicine due to its marked toxicity and medicinal value shown by them: [1-3]

G. glauca - Fish poison, insecticide, diuretic, stimulant, treatment for indigestion, contusion and swelling, anti-neoplastic activity.

G. kraussiana - Molluscicidal agent, fish and arrow poison, homicidal agent, treatment for abdominal pain and snake bites.

G. chrysantha - Abortifacient, Laxative/purgative.

G. glabra and *G. latifolia* – Laxative/purgative

The chemistry of *G. glauca* has been extensively investigated. Compounds belonging to several chemical groups have been isolated from *G. glauca.* These include coumarins, bicoumarins, flavonoids, anthocyanins, lignans, sterols, sesquiterpenoids, diterpenoids, lipids, organic acids, amino acids, lactones and sugars. [3-5]

The fresh stem bark and leaves of *G. glauca* is widely used by farmers in remote areas in Sri Lanka as a traditional insecticide in paddy cultivation.

ii. Scientific scope of the project (overall and specific objectives)

The only larvicidally active compound isolated from *G. glauca* has been a weakly larvicidal (LC_{50}=13.25 ppm against 2nd instar larva of *Aedes egypti*) flavanoid, 3'-methoxyflavone, isolated from its dichloromethane extract.[6] Since the procedure adopted by farmers is to allow water to pass into the paddy field over fresh *G. glauca* bark and leaves, the present work was initiated in order to determine whether it would be possible to identify more polar water soluble larvicidal materials present in the extract with broad insecticidal properties. It was also hoped that because of the history of traditional use by farmers, they would be environmentally friendly and can ultimately be developed into a new family of bio-friendly insecticides.

iii. Materials and methods (including statistical methods)

Plant materials were collected from Madugoda, in the eastern part of central province of Sri Lanka in September 2005 and thereafter at regular intervals when required for research purposes. Both samples of the bark and the stem dried in the shade and the fresh samples of leaves, bark and stem of *G. glauca* were used in the work. Fresh and dried samples were separately ground into powder and sequentially extracted into hexane, dichloromethane and methanol (10 L each) using a bottle shaker at room temperature. The solvent was removed under reduced pressure using a rotavapor while maintaining temperature below 30 °C. The mosquito larvicidal activity of each crude extract was assessed against second instar larvae of *Aedes*

egypti in order to identify larvicidally active crude extracts. The second instar larvae of *A.egypti* were obtained from eggs of mosquito kept in a desiccator in the laboratory which hatched 24 hours after being placed in water. After a further 24 hours the second instar appeared and was available for the experiment. The active crude extracts were repeatedly subjected to bio-activity guided fractionation using activity against second instar larvae of *A.egypti* as a guide. Fractionation involved column chromatography, medium pressure liquid chromatography, flash chromatography and thin layer chromatography. Pure compounds were subjected to spectroscopic analysis using Ultraviolet, Infrared and ^{1}H- and ^{13}C-Nuclear Magnetic Resonance Spectroscopy

iv. Results/outputs and Discussion

The crude extracts of shade dried plant materials (leaves, bark and stem) of *G.glauca* were not found to be active, while those of fresh samples of leaves, bark and stem were found to be active. The activity of crude extracts of fresh samples of *G. glauca* (Table 1) was as follows:

Table 1: Activity of fresh G. glauca against A. aegypti 2nd instar larvae.

Extract		% Mortality in 24 hrs		
		500 ppm	250 ppm	125 ppm
Leaves	Hexane	50	30	10
	Dichloromethane	90	75	05
	Methanol	20	05	05
Bark	Hexane	100	100	100
	Dichloromethane	100	65	20
	Methanol	100	100	100
Stem	Hexane	100	40	10
	Dichloromethane	100	100	55
	Methanol	0	0	0

The active dichloromethane extract of the fresh bark yielded five pure compounds of which only one compound showed extremely weak activity with 40 % larval mortality shown at 25 ppm.

The activity of several combined fractions of dichloromethane extract of fresh bark showed loss in activity after about three months while most of the active crude extracts of fresh stem and leaves showed an activity loss after a similar period of time. In five months all activity was lost (Table 2).

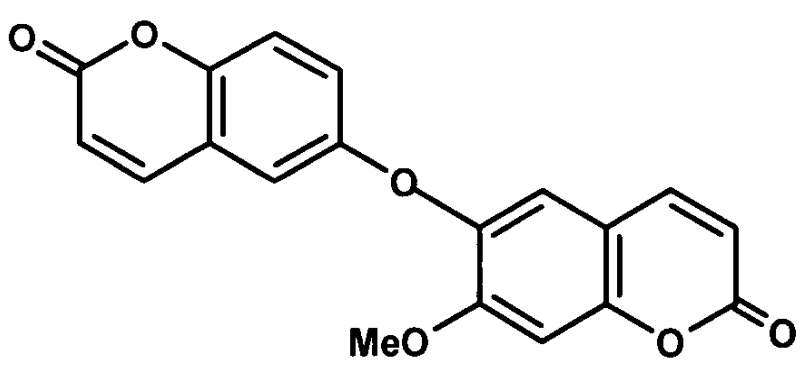
A combined fraction from the dichloromethane extract of fresh bark exhibited 100% mortality of 2nd instar *A. egypti* larvae within 24 hours of exposure. This combined fraction was subjected to mosquito larvicidal bioassay guided fractionation in order to isolate compounds with mosquito larvicidal activity. Two pure compounds, which were present in higher amounts in the combined fraction, were isolated, but they were found to not possess mosquito larvicidal activity. A mixture of these two pure compounds was also inactive against second instar larvae of *A.egyptii*, ruling

out a synergestic effect. The larvicidal activity of this combined fraction may have been due to the minor compounds present in trace amounts, a large number of which were present in the combined fraction. Attempts made to isolate bioactive compounds from this combined fraction proved unsuccessful.

Table 2: Activity of 3-month old extracts of fresh G. glauca against A. aegypti 2nd instar larvae.

Extract		% Mortality in 24 hrs
		500 ppm
Bark	Hexane	0
	Dichloromethane	35
	Methanol	0
Stem	Hexane	0
	Dichloromethane	0
	Methanol	0

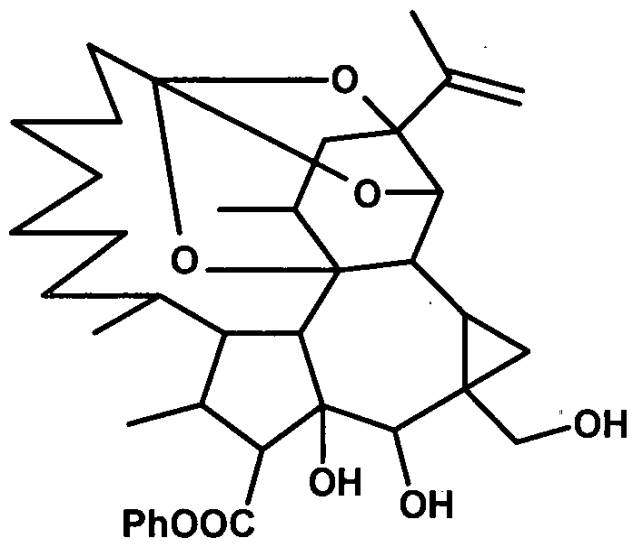
Two other compounds isolated from different combined fractions were identified. One of them was isolated from combined fraction 4 after medium pressure liquid chromatography. Repeated bioassay guided fractionation using medium pressure liquid chromatography gave an active fraction containing only one compound from thin layer chromatography analysis. This fraction was subjected to column chromatography to isolate the pure compound which was shown to be only weakly insecticidal. It was characterized as a bicoumarin, 6-(2-oxochromene-6-yloxy)-7-methoxychromene-2-one (1). The presence of a methoxy singlet and nine aromatic protons in its 1H NMR spectrum gave evidence for its structure. The ^{1}H NMR spectrum also showed a trisubstituted benzene ring with an AMX coupling system and a second benzene ring which was tetra substituted with two of its protons having a para-relationship in keeping with the proposed structure. The ^{13}C NMR spectrum with its signals for two carbonyl carbons and signals showing them to be part of an α,β-unsaturated carbonyl system and the corresponding ^{1}H NMR signals for alkene protons suggested that the two benzene rings were part of two coumarin nuclei attached to each other through the benzene rings. The DEPT spectrum of the bicoumarin showed 9 quaternary C atoms, 9 CH carbons and 1 CH_3 carbon in keeping with the proposed structure. The COSY and HMBC spectra of the compound permitted the assignment of the proton and carbon NMR signals to the protons and carbon atoms in the compound.



6-(2-oxochromene-6-yloxy)-7-methoxychromene-2-one (1)

This bicoumarin, 6-(2-oxochromene-6-yloxy)-7-methoxychromene-2-one (1) has previously been isolated from a related species, *Gnidia socotrana.* The bicoumarin was weakly active (LC_{50}=16.4 ppm) against the 2nd instar larva of *A.egypti.*

The second compound was isolated from combined fraction 1 from the medium pressure liquid chromatography of the bark dichloromethane extract. Repeated bioassay guided fractionation using medium pressure liquid chromatography gave an active fraction containing only one compound from thin layer chromatography analysis. This fraction was subjected to column chromatography to isolate the pure compound which was shown to be inactive. It had the spectral characteristics of Pimelea factor P_2, a diterpene with a daphnane skeleton (2), previously isolated from other *Gnidia* species[7] and from *Wikstroemisa retusa,*[8] also a member of Family Thymeliaceae. Its ^{1}H and ^{13}C NMR data were identical with those reported for Pimelea factor P_2 and its 1H-1H COSY spectrum was in keeping with the proposed structure. Pimelia factor P_2 although isolated from an active fraction did not show insecticidal activity against 2nd instar larva of *A.egypti.*



Pimelea Factor P_2 (2)

v. Conclusions

The work showed that fresh plant samples of *Gnidia glauca* showed greater insecticidal activity against the 2nd instar larvae of *A. egypti* than dried plant samples. This probably explained the failure to isolate the insecticidal compounds present in the plant although the plant is routinely used by farmers for this purpose by allowing water entering paddy fields to pass through a channel containing a *G.glauca* branch. The work showed that the stem and bark of *G.glauca* were more active than the leaves and that the activity of the stem was due to less polar compounds as its methanol extract was inactive. Furthermore the extracts themselves were shown to rapidly lose activity, the only extract showing larvicidal activity after three months of storage at ambient temperature being the dichloromethane extract of *G.glauca* bark which had by then lost about two-thirds of its activity. Activity was also lost during bioassay directed fractionation with very few of the isolates showing the activity shown by the fractions. This could be due to thermolability or decomposition of the active compounds or due to synergism contributing to activity. However we were unable to observe synergism when inactive compounds isolated from a particular active fraction showing loss of

activity were mixed and their activity determined. In many such experiments, no activity or improved activity was seen giving little evidence for synergism.

vi. References

1. Borris, R.P., Blasko, G., and Cordell, G.A., *J. Ethnopharm.*, 1988, **24**, 41-91
2. Borris, R.P. and Cordell, G.A., *J. Nat. Prod.*, 1984, **47**, 270-278
3. Franke, K., Porzel, A., Schmidt, J., *Phytochemistry*, 2002, **61**, 873-878.
4. Kupchan, S.M., Sweeny, J.G., Baxter, R.L., Murae, T., Zimmerly, V.A., and Sickles, B.R., *J. Amer. Chem. Soc.* 1975, **97**, 672-673.
5. Ferrari, J., Terreaux, C., Sahpaz, S., Msonthi, J.D., Wolfenden, J., Hostettmann, K., *Phytochemistry*, 2000, **54**, 883-889.
6. Alagesan, K., 1999, *Ph.D Thesis*, University of Peradeniya, p.123-127.
7. Kupchan, S.M., Shizuri, Y., Sumner, Jr., W.C., Haynes, H.R., Leighton, A.P. and Sickles, B.R., *J. Org. Chem.*, 1976, **41**, 3850-3853
8. Yaga, S., Kinjo, K., Hayashi, H. Matsuo, N., Abe, F. and Yamauchi, T., *Phytochemistry*, **32**, 141-143

vii. Problems if any, encountered during the implementation of the project

The major problem was the rapid decomposition of active constituents in the extracts and active fractions of *G.glauca* leaves stem and bark which made it extremely difficult to isolate active constituents.

It may have helped if a Chemistry Special degree graduate had been appointed as Research Assistant as this would have improved productivity by saving time spent on training in research.

The Co-Investigator Anoma Mudalige shortly after her PhD done under my supervision applied for this grant. Since NSF rules did not permit her to be Principal Investigator, I reluctantly agreed to be PI for the grant on the understanding that she would do most of the supervision as I would not be able to spend much time on it as I was Dean of Faculty. In 2006 halfway on the project she migrated to Canada promising me that she would continue supervision using e-mail facilities and run the grant from Canada. However this arrangement was not altogether satisfactory and the project suffered.

viii. Major findings and follow up activities.

It was found that shade dried leaves, bark and stem of G.glauca were inactive against the 2nd instar larvae of *A.egypti*, while the fresh plant material were active. The activity of the leaf extract was much less than that of the bark and stem. All the extracts and the active fractions obtained by bioassay guided fractionation were found to gradually lose their activity with much of the material losing activity in three months of storage and all activity being lost within five months. The dichloromethane extract of the bark gave on bioassay guided fractionation two compounds characterized as a bicoumarin and a diterpene. While the former showed weak larvicidal activity, the latter did not show any activity.

As a follow up it may be worth studying water extracts of *G.glauca* to determine whether they contain any insecticidal compounds

Section 4: Impact of Research Results

i. *Relevance of results achieved to scientific advancement*

It has been shown that larvicidal activity is shown by plant parts of *G. glauca* only if fresh material is used. Material dried in the shade is found to be inactive. Extracts of fresh plant material and active fractions were found to lose activity in around three months. Two compounds were isolated and characterized from active fractions using bioassay assisted fractionation.. The bicoumarin was shown to have only weak larvicidal activity while the diterpene did not show any activity..

ii. *Relevance of results achieved to national/socio-economic development*

The importance of the farmer technique of using fresh plant material when using *G. glauca* for plant protection was shown

iii. *Dissemination/application of research output*

Further work such as a study of the water extract of *G.glauca* or cold room experiments to prevent decomposition of active compound would be required to obtain results which would be applicable, Dissemination of results would also require further development.

Section 5: Miscellaneous

i. *List of major equipment acquired during the project period and their functionality:*

None

ii. *List of publications/communications arising from the project and/or presentations made at seminars, workshops etc. (Please attach copies)*

Amarajeewa, B.W.R.C., Mudalige, A.P. and Kumar, V., 2007. Chemistry and mosquito larvicidal activity of *Gnidia glauca*. *Proceedings of the Peradeniya University Research Sessions (PURSE)* **12(1)**: 101–102.

Section 6: Summary Statement of Expenditure - NSF Project RG/2004/C/01

Total Grant received from NSF		Rs. 598,000.00
Expenditure:		
Personnel	Rs. 278,500.00	
Equipment	Rs. -	
Consumables	Rs. 278,325.13	
Travel	Rs. 2,613.00	
Subsistence	Rs. -	
Miscellaneous	Rs. 15,758.00	Rs. 575,196.13
Balance (with University – Refund to NSF requested)		Rs. 22,803.87

Section 7

i. Grantees' signatures

V. Kumar

ii. Comments of the Head of the Department/signature

Work completed satisfactorily. Findings have been published.

Head
Department of Chemistry
University of Peradeniya

iii. Head of the Institution's signature

Vice - Chancellor
University of Peradeniya
Peradeniya
Sri Lanka

Revised date: 17/08/2010

NATIONAL SCIENCE FOUNDATION

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Completed Research Projects

Topic

Insecticidal Compounds from Gnidia glauca to develop bio-friendly insecticides

Grant no.: RG/2004/C/01

Names and affiliations of Investigators

PI: Dr. Vijaya Kumar
Department of Chemistry,
University of Peradeniya.

Co-Is:
Dr. Anoma P. Mudalige
Department of Chemistry,
Open University of Sri Lanka

Gnidia glauca tree

INTRODUCTION

The fresh leaves and twigs of *Gnidia glauca*, a small tree found in the Central province is used by farmers in Sri Lanka to control pests in paddy cultivation. The work aimed at identifying the active compounds so that they could be developed into environmentally friendly crop protection agents.

PROJECT ACHIEVEMENTS

Only fresh plant samples of *Gnidia glauca* shown to have larvicidal activity, not dried samples and stem and bark showed more activity than leaves. Extracts and active fractions lost their activity in about three months. This could be due to thermolability or decomposition of the active compounds or activity being due to synergism, although no evidence of the latter was seen. Two compounds, a weakly active bicoumarin, and an inactive diterpene, were isolated

Gnidia glauca leaves and flower

RELEVANCE TO SOCIO ECONOMIC DEVELOPMENT OF THE COUNTRY

The importance of the farmer technique of using fresh plant material when using *Gnidia glauca* for plant protection was shown.

FOLLOW-UP

It may be worth studying water extracts of G.glauca to determine whether they contain any insecticidal compounds

For more details of the project, contact:
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Chemistry and Mosquito Larvicidal Activity of *Gnidia glauca*

B.W.R.C. Amarajeewa[1], A.P. Mudalige[2] and V. Kumar[1*]

[1]*Department of Chemistry, Faculty of Science, University of Peradeniya*
[2]*Department of Chemistry, Open University, Nawala*

Introduction

Gnidia glauca is a small bushy tree growing to about 3 m in height. It belongs to the family Thymelaeaceae. The genus *Gnidia* possesses wide variety of folkloric as well as traditional phytomedicinal and agrochemical applications. It is used as traditional African medicine for cancers, sore throat, abdominal pain, wounds, burns and snake bites. It is also used as molluscicidal, insecticidal, piscicidal and even homicidal agents and as arrow poisons (Borris and Cordell, 1984; Franke *et al.*, 2002). It has been shown that several *Gnidia* species possess remarkable antineoplastic activity (Kupchan *et al.*, 1976).

Fresh, ground whole plant of *G. glauca* is currently used as an effective insecticidal and traditional piscicidal agent in fishing by remote framers, especially in the Knuckles region. The present study is an attempt to identify the insecticidal constituents of *G. glauca* using bioassays directed against the second instar larvae of *Aedes aegypti*. Plant extracts found to be active were separated using bioactivity directed fractionation in order to isolate pure active compounds which were then subjected to chemical analysis.

Materials and methods

Plant materials were collected from Madugoda in Udadumbara area in September 2005. Both dried and fresh plant material were used. Air dried plant materials were ground into powder and fresh plant materials into a paste using a grinder. They were then separately and sequentially extracted into hexane, dichloromethane (CH_2Cl_2) and methanol (MeOH) using a bottle shaker at 24 °C. The extracts were concentrated (< 30 °C) *in vacuo* using a rotavapor. All crude extracts were subjected to mosquito larvicidal bioassay against second instar larvae of *A. aegypti* in order to assess the larvicidal activity of the extract. Active crude extracts were further subjected to bio-activity guided fractionation against second instar larvae of *A. aegypti* in order to isolate pure compounds. Fractionation involved the use of MPLC and flash (FC) chromatography using Merck 9385 Silica gel (40-63 μm), gravity column (CC) chromatography using 7734 Merck Silica gel (63-200 μm), and analytical thin layer chromatography (TLC) using Merck Silica gel 5554 plates F_{254} on aluminium foil. A solvent gradient of increasing polarity using mixtures of hexane, ethyl acetate and methanol was used for MPLC and suitable mixtures of these solvents were used for FC, CC and TLC.

Structure elucidation of pure compounds isolated was carried out by NMR (^1H, ^{13}C, COSY, DEPT, HMBC and HMQC), IR and UV spectroscopy.

Results

Although hexane and CH_2Cl_2 extracts of dried bark of *G. glauca* showed moderate mosquito larvicidal activity, extracts of fresh bark exhibited high activity. Table 1 shows % mortality of second instar larvae of *A. aegypti* when treated with fresh plant material extracts.

Table 1. Mortality of A. aegypti larvae on treatment with G. glauca fresh plant extracts

		% Mortality after 24 hours		
Extract		500 ppm	250 ppm	125 ppm
fresh leaves	Hexane	25	10	10
	CH_2Cl_2	75	55	5
	MeOH	5	0	0
fresh bark	Hexane	100	100	90
	CH_2Cl_2	100	100	100
	MeOH	100	100	80
fresh stem	Hexane	50	35	5
	CH_2Cl_2	25	10	10
	MeOH	25	10	0

The fresh bark and stem extracts lost their activity in three months. Table 2 shows the mortality of second instar larvae of *A. aegypti*

when treated with 500 ppm three month old extracts of fresh

Table 2. Mortality of *A. aegypti* larvae on treatment with 3 month old *G. glauca* extracts

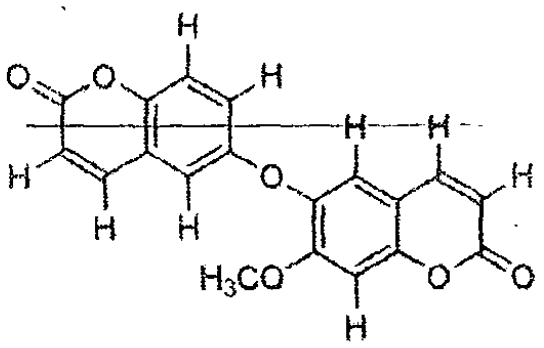
Extract		% Mortality
fresh bark	CH_2Cl_2	35
	MeOH	0
fresh stem	CH_2Cl_2	0
	MeOH	0

Bioactivity guided fractionation of the crude CH_2Cl_2 extracts of fresh bark of *G. glauca* using MPLC, FC, CC and TLC yielded two pure compounds. One was weakly active against second instar larvae of *A. aegypti*.

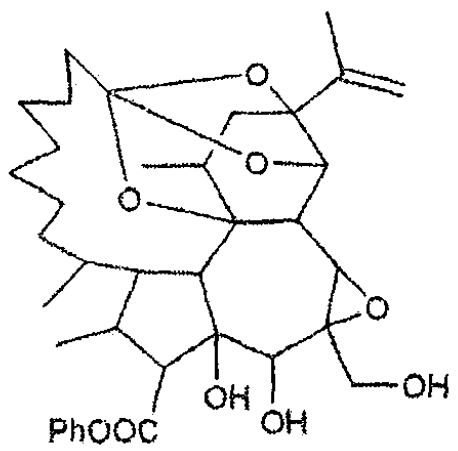
Discussion

Hexane, CH_2Cl_2 and MeOH extracts of fresh bark of the plant exhibited strong larvicidal activity against second instar larvae of *A. aegypti*. The CH_2Cl_2 extract of fresh bark exhibited 100 % mortality within few minutes. Bioactivity guided fractionation of the active crude extracts revealed that the active compounds were present only in trace amounts in the plant. Furthermore, active extracts lost their activity rapidly. This suggests the photolability of the active compounds present in the extract.

NMR, UV and IR data revealed compound (**1**) to be a bicoumarin. This compound was weakly active against second instar larvae of *A. aegypti*. NMR data of compound (**2**) showed it to be identical with Pimelea factor P_2 (Kupchan *et al.*, 1976; Yaga *et al.*, 1993) isolated from other members of Family Thymeliaceae. Compound (**2**) is a diterpene and it is known to possess strong antineoplastic activity (Kupchan *et al.*, 1976).



Compound 1



Compound 2

Acknowledgement

NSF financial support is gratefully acknowledged.

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Yaga, S., Kinjo, K., Hayashi, H., Matsuo, N., Abe, F. and Yamauchi, T. (1993) Diterpenoids with the Daphnane Skeleton from *Wikstroemia retusa, Phytochemistry*, 32, 141-143.

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