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Isolation of a Potent Mosquito Repellent from *Vitex negundo* L.: An Alternative Source of Rotundial

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Abstract – The chloroform fraction of the aqueous extract of the fresh leaves of *Vitex negundo* by bio-activity guided isolation yielded a pure compound, rotundial (**1**) which has shown potent mosquito repellent activity. Using spectral data (UV, IR, ^{1}H & ^{13}C NMR and MS) its structure has been elucidated.

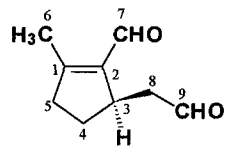
Key words – Rotundial, mosquito repellent, *Aedes aegypti and Vitex negundo*

Introduction

Blood sucking insects transmit many diseases throughout the world, one of the most important of these being malaria (*Plasmodium* sp.; Vector *Anopheles* sp.) (Anon., 1987) in the tropics. The vector bites may cause skin irritation, allergic reactions and secondary infections. To avoid these problems repellents play an important role in providing personal protection against blood sucking insects as they can be used at any place and any time. These repellents are recommended by the WHO for personal protection against malaria because of increasing resistance of *Plasmodium falciparum* to antimalarial drugs (Anon., 1988). One of the most important and widely used synthetic compound, *N,N*-diethyl *m*-toluamide (DEET) which was discovered in 1954, has a broad spectrum of efficacy against biting arthropods. DEET exhibits an undesirable effect of causing irritation to skin and mucous membranes, moreover it is not compatable with other synthetic compounds (Boeckh *et al.*, 1996).

The plant materials of the genus *Vitex* are of prolific occurrence and about 15 species of them have been chemically examined. These species produce a wide range of metabolites which includes mono, sesqui, di and triterpenoids, flavonoids, steroids, iridoids and their glycosides, ecdysteroids, lignans, alkaloids, anthocyanidins and aromatic compounds. The chemical constituents and their biological activity of *V. negundo* was reviewed recently (Das and Das, 1994). Although the volatile oil from the leaves has shown mosquito repellent activity, no active principle has been isolated (Hebbalkar *et al.*, 1992). The leaf extract possesses an antiarthritic effect (Chaturvedi and Singh, 1965) and smoke of its leaves is used to get relief from headaches.

In search of new mosquito repellents from Indian plants, we examined the leaves of *V. negundo*, which is known to have potential for pest control and insect repellency. In the present study we report the isolation of a potent mosquito repellent, rotundial (**1**) from *V. negundo* for the first time *via* aqueous extract of its fresh leaves.



Rotundial (1)

Experimental

Materials and Methods – UV and IR spectra were recorded in chloroform on Perkin Elmer Lambda 2S and Perkin Elmer model 1310 spectrophotometer, respectively. ^{1}H NMR (200 MHz) and ^{13}C NMR (50 MHz) were recorded on Bruker Advance DPX-200 using $CDCl_3$ as solvent and tetramethylsilane as internal standard. GC-MS was recorded on Shimadzu QP-2000, operating at 70 eV with capillary column DB-1

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(30 m × 0.03 mm id) and helium was used as carrier gas. The optical rotation was on Jasco DP-1000 instrument in chloroform. Silica gel was obtained from Acme's (100~200 mesh) chemical company, Bombay and all solvents were obtained from SD Fine Chemicals, Bombay and distilled before use. TLC's were recorded on precoated TLC plates (0.2 mm) and obtained from E. Merck (India) Ltd., Bombay. The fresh leaves of *V. negundo* were collected from Boodigiri, Bangalore, India and identified by Balakrishna Gowda, Department of Botany, School of Basic Sciences, University of Agricultural Sciences, Bangalore. The voucher specimen (EID-05/98) was preserved at R & D Centre, EID Parry (I) Ltd., Bangalore.

Extraction and Isolation – The fresh leaves of *V. negundo* (3 Kg) were collected from Boodigiri, Bangalore, India. The voucher specimen was preserved at R & D centre, EID Parry (I) Ltd., Bangalore. The leaves were cut into pieces, powdered and extracted with distilled water (5 L × 2). The aqueous extract was fractionated with chloroform (2 L), concentrated under reduced pressure to get 3 g of yellow syrup. The $CHCl_3$ extract was chromatographed over a column of silica gel using solvent of increasing polarity of hexane and hexane: ethyl acetate mixtures. The active fraction II was eluted with hexane: ethyl acetate (9:1) which was further rechromatographed and the active compound was eluted with hexane: ethyl acetate (98:2) to yield 450 mg of pale yellow oil. The yellow oil was subjected to preparative TLC to obtain 300 mg (0.01%) of pure compound.

Rotundial (1) – Pale yellow oil, R_f 0.53 (hexane: ethyl acetate 1:1); $C_9H_{12}O_2$; IR (ν_{max}) cm^{-1}: 2940, 1720, 1665, 1615, 1260 and 1190; UV(λ_{max}): 250 nm; $[\alpha]_D^{25}$: + 40.6 (c=1.0 $CHCl_3$); MS m/z 152, 137(M^+-Me), 108(M^+- CH_3CHO); ^1H NMR($CDCl_3$): δ 9.98 (1H, s, H-7), 9.76 (1H, t, J=2.0 Hz, H-9), 3.46 (1H, m, H-3), 2.95 (1H, ddd, J=1.6, 4.2 & 16.7 Hz, H-8), 2.55 (2H, m, H-5), 2.38 (1H, ddd, J=2.2, 9.1 & 16.8 Hz, H-8), 2.20 (1H, m, H-4), 2.15 (3H, d, J=1.3 Hz, H-6), 1.52 (1H, m, H-4); ^{13}C NMR: δ 164.3 (C-1), 138.9 (C-2), 38.0 (C-3), 28.0 (C-4), 38.9 (C-5), 14.3 (C-6), 187.9 (C-7), 47.7 (C-8), 201.9 (C-9).

Results and Discussion

The residue from the cold aqueous percolate of the leaves was reextracted with chloroform to obtain chloroform extract (3 g) as syrup. This extract showed 100% repellency till 4th hr and thereafter decreased to 87% at end of 6th hr at the application rate of 1.5 mg/cm^2. It is worth while to mention that the oil obtained by the steam distillation of *V. negundo* leaves showed much lower repellency than chloroform fraction of the aqueous extract in our hand (Table 1). Bioactivity guided fractionation of chloroform extract with hexane, hexane: ethyl acetate (9:1, 8:2, 7:3 and 1:1 ratio's) and ethyl acetate over silica gel to afford 6 fractions. Out of which fraction II obtained from hexane: ethyl acetate (9:1) was found to be most active and had shown 90% and 77% repellency till 4th hr and 80% and 69% at 6th hr at the application dose of 0.15 and 0.08 mg/cm^2. The fraction II was rechromatographed over silica gel to obtain a light yellow oil which was further purified by preparative TLC to obtain pure compound (**1**) as pale yellow oil, $C_9H_{12}O_2$, M^+ 152. The compound (**1**) had shown 94% repellency upto 4th hr and maintained 82% at 6th hr at application dose of 0.15 mg/cm^2 (Table). It was recognized as a ketonic compound by spray with 2, 4-dinitrophenylhydrazine solution. The UV spectrum (λ_{max}: 250 nm) showed the presence of an α, β-unsaturated conjugation in the molecule.The IR spectrum showed the bands at 1720 and 1735 cm^{-1} indicating the molecule having two carbonyl groups.

Table 1. Mosquito repellent activity of extracts and fractions of *V. negundo*

Compound/extract	Dosage (mg/cm^2)	%Repellency after treatment of						
		0	1	2	3	4	5	6hrs.
V. negundo oil	1.50	85	42	46	24	24	22	21
V. negundo aq. ext.	1.50	100	100	100	100	91	88	87
Fraction II	0.15	99	96	97	94	90	89	80
Fraction II	0.08	89	86	79	79	77	70	69
Rotundial	0.15	100	98	97	97	94	88	82
DEET	0.15	100	100	100	100	100	90	87

The ^{1}H NMR spectrum showed clearly two aldehydic protons, one proton at δ 9.76 as a triplet indicating that it was connected with neighboring methylene group and other proton at δ 9.98 as a singlet. The two protons showed double double doublets at δ 2.95 and 2.38 assigned for the methylene protons α-to the simple aldehyde group. One proton signal appeared at δ 3.46 as a multiplet that could be explained to β-to the aldehydic groups. Further the spectrum showed a signal at δ 2.15 (s, 3H) explained as a methyl on trisubstituted double bond, two more low filed signals appeared at δ 2.20 and 1.52 (m) explained to methylene protons at C_4 and 2.55 (m) explained to methylene protons at C_5. The ^{13}C NMR spectrum showed a total of nine carbon signals, out of which two carbonyl carbons (δ 201.9 & 187.9) and two olefinic carbons (δ 164.3 & 138.9). Based on the above data, a literature search revealed that the physical and spectral data of the compound (**1**) agreed perfectly with rotundial reported from *V. rotundifolia* in 0.005% yield (Watanabe *et al.*, 1995) while the present report describes its alternative source by different extraction procedure from *V. negundo* for the first time in better yield (0.01%).

Bioassay – The assay for repellent activity was carried out using 3-6 day old blood starved, sucrose fed *Aedes aegypti* drawn from well established laboratory colony as described earlier (Hebbalkar *et al.*, 1992). Human hand covered with snugly fitting polythene bag was introduced in the cage (30 × 30 × 30 cm) containing about 250 hungry female mosquitoes and mosquitoes were allowed to bite on back of the hand through black muslin cloth screen stuck over a small area (40 cm^2) cut in the polythene bag. Known quantity of the extracts and fractions of *V. negundo* were loaded on the muslin cloth to give a deposition rate of 0.08~1.5 mg/cm^2. The muslin cloth was first treated with the extract and solvent was allowed to evaporate before use. Control muslin cloth screen was treated with solvent alone. After introduction of the hand covered with the polythene bag with muslin screen in the mosquito cage, the number of mosquitoes landing and probing on the treated or control arm is counted for 2 min. and subsequent observations were made every hr till 6 hrs. Percentage repellency is calculated by using the following formula:

$$\% \text{ Repellency} = (1-A/B) \times 100$$

where A is number of mosquitoes landing/bites on treated surface and B is the number of mosquitoes landing/bites on solvent treated surface.

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