New Insecticidal Amides from Petroleum Ether Extract of Dried *Piper nigrum* L. Whole Fruits

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The petroleum ether extract of dried ground whole fruits of *Piper nigrum* L. afforded 20 compounds (1—20) including two new insecticidal amides named as pipnoohine (1), and pipyahyine (2), seven reported for the first time from this plant (12, 13, 15—17, 19, 20), and eleven known compounds (3—11, 14, 18). The structure of 1 has been elucidated as (2*E*,4*E*,12*Z*)-*N*-(4-methylpentyl)octadeca-2,4,12-trienamide and that of 2 as (2*E*,4*E*,11*E*)-12-(benzo[1,3]dioxol-5-yl)-*N*-(3-methylbutyl)dodeca-2,4,11-trien-amide through extensive ID-, 2D-NMR spectral studies and chemical reactions. The known compounds have been identified through comparison of their spectral data with those reported in literature. 1 and 2 exhibited toxicity at 35.0 and 30.0 ppm respectively against fourth instar larvae of *Aedes aegypti* L. by WHO method.

Key words *Piper nigrum* L.; pipnoohine; pipyahyine; pesticidal activity; *Aedes aegypti*

Piper nigrum L., (black pepper) belongs to the family Piperaceae. Various *Piper* species have been used as spice and in folk medicine due to the attributed physiological activities and thus bear a great commercial, economical and medicinal potential.[1—5] In search of plant-based potent insecticides,[6—10] current investigations on the petroleum ether extract of the dried whole fruits of *P. nigrum* have resulted in the isolation of two new, seven hitherto unreported from this plant and eleven known compounds. Compounds isolated from petroleum ether extract included stigmastanol (**3**),[11] β-sitosterol (**4**)[12] stigmasterol (**5**),[12] stigmastanol 3-*O*-β-D-glucopyranoside (**6**),[11,13] β-sitosterol 3-*O*-β-D-glucopyranoside (**7**),[13—16] [(2*E*,4*E*)-octadienoyl]-*N*-isobutylamide (**8**),[17] sarmentine (**9**),[18] [(2*E*,4*E*)-dodecadienoyl]-*N*-isobutylamide (**10**),[19] [(2*E*,4*E*)-dodecadienoyl]pyrrolidine (**11**) [4], hexadecanoic ethyl ester (**12**),[20] octadecanoic acid (**13**),[21] pellitorine (**14**) [18], hexadecanoylpyrrolidine (**15**),[22] [(2*E*)-octadecanoyl]pyrrolidine (**16**),[22] 1-[(2*E*,4*E*,12*Z*)-octadecatrienoyl]-*N*-isobutylamide (**17**),[23] piptaline (**18**),[24] 1-[7-(3,4-methylenedioxyphenyl)-(2*E*,4*E*)-heptadienoyl]-*N*-isobutylamide (**19**),[25] 1-(3,4-methylenedioxyphenyl)-(1*E*)-tetradecene (**20**),[18] using different chromatographic techniques. The toxicities of **8**—**11**, **14**—**17** and **19** were determined against fourth instar larvae of *Aedes aegypti* by WHO method[26] and were found to be 23.0, 27.0, 26.0, 29.0, 20.0, 75.0, 64.0, 29.0, and 13.0 ppm respectively. In continuation of our program towards obtaining pure compounds with potential biological activity from indigenous medicinal plants pesticidal activity against *Aedes aegypti* and *Anopheles stephensi* have been reported from this laboratory time to time.[27,28]

The EI-MS and HR-EI-MS of pipnoohine (**1**), showed the molecular ion peak at *m*/*z* 361 and 361.3353 (calculated 361.3345), respectively, accounting for the elemental composition of $C_{24}H_{43}NO$, possessing four degrees of unsaturation. The IR spectrum showed absorption bands for a secondary amide NH functionality (3275 cm^{-1}) and for amide C=O (1657 cm^{-1}). The UV absorption at 261.3 nm (ε=24500) suggested the presence of an unsaturated amide with extended conjugation. The ^{1}H-NMR spectrum supported the presence of conjugated dienamide by the presence of a d at δ (ppm) 5.73 (J=15.0 Hz, H-2), two dd at 7.15 (J=15.0, 9.5 Hz, H-3) and 6.13 (J=15.7, 9.5 Hz, H-4) and a dt at 6.03 (J=15.7, 6.7 Hz, H-5). These protons displayed HMQC connectivities with carbons at δ (ppm) 120.9 (C-2), 142.1 (C-3), 127.3 (C-4), and 140.5 (C-5) respectively. H-2 and H-3 also showed HMBC connectivities with 164.7 (C-1). These data indicated the presence of *trans*-$\alpha,\beta,\gamma,\delta$-double bonds conjugated with a carbonyl group. The signals for one more double bond were observed at δ 5.31 (2H, J=5.0 Hz, H-12, H-13) as a t in the ^{1}H-NMR (Table 1). These *cis*-olefinic protons showed HMQC interactions with carbons at δ 129.9 (C-12, C-13). The *cis* nature of isolated double bond was evident from the same chemical shift of H-12/H-13 and by the upfield ^{13}C-NMR shifts of C-11 and C-14 between δ 26.5—27.1, due to *cis* shielding.[29] The position of the isolated double bond at C-12/C-13 was determined by EI-MS and HR-EI-MS and confirmed by the reaction of **1** with OsO_4 and product analysis which yielded hexanal as identified in the reaction mixture by GC and GC/MS analysis[7,23] The mass

Table 1. ^{1}H- and ^{13}C-NMR Spectral Data of **1** (in $CDCl_3$)

No.	**1**	
	δ_C	δ_H (J=Hz)
1	164.7	—
2	120.9	5.73, d (15.0)
3	142.1	7.15, dd (15.0, 9.5)
4	127.3	6.13, dd (15.7, 9.5)
5	140.5	6.03, dt (15.7, 6.7)
6	32.3	2.15, q (6.7)
7—10, 15, 16	28.3—29.1	1.19—1.34, br s
11, 14	26.5—27.1	2.21, m
12, 13	129.9	5.31, t (5.0)
17	22.3	1.19—1.34, br s
18	14.1	0.86, t (6.7)
1′	46.3	3.14, q (6.5)
2′, 3′	28.3—29.1	1.41—1.47, m
4′	28.1	1.79, m
5′, 6′	19.5	0.90, d (6.7)
NH	—	5.43, br s

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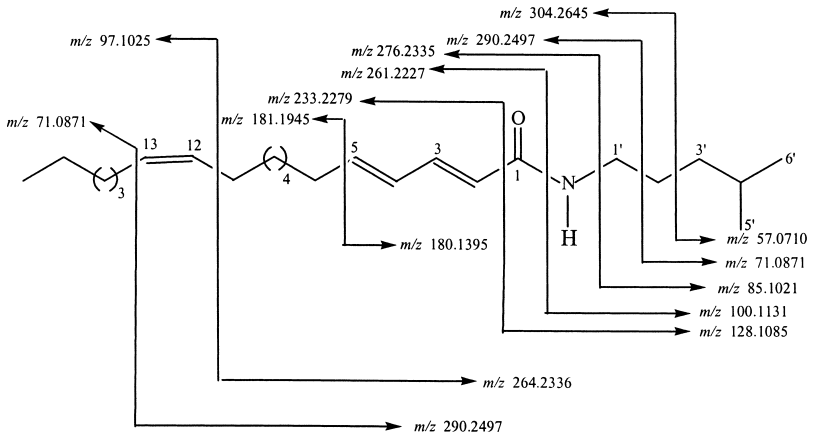

Fig. 1. Mass Fragments of **1**

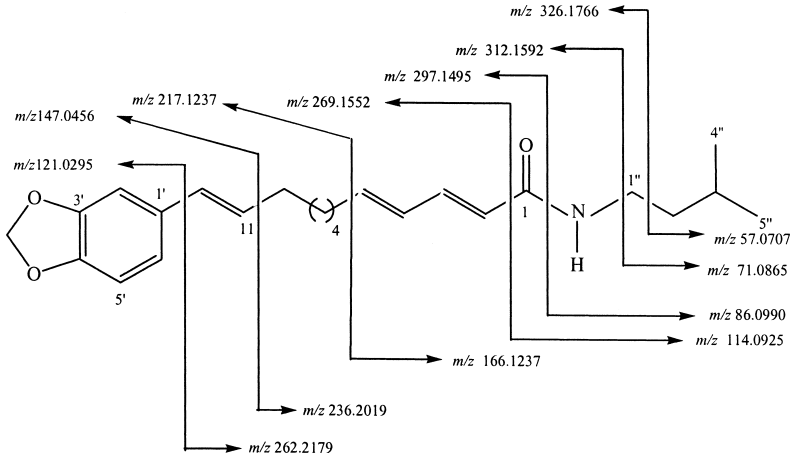

Fig. 2. Mass Fragments of **2**

fragments with a difference of 14 mass units were observed from *m/z* 180.1395 ($C_{11}H_{18}NO$) to *m/z* 264.2336 ($C_{17}H_{30}NO$) following *m/z* 290.2497 ($C_{19}H_{32}NO$) rather 292 also indicated the presence of a double bond at C-12.[7,23] The ^{1}H-NMR spectrum further showed a two-proton q at δ 2.15 (J=6.7 Hz, H-6). It had a cross peak at δ 32.3 in the HMQC spectrum. A broad s of fourteen protons ranging between δ 1.19—1.34 was attributable to CH_2 (7—10, 15—17). C-18 appeared at δ 0.86 as a t (J=6.7 Hz) and showed connectivity with a carbon at δ 14.1 in HMQC a carbon at δ 22.3 (C-17) in HMBC. Again HR-EI-MS, GC and GC-EI-MS analysis provided conclusive evidence in the favour of isohexyl amine moiety.[7, 30] The fragment ion peaks at *m/z* 71.0871 (C_5H_{11}), 85.1021 (C_6H_{13}), 100.1131 ($C_6H_{14}N$), 128.1085 ($C_7H_{14}NO$), 233.2279 ($C_{17}H_{29}$), 261.2227 ($C_{18}H_{29}O$) and 276.2335 ($C_{18}H_{30}NO$) clearly confirmed isohexyl amide moiety in the molecule. Additional support was obtained by ^{1}H- and ^{13}C-NMR spectrum by the signals of a q at δ 3.14 (J=6.5 Hz, H-1′), a broad s ranging between 1.41—1.47 (H-2′, H-3′), a m at 1.79 (H-4′), a d at 0.90 (J=6.7 Hz, H-5′, H-6′). The respective carbons for these functionalities were observed at δ 46.3, 28.3—29.1, 28.1 and 19.5 in the HMQC plot. Acid hydrolysis of **1** with concentrated HCl and work up[7,30] yielded (4-methylpentyl)amine, as found by GC (co-injection of authentic samples of similarly branched amines). In the light of above discussion, the structure of **1** was elucidated as (2*E*,4*E*,12*Z*)-*N*-(4-methylpentyl)octadeca-2,4,12-trienamide.

Pipyahyine (**2**) was obtained as white needles (petroleum ether–AcOEt (7 : 3)), mp 109—110.5 °C. The EI-MS showed molecular ion peak at *m/z* 383 and HR-EI-MS at *m/z* 383.2455 (Calcd. 383.2460) corresponding to the molecular formula $C_{24}H_{33}NO_3$ with nine unsaturations in the molecule. Four degrees of unsaturation were accounted for by the aromatic ring, one by the carbonyl function, while remaining by the three double bonds and methylenedioxyphenyl group (*vide* infra). The IR spectrum showed absorptions for a secondary amide N–H at 3333 cm^{-1}, an amide C=O at 1656 cm^{-1}, aromatic and aliphatic C=C ranging from 1615—1405 cm^{-1}. Characteristic absorption bands in the UV spectrum at 315 nm (ε=3500) were indicative of a conjugated benzo[1,3]dioxol moiety[31] and 265 nm (ε=38500) for conjugated amide C=O. A q in the ^{1}H-NMR spectrum at δ (ppm) 3.08 (J=6.5 Hz, H-1″), a m at 1.75 (H-3″) and a six-proton d at 0.85 (J=6.7 Hz, H-4″, H-5″) supported the presence of isopentyl group which was substantiated by fragments in HR-EI-MS at *m/z* 57.0707 (C_4H_9), 71.0865 (C_5H_{11}), 86.0990 ($C_5H_{12}N$), 114.0925 ($C_6H_{12}NO$), 269.1552 ($C_{18}H_{21}O_2$) and 297.1495 ($C_{19}H_{21}O_3$). The presence of isopentyl amide (3-methylbutyl amide) which was chemically confirmed by acidic hydrolysis of **2** with HCl to yield isopentyl amide (on usual workup) identified through GC and GC/MS.[7,30] The ^{1}H-NMR further showed two six-proton

Table 2. ^{1}H- and ^{13}C-NMR Spectral Data of **2** (in $CDCl_3$)

No.	**2**	
	δ_C	δ_H (J=Hz)
1	164.9	—
2	120.9	5.73, d (15.1)
3	141.9	7.13, dd (15.0, 10.1)
4	127.5	6.13, dd (15.5, 10.1)
5	139.8	6.04, dt (15.5, 6.7)
6	32.7	1.49—2.23, br s
7—9	28.7—31.5	1.19—1.31, br s
10	31.9	1.49—2.23, br s
11	128.9[a)]	6.01, dt (15.7, 6.7)
12	129.1[a)]	6.21, d (15.7)
1′	131.9	—
2′	104.9	6.82, d (1.3)
3′	146.5	—
4′	145.3	—
5′	107.8	6.65, d (8.0)
6′	119.7	6.71, dd (8.0, 1.3)
1″	46.5	3.08, q (6.5)
2″	28.7—31.5	1.49—2.23, br s
3″	28.3	1.75, m
4″, 5″	19.9	0.85, d (6.7)
–OCH_2O–	100.7	5.85, s
NH	—	5.44, br s

a) Assignments are interchangeable.

br singlets; one between δ 1.19—1.31 and other between 1.49—2.23 accounting for H-7, H-8, H-9 and H-2″, H-6, H-10 respectively. The respective carbons for these protons were observed between δ 28.7—31.5 C(2″,7,8,9), 32.7 C(6), 31.9 C(10) (BB, DEPT, HMQC, HMBC: Table 2). Two of the olefinic signals appeared as a d at δ 5.73 (J=15.1 Hz, H-2), and as a dd at 7.13 (J=15.1, 10.1 Hz, H-3) and were attributed to a *trans*-α,β-unsaturated carbonyl system. Further signals of dd were observed at δ 6.13 (J=15.5, 10.1 Hz, H-4) and of a dt at δ 6.04 (J=15.5, 6.7 Hz, H-5) which were attributed to the *trans*-oriented proton on a second double bond conjugated with Δ^2. The assignments of these protons were established by ^{1}H–^{1}H-COSY. The carbons correlated with these protons were observed at 120.9 (C-2), 141.9 (C-3), 127.5 (C-4), and 139.8 (C-5) ppm in the HMQC alongwith a quaternary carbon at δ 164.9 (C-1) in the broad-band decoupled ^{13}C-NMR spectrum. The ^{1}H-NMR spectrum of **2** (Table 2) also contained signals for a benzo[1,3]dioxol moiety conjugated with a double bond as manifested by two one-proton signals at δ 6.21 as a d (J=15.7 Hz, H-12) and at 6.01 as a dt (J=15.7, 6.7 Hz, H-11), and three aromatic protons at δ 6.82 (d, J=1.3 Hz, H-2′), 6.65 (d, J=8.0 Hz, H-5′) and at 6.71 (dd, J=8.0, 1.3 Hz, H-6′) in addition to a two-proton s at δ 5.85 manifesting the presence of a methylenedioxy moiety attached to an aromatic ring. These protons had cross peaks at δ 104.9 (C-2′), 107.8 (C-5′), 119.7 (C-6′) and δ 100.7 (OCH_2O) in the HMQC. The broad band decoupled ^{13}C-NMR spectrum also had signals for quaternary carbons at 131.9 C(1′), 146.5 C(3′) and 145.3 C(4′). These data led to assign the structure of pipyahyine **2** as (2*E*,4*E*,11*E*)-12-(benzo[1,3]dioxol-5-yl)-*N*-(3-methylbutyl)dodeca-2,4,11-trienamide.

1 and **2** exhibited toxicity at 35.0 and 30.0 ppm respectively against fourth instar larvae of *Ae. aegypti* L. determined by WHO method.

Experimental

General Experimental Procedures Column chromatography (CC): silica gel 9385 (Merck, 0.040—0.063 mm). Prep. TLC (PTLC): silica gel 60 PF_{254} (Merck). GC: Shimadzu-GC-9A gas chromatograph, FID at 260 °C, N_2 at 1.0 ml/min, SPB-5® capillary column (30 m×0.53 mm ID; 0.3 μ df); split ratio 1 : 30 injector temp. was 240 °C; the column temp. was maintained at 50 °C for the first 5 min and then raised to 240 °C (3 °C/min) followed by 5 min at 240 °C. UV spectra: Hitachi U-3200 spectrophotometer; λ_{max} in nm. IR spectra: Jasco A-302 spectrophotometer; ν in cm^{-1}. ^{1}H-NMR spectra (COSY, NOESY, and *J*-resolved): Bruker, 500 MHz; chemical shifts δ in ppm, coupling consants J in Hz, referenced to residual solvent signals. ^{13}C-NMR: Bruker, 125 MHz. The assignments of ^{1}H- and ^{13}C-NMR data (Tables 1, 2) are based on ^{1}H-, ^{1}H COSY-45, *J*-resolved, and ^{13}C-NMR (broad-band decoupled and DEPT), HMQC, and HMBC spectra. EI-MS: Finnigan-Mat-311A; source at 250 °C and 70 eV; *m/z* (%). HR-EI-MS: Jeol JMS-HX-110, source at 250 °C and 70 eV; *m/z* (%). Plates were visualized under UV light (254, 366)/I_2 vapors.

Extraction and Purification The black whole dried fruits of *P. nigrum* were purchased from the local market in Karachi. Air-dried, ground fruits of *P. nigrum* (5 kg) were extracted with petroleum ether (3×10 l) at room temperature for 72 h. Evaporation of the combined extracts *in vacuo* afforded a dark brown viscous residue (102.1 g). A portion (91.8 g) of this extract was partitioned between petroleum ether and 90% MeOH. The petroleum ether phase was dried (Na_2SO_4 anhydrous) and the solvent evaporated *in vacuo* to give a brownish syrupy concentrate (PEP, 63 g). A part (PEP, 21.0 g) was subjected to a silica gel CC which was eluted with petroleum ether>cyclohexane>cyclohexane/$CHCl_3$>$CHCl_3$>$CHCl_3$/AcOEt>AcOEt>MeOH in order of increasing polarity to obtain twenty five fractions. Fr-12 (1.8 g) was subjected to PTLC in solvent system petroleum ether–AcOEt (8.0 : 2.0) when **3** (2.9 mg) and **4** (3.1 mg) were obtained. Compound **5** (2.3 mg) was obtained on PTLC of fr-13 (cyclohexane–$CHCl_3$, 2 : 8 eluate; 2.3 g) in petroleum ether–AcOEt (7.5 : 2.5). Fr-15 ($CHCl_3$–AcOEt, 1 : 1 eluate; 9.4 g) was subjected to silica gel CC and eluted with petroleum ether>petroleum ether/AcOEt>AcOEt> $CHCl_3$>$CHCl_3$/MeOH>MeOH); to obtain fifteen fractions. Fr-3′ (petroleum ether–AcOEt, 8.5 : 1.5 eluate; 1.8 g) gave thirty five sub-fractions on small CC (silica gel) eluted with petroleum ether>petroleum ether/AcOEt>AcOEt. Fr-14″ (petroleum ether–AcOEt, 8.5 : 1.5 eluate; 54 mg) was purified on PTLC to get stigmastanol glucoside (2.7 mg) in solvent system petroleum ether–AcOEt (7.5 : 2.5). Fr-19″ (petroleum ether–AcOEt, 8 : 2 eluate; 79 mg) furnished **7** (1.9 mg) in solvent system petroleum ether–AcOEt (7.5 : 2.5), on purification on PTLC. Fr-21″ (petroleum ether–AcOEt, 7.8 : 2.3 eluate; 125 mg), fr-23″ (petroleum ether–AcOEt, 7.5 : 2.5 eluate; 239 mg) and fr-31″—35″ (petroleum ether–AcOEt, 7 : 3→6 : 4 eluate; 191 mg) all consisted of many bands when subjected to PTLC and finally furnished **8** (2.9 mg), **9** (3.5 mg), **10** (3.1 mg), **11** (2.5 mg), **12** (2.9 mg), and **13** (2.3 mg), in the order of polarity on repeated PTLC in solvent system petroleum ether–AcOEt (7.5 : 2.5→7.0 : 3.0). Fr-4′ (petroleum ether–AcOEt, 8 : 2 eluate; 0.2 g) on repeated PTLC in solvent system petroleum ether–AcOEt (7.5 : 2.5) gave **14** (7.3 mg) and **1** (15.3 mg) in order of polarity. Fr-5′ (petroleum ether–AcOEt, 7.8 : 2.3 eluate; 0.9 g) on repeated PTLC in solvent petroleum ether–AcOEt (7.5 : 2.5) yielded **15** (3.2 mg), **16** (4.1 mg), **17** (6.4 mg) and **18** (4.7 mg), in the order of polarity. Fr-6′ (petroleum ether–AcOEt, 7.5 : 2.5 eluate; 3.1 g) again on repeated PTLC in solvent petroleum ether–AcOEt (7 : 3) yielded **20** (5.6 mg), **19** (4.9 mg), and **2** (11.5 mg) in order of polarity.

Pipnoohine (=(2*E*,4*E*,12*Z*)-*N*-(4-methylpentyl)octadeca-2,4,12-trienamide; **1**): Amorphous powder; IR ν_{max}^{KBr} 3275, 2950, 1657, 1629, 1610, 1559, 1239, 1135 cm^{-1}; UV λ_{max}^{MeOH} 261.3 (ε=24500) nm. ^{13}C- (125 MHz, $CDCl_3$) and ^{1}H-NMR data (500 MHz, $CDCl_3$) Table 1. EI-MS 361 (39), 334 (15), 304 (12), 290 (41), 276 (35), 264 (19), 261 (21), 233 (15), 208 (25), 207 (19), 180 (65), 154 (25), 128 (100), 100 (27), 97 (69), 85(33), 71 (85), 57 (69). HR-EI-MS: 361.3353 (M^+, $C_{24}H_{43}NO$: calcd. 361.3345), 304.2645 $(C_{20}H_{34}NO)^+$, 290.2497 $(C_{19}H_{32}NO)^+$, 276.2335 $(C_{18}H_{30}NO)^+$, 264.2336 $(C_{17}H_{30}NO)^+$, 261.2227 $(C_{18}H_{29}O)^+$, 233.2279 $(C_{17}H_{29})^+$, 208.1699 $(C_{13}H_{22}NO)^+$, 207.2101 $(C_{15}H_{27})^+$, 181.1945 $(C_{13}H_{25})^+$, 180.1395 $(C_{11}H_{18}NO)^+$, 128.1085 $(C_7H_{14}NO)^+$, 100.1131 $(C_6H_{14}N)^+$, 97.1025 $(C_7H_{13})^+$, 85.1021 $(C_6H_{13})^+$, 71.0871 $(C_5H_{11})^+$, 57.0710 $(C_4H_9)^+$.

Pipyahyine (=(2*E*,4*E*,11*E*)-12-(benzo[1,3]dioxol-5-yl)-*N*-(3-methylbutyl)-dodeca-2,4,11-trienamide; **2**): White needles (petroleum ether–AcOEt (7 : 3)); mp 109—110.5 °C; IR $\nu_{max}^{CHCl_3}$ 3333, 3055, 2950, 1656, 1630, 1615—1405, 1239, 1135 cm^{-1}; UV λ_{max}^{MeOH} 315 nm (ε=3500), 265 (δ=38500) nm. ^{13}C- (125 MHz, $CDCl_3$) and ^{1}H-NMR data (500 MHz, $CDCl_3$) Table 2. EI-MS 383 (21), 326 (15), 312 (51), 297 (41), 269 (17), 262 (15), 243 (21), 236 (27), 166 (25), 161 (71), 147 (33), 140 (21), 135 (59), 131 (65), 121 (69),

114 (29), 103 (100), 86 (79), 71 (55), 57 (73). HR-EI-MS: 383.2455 (M^+, $C_{24}H_{33}NO_3$: calcd. 383.2460), 326.1766 $(C_{20}H_{24}NO_3)^+$, 312.1592 $(C_{19}H_{22}NO_3)^+$, 297.1495 $(C_{19}H_{21}O_3)^+$, 269.1552 $(C_{18}H_{21}O_2)^+$, 262.2179 $(C_{17}H_{28}NO)^+$, 243.1377 $(C_{16}H_{19}O_2)^+$, 236.2019 $(C_{15}H_{26}NO)^+$, 217.1237 $(C_{14}H_{17}O_2)^+$, 166.1237 $(C_{10}H_{16}NO)^+$, 161.0597 $(C_{10}H_9O_2)^+$, 147.0456 $(C_9H_7O_2)^+$, 140.1085 $(C_8H_{14}NO)^+$, 135.0447 $(C_8H_7O_2)^+$, 131.0509 $(C_9H_7O)^+$, 121.0295 $(C_7H_5O_2)^+$, 114.0925 $(C_6H_{12}NO)^+$, 103.0548 $(C_8H_7)^+$, 86.0990 $(C_5H_{12}N)^+$, 71.0865 $(C_5H_{11})^+$, 57.0707 $(C_4H_9)^+$.

Pesticidal Activity Rearing Technique: The 4th instar larvae of *Ae. aegypti* L. (O.T. wild strain), a yellow fever mosquito, were collected from semi-natural pond, especially established for this research work. The size of this pond was 8×4 feet with a depth of 2 feet. The egg strips of identified mosquito *i.e. Ae. aegypti* was dipped into the pond. The larvae in the pond were fed by dried and grinded prawns as a powder. The pond was covered with a net having small holes so that the inter mixing of other mosquito species may be avoided and the release of mosquitoes from pond into the environment may be checked. The pupae from the pond were collected daily and kept in mosquito cages for adult emergence. These adults were fed by Albino rats twice a week and the filter paper strips were kept in bowls of 6″ diameter. The egg strips were dried for one day and then dipped into the pond for larvae hatching.

Biological test (screening procedure): Ten young 4th instar larvae of *Ae. aegypti* were collected in 250 ml beaker having 5 ml of tap water separately and the beaker was filled upto the level of 200 ml. The fractions (or compounds) were tested at 28±1 °C at five final concentrations. The control and check were also set. The observation was recorded after 24 h.

Accurate Tests: The W.H.O. method was followed for the application. A group of 7 beakers was set up, five for different concentrations and one each for control and check, separately for *Ae. aegypti*. Each experiment was repeated five times. The experiment was discarded if the mortality was found more than 10% in control. The mortality was recorded after 24 h and readings were subjected to Abbot's formula.[32]

Calculations of LC_{50}: The lethal concentrations (LC_{50}) were calculated using PROBIT analysis for *Ae. aegypti*.[33]

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