*INT. J. BIOL. BIOTECH*., 3 (1): 81-85, 2006.

# TOXICITY OF VARIOUS FRACTIONS AND SUBFRACTIONS FROM FRUIT COATS OF NEEM (*AZADIRACHTA INDICA* A. JUSS.) AGAINST *ANOPHELES STEPHENSI* LISTON

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## ABSTRACT

The extract of fruit coats (RB-b) and its various fractions i.e. EtOAc phase of RB-b, RB-b 'A' (Acidic fraction of EtOAc phase), RB-b 'N' (Neutral fraction of EtOAc phase), PE 'S' (petrol-ether soluble fraction of RB-b 'N'), PE 'I' (petrol-ether insoluble fraction of RB-b 'N'), 'ES' (Ether soluble fraction of PE 'S') and 'EI' [Ether insoluble fraction of PE 'I', (Exclusively consisted of azadiradione (pure compound)] of neem (*Azadirachta indica* A. Juss.) were tested against the 4th-instar larvae of *Anopheles stephensi* Liston. The results of these were compared with permethrin (25 EC). The $LC_{50}$ value of the extract and its various fractions was found 290, 165, 142, 43, 159, 154, 106 and 15 ppm, respectively. The fraction 'ES' was further subfractionated into 13 VLC-fractions (A-M) and $LC_{50}$ values of these VLC-fractions were 100(A), 200(B), 150(C), 215(H), 107(I), 136(J), 212(K), 66(L) and 182(M) ppm, while that of permethrin used as standard, it was 0.120 ppm.

**Key words:** Toxicity, neem fruit-coats extract (RB-b), permethrin, *Anopheles stephensi* Liston.

## INTRODUCTION

A huge work has been done on chemical nature of different parts of neem tree (Chaterji and Ray, 1917; Troup, 1921; Lavie and Jain, 1967; Lavie *et al*., 1971; Butterworth and Morgan, 1971; Nakanishi, 1975; Ahmed *et al*., 1984; Khalid *et al*., 1986; 1989; Zebitz, 1987; Siddiqui *et al*., 1986, 1991a, b; Siddiqui *et al*., 1992; Naqvi and Schmidt, 1993; Ascher, 1993; Tariq *et al*., 1994; Muse *et al*., 1996; Siddiqui *et al*., 1999, 2000, 2002, 2003, 2004; Tariq *et al*., 2001, 2002, 2004) but only a little work has been reported on neem fruit coats, only from Pakistan and that is from the same source. Therefore in the present investigation a detail work was carried out on neem fruit coats (RB-b), its main fractions and sub-fractions and examined their effects against *A. stephensi*.

## MATERIALS AND METHODS

### Collection of mosquito larvae (*Anopheles stephensi*)

The fourth instar larvae of *Anopheles stephensi* Liston (Orangi Town wild-strain) were collected directly from the semi-natural environment in Orangi Town Pakistan. The size of this pond was 8x4 sq.ft., having a depth of 2 feet. The larvae in this pond were fed by dried prawns in grinded condition as a powder. The pupae from the pond were collected daily and kept in cages for research work and were not left to emerge in pond, to prevent the release of malaria mosquitoes in neighbouring environment. The pond was also covered with net so that the egg laying of other mosquitoes into the pond may be prevented to avoid the mixing of other species or genera. The female *Anopheles* mosquito in cage (16x12x12) were fed thrice a week by rat feeding at evening time. The male *Anopheles* mosquito were fed on 5% glucose solution soaked in cotton pad. The bowl of 6" diameter filled with pond water were kept in the cages, so that the female *Anopheles* may lay their eggs in these bowls. In the morning time these bowls were checked and the eggs were released into the pond to rear the mosquito progeny. Identification of mosquito species was done according to Amerasinghe *et al*. (2002).

### Toxicity determination

A group of ten (5 male, 5 female), fourth instar larvae of *A. stephensi* having uniform age and size was released in 250 ml beakers using WHO method (1970). The insects were treated with respective neem products, and standard (permethrin) having different concentrations. A set of seven beakers was set up for each neem product in duplicate, five for five different concentrations, one for check (ethanol) and one for control. For standard (permethrin), six beakers were set up, five for five different concentrations and one for control, but the 7th beaker for check was not

set up because permethrin was diluted in distilled water. Mortality counts were made after 24 hours. Each experiment was repeated 5 times. Moribund larvae were counted as dead. The observations were analysed statistically according to Abbot's formula (1925).

Average values were calculated and mortality curve was drawn on log-log graph paper to find out $LC_{50}$ by taking dose on x-axis whereas the percent mortalities on y-axis for each compound. Data was analysed statistically.

**Chemicals:**

The extract of neem fruit coats (RB-b) and its various fractions i.e. EtOAc phase of RB-b, RB-b 'A' (Acidic fraction of EtOAc phase), RB-b 'N' (Neutral fraction of EtOAc phase), PE 'S' (petrol-ether soluble fraction of RB-b 'N'), PE 'I' (petrol-ether insoluble fraction of RB-b 'N'), 'ES' Ether soluble fraction of PE 'S'), 'EI' [Ether insoluble fraction of PE 'I', (Exclusively consisted of azadiradione (pure compound)], and 13 VLC-fractions (A-M) were obtained from HEJ Research Institute of Chemistry, the International Centre for Chemical Sciences, WHO Collaborating Centre, Karachi, Karachi-75270, Pakistan.

One gram (1 gm) of each fraction was diluted in 100 ml ethanol, preparing 1% stock solution. The stock solution was diluted further if required.

## RESULTS AND DISCUSSION

The 4th-instar larvae of malaria vector mosquito *A. stephensi* Liston (Orangi Town wild-strain) were treated with the extract of neem fruit coats (RB-b) and its various fractions, i.e. EtOAc phase of RB-b, RB-b 'A' (Acidic fraction of EtOAc phase), RB-b 'N' (Neutral fraction of EtOAc phase), PE 'S' (petrol-ether soluble fraction of RB-b 'N'), PE 'I' (petrol-ether insoluble fraction of RB-b 'N'), 'ES' (Ether soluble fraction of PE 'S') and 'EI' [Ether insoluble fraction of PE 'I', (Exclusively consisted of azadiradione (pure compound)] of indigenous (Pakistani) neem tree (*Azadirachta indica* A. Juss.). The results of these were compared with permethrin (25 EC), used as standard. The larval mortality of *An. stephensi* gradually increased with the increase of dose. The $LC_{50}$ value of extract of neem fruit coats (RB-b), its fractions and subfractions are shown in the List # 1, including the standard (permethrin) as well.

Table 1. Various fractions and subfractions from the extract of fruit coats (RB-b) tested against 4$^{th}$-instar larvae of *Anopheles stephensi* Liston showing their $LC_{50}$ values.

**A. MAIN FRACTIONS**

| No. | Fractions | $LC_{50}$ (ppm) | No. | Fractions | $LC_{50}$ (ppm) |
|---|---|---|---|---|---|
| 1. | Permethrin (standard) | 0.120 | 6. | PE 'S' (Petrol-ether soluble fraction of RB-b 'N') | 159 |
| 2. | RB-b (Fresh fruit coats extract) | 290 | 7. | PE 'I' (Petrol-ether insoluble fraction of RB-b 'N') | 154 |
| 3. | EtOAc phase of FFC (RB-b) after liquid liquid partitioning. | 165 | 8. | 'ES' (Ether soluble fraction of PE 'S') | 106 |
| 4. | RB-b 'A' (Acidic fraction of EtOAc phase) | 142 | 9. | 'EI' (Ether insoluble fraction of PE 'I')(Exclusively consisted of azadiradione: 1) | 15 |
| 5. | RB-b 'N' (Neutral fraction of EtOAc phase) | 43 | | | |

**B. VLC-FRACTIONS FROM 'ES' AND THEIR $LC_{50}$ VALUES**

| | | | | | |
|---|---|---|---|---|---|
| 10. | Fraction 'A' | 100 | 15. | Fraction 'I' | 107 |
| 11. | Fraction 'B' | 200 | 16. | Fraction 'J' | 136 |
| 12. | Fraction 'C' | 150 | 17. | Fraction 'K' | 212 |
| 13. | Fraction 'D' through 'G' | * | 18. | Fraction 'L' | 66 |
| 14. | Fraction 'H' | 215 | 19. | Fraction 'M' | 182 |

* These fractions were mixture of four compounds therefore these fractions were not assayed.

Table 2. Toxic effects of extract of neem fruit coats alone (RB-b), its various fractions and subfractions as compared to permethrin, showing mean mortalities at different doses with SD value.

| Entry | Dose [ppm] | Mean mortality [% + SD]*) | Dose [ppm] | Mean mortality [% + SD]*) | Dose [ppm] | Mean mortality [% + SD]*) |
|---|---|---|---|---|---|---|
| | (1) permethrin | | (2) RB-b | | (3) EtOAc phase of RB-b | |
| 1 | 0.0312 | 22 ± 4 | 100.0 | 20 ± 7 | 80.0 | 20 ± 7 |
| 2 | 0.0625 | 36 ± 5 | 200.0 | 34 ± 5 | 120.0 | 34 ± 5 |
| 3 | 0.1250 | 52 ± 8 | 300.0 | 52 ± 4 | 160.0 | 42 ± 4 |
| 4 | 0.1875 | 66 ± 5 | 400.0 | 82 ± 4 | 200.0 | 66 ± 5 |
| 5 | 0.2500 | 77 ± 4 | 500.0 | 98 ± 4 | 240.0 | 82 ± 4 |
| | (4) RB-b'A' | | (5) RB-b 'N' | | (6) PE 'S' | |
| 1 | 125.0 | 36 ± 5 | 20.0 | 8 ± 4 | 80.0 | 22 ± 4 |
| 2 | 150.0 | 54 ± 5 | 30.0 | 28 ± 4 | 120.0 | 36 ± 5 |
| 3 | 175.0 | 72 ± 4 | 40.0 | 52 ± 8 | 160.0 | 44 ± 5 |
| 4 | 200.0 | 84 ± 5 | 50.0 | 62 ± 4 | 200.0 | 72 ± 4 |
| 5 | 225.0 | 92 ± 4 | 60.0 | 76 ± 5 | 240.0 | 88 ± 4 |
| | (7) PE 'I' | | (8) 'ES' | | (9) 'EI' | |
| 1 | 80.0 | 24 ± 5 | 75.0 | 44 ± 5 | 10.0 | 34 ± 5 |
| 2 | 20.0 | 36 ± 5 | 125.0 | 54 ± 5 | 15.0 | 50 ± 7 |
| 3 | 160.0 | 55 ± 5 | 175.0 | 66 ± 5 | 20.0 | 68 ± 4 |
| 4 | 200.0 | 74 ± 5 | 225.0 | 76 ± 5 | 25.0 | 84 ± 5 |
| 5 | 240.0 | 98 ± 4 | 275.0 | 98 ± 4 | 30.0 | 92 ± 4 |
| | (10) VLC-fraction 'A' | | (11) VLC-fraction 'B' | | (12) VLC-fraction 'C' | |
| 1 | 55.5 | 8 ± 4 | 110.2 | 2 ± 4 | 92.2 | 14 ± 5 |
| 2 | 74.0 | 26 ± 5 | 147.0 | 8 ± 4 | 123.0 | 22 ± 4 |
| 3 | 92.5 | 48 ± 4 | 183.7 | 36 ± 5 | 153.7 | 52 ± 8 |
| 4 | 111.0 | 78 ± 4 | 220.5 | 56 ± 5 | 184.5 | 76 ± 5 |
| 5 | 229.5 | 92 ± 4 | 257.2 | 82 ± 8 | 215.2 | 94 ± 5 |
| | (13) VLC-fraction 'H' | | (14) VLC-fraction 'I' | | (15) VLC-fraction 'J' | |
| 1 | 50.0 | 8 ± 4 | 50.0 | 28 ± 4 | 50.0 | 24 ± 5 |
| 2 | 100.0 | 14 ± 5 | 100.0 | 48 ± 4 | 100.0 | 36 ± 5 |
| 3 | 150.0 | 16 ± 5 | 150.0 | 72 ± 4 | 150.0 | 56 ± 5 |
| 4 | 200.0 | 44 ± 5 | 200.0 | 88 ± 4 | 200.0 | 62 ± 4 |
| 5 | 250.0 | 66 ± 5 | 250.0 | 96 ± 4 | 250.0 | 98 ± 4 |
| | (16) VLC-fraction 'K' | | (17) VLC-fraction 'L' | | (18) VLC-fraction 'M' | |
| 1 | 50.0 | 8 ± 4 | 25.0 | 20 ± 7 | 50.0 | 22 ± 4 |
| 2 | 100.0 | 14 ± 5 | 50.0 | 44 ± 5 | 100.0 | 34 ± 5 |
| 3 | 150.0 | 34 ± 5 | 100.0 | 62 ± 4 | 150.0 | 42 ± 4 |
| 4 | 200.0 | 46 ± 5 | 150.0 | 76 ± 5 | 200.0 | 56 ± 5 |
| 5 | 250.0 | 62 ± 4 | 200.0 | 96 ± 4 | 250.0 | 76 ± 5 |

Check for all = 2±4.48 and control = 0+0.

**Note:** VLC-fraction D,E,F&G were the mixture of various compounds therefore they were not assayed as such.

In the present investigation the work was carried out on the extract of neem fruit coats (RB-b) and its various fractions and subfractions as compared to permethrin (25 EC), against the 4$^{th}$-instar larvae of malaria vector mosquito *Anopheles stephensi* Liston (Orangi Town wild-strain). The extract of neem fruit coats alone (RB-b) was fractionated into various fraction i.e., EtOAc, Rb-b 'A', RB-b 'N', PE 'S', PE 'I', 'ES' and 'EI'. The $LC_{50}$ of RB-b (Mother fraction) and its fractions in sequence was 290,165,142,43,159,154,106 and 15 ppm. The fraction 'ES' was further fractionated into 13 subfraction (A-M). The $LC_{50}$ value of these fractions was found to be 100(A), 200(B), 150(C), 215(H), 107(I), 136(J), 212(K), 66(L) and 182 (M), whereas the $LC_{50}$ of D,E,F&G was not assayed as such because they were mixture of different compounds.

Zebitz (1987) reported the $LC_{50}$ value of neem seed kernel extract (NSKE), AZT-VR-K-E and MTB/$H_2O$-K-NR-E against four mosquito species but the $LC_{50}$ against *An. stephensi* was not reported because it was not tested (Please see Int. 3rd Neem Conf. Nairobi-1986 published in 1987, P. 555-573), but in the present work various fractions and subfractions from neem fruit coats alone were tested against *An. stephensi* Liston. The $LC_{50}$ value of these fractions were found as 290,165,142,43,159,154,106 and 15 ppm, whereas the $LC_{50}$ value of subfractions from 'ES' fraction were 100(A), 200(B), 150(C), 215(H), 107(I), 136(J), 212(K), 66(L) and 182 (M) ppm. Hence the present report is the 1st report on the activity of neem fruit coats against the 4th-instar larvae of malaria vector mosquito *A. stephensi* Liston. Naqvi and Schmidt (1993) reported the comparison of RB-a (also called FFS or SDS), RB-b (also called FFC or SDC) and margosan O™ against *Musca domestica* L. larvae. They reported that RB-b was more effective as compared to RB-a, and this neem fruit coats RB-b was nearly more or less equal to the margosan O™, a purified pure azadirechtin from RB-a i.e. from seed: (Kernel). It was the first report of neem fruit coats against the larvae of house flies *Musca domestica* L. In the present investigation neem fruit coats alone (extract) was tested against the 4th-instar alone (extract) was tested against the 4th-instar larvae of malaria vector mosquito *A. stephensi* Liston, as compared to a synthetic pyrethroid permethrin (25 EC). The $LC_{50}$ of RB-b and permethrin was found to be 290 and 0.120 ppm respectively.

Naqvi *et al*. (1994) reported the toxicity and IGR effects caused by neem fraction RBU-9 (whole ripe berries dried under UV light), FFC (RB-b) and margosan $O_{TM}$ against 4th-instar larvae of *Aedes aegypti* L. (PCSIR-strain). They reported the $LC_{50}$ of RBU-9 as 380, FFC as 490 ppm and that of margosan O™ 340 ppm, showing less effectiveness of RB-b (FFC) as compared to margosan O™. In the present work the $LC_{50}$ of RB-b against *A. stephensi* Liston was found to be 290 ppm as compared to earlier reporter i.e. 490 ppm. The difference in $LC_{50}$ may be due to the difference in mosquito genous, i.e. *Aedes aegypti* and *A. stephensi*.

Ahmed *et al*. (1996) reported the efficacy of RB-a and RB-b, in comparison with *Nerium indicum* and cypermethrin against Legume bug Piezodorus hybneri (Gmelin). The $LC_{50}$ for RB-a, RB-b, *N. indicum* and cypermethrin was found to be 3.6, 1.6, 0.937 and 0.0014%. In the present work, more advanced studies on RB-b were carried out due to the fact that it was found more effective than RB-a as in the above case.

Ahmed *et al*. (1998) reported the toxic effects of RB-a, RB-b and B.B (bakayan berries) against Ladybird beetle, (Coccinella spp), by filter paper impregnation method. The LC50 of RB-a, Rb-b and B.B was found to be 7.8, 7.4 and 2.6%.

Tariq *et al*. (2001) reported the toxic effects of RB-a and RB-b against yellow fever mosquito *Aedes aegypti* L., on its 4th instar larvae. They reported the $LC_{50}$ value of RB-a and RB-b as 446 and 319 ppm respectively. In the present studies further work was carried out on RB-b due to its more effectiveness as noted above.

Tariq *et al*. (2002) reported the comparative toxic effects of RB-a and RB-b against 4th-instar larvae of *Anopheles stephensi* Liston by W.H.O method. They reported the $LC_{50}$ of RB-a and RB-b as 784 and 290 ppm respectively. In present work more advanced studies were carried out due to the more effectiveness of RB-b as noted above.

Tariq *et al*. (2004) reported the toxicity of sixteen pure compounds from the fruit coats of neem tree against *Anopheles stephensi*, but in the present work the toxicity of various fractions and subfractions from the fruit coats have been described.

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(*Accepted for publication December 2005*)