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Combined Larvicidal Efficacy of Rhinacanthin-C, Luteolin, Quercetin, and Binary Mixtures of *Rhinacanthus nasutus*, *Andrographis paniculata* and *Vernonia cinerea* Extracts against *Aedes aegypti* Mosquito

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Abstract Phytochemicals have been shown to possess insecticidal activities against insects and mosquitoes. We tested toxicities of the mixtures of *Rhinacanthus nasutus*, *Andrographis paniculata* and *Vernonia cinerea* extracts together with their major compounds against *Aedes aegypti* mosquito, collected from Nakhon Pathom, Thailand. The mosquitoes were observed tolerant to pyrethroids and had increased activities of detoxification enzymes compared to the susceptible Bora strain, indicating detoxicification system could be responsible for tolerance to pyrethroid insecticides. Among the extracts, *R. nasutus* hexane extract showed the highest larvicidal activity toward *Ae. aegypti* fourth-instar larvae (LC_{50}=68.52 µg/ml). Synergism in toxicity was observed on binary mixtures of *R. nasutus* with *A. paniculata* hexane fraction and of *R. nasutus* hexane with *V. cinerea* ethyl acetate fraction. Mixtures of rhinacanthin-C and luteolin or quercetin flavonoids, the major compounds of *R. nasutus* and *V. cinerea*, respectively, revealed potent larvicidal activity. Effect of those extracts and compounds against detoxification enzyme activities suggested that the observed larvicidal synergism might be due to the combination of insecticidal property of rhinacanthin-C and inhibition of insecticide detoxification enzymes by the respective phytochemical compounds. This study should have an implication in development of eco-friendly strategy in resistance mosquito vector control.

Keywords: Rhinacanthin-C, Luteolin, Larvicidal synergism, phytochemical compounds, *Aedes aegypti*

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Introduction

Aedes aegypti is the main mosquito vector of many serious viral infectious diseases in human such as dengue fever, yellow fever, and Chikungunya viral diseases. Moreover, *Ae. aegypti* is also suspected to be incriminated in transmission of the Zika virus (Ayres, 2016; Marcondes and Ximenes, 2015). Though many approaches have been developed to control mosquitoes, the chemical-based protocol remains a dominant control tool for prevention of disease transmission and epidemic outbreaks both in household and agricultural areas despite their adverse environmental and harmful effects on non-target organisms. In addition, long-term use of chemical insecticides has created insecticide resistance in mosquito vectors, especially *Ae. aegypti* in many regions of the world (Jirakanjanakit *et al*., 2007a, b; Lliná s *et al*., 2010; Marcombe *et al*., 2012). A primary mechanism of insecticide resistance in mosquitoes has been indicated by the increase in detoxification of insecticides mediated by detoxification enzymes which are mixed function oxidases (MFOs) that include cytochrome P450 monooxygenases (P450s), glutathione S-transferases (GSTs), and α- and β-esterases (Hemingway and Ranson, 2000).

Alternatively, the naturally occurring products could be used as insecticides and insecticide synergists against mosquitoes, as they are considered safe for environments and are easily degraded. Insecticidal properties of plant extracts and their bioactive compounds have been extensively investigated on *Aedes aegypti* (Komalamisra *et al*., 2005; Shaalan *et al*., 2005). The mosquitocidal compounds isolated from plant extracts constitute diverse structural groups in which they could possess different in mode of action and using in combinations could thus produce higher toxicity (Geris *et al*., 2012; Kishore *et al*., 2014; Rongnoparut *et al*., 2016). Synergistic effects of mixtures of phytochemicals or plant extracts against insects have also been reported. Studies have shown that leaf extracts of *Andrographis echioides* and *Cadaba trifoliata* acted synergistically in toxicity against mosquito larvae (Rajkumar *et al*., 2012), and adillapiol compound isolated from *Piper aduncum* could synergize the insecticidal activity of the pyrethrum extract against the Colorado potato beetle (Liu *et al*., 2014).

Rhinacanthus nasutus (Acanthaceae) is a shrub commonly found in Southeast Asia and has been used in traditional medicine. Larvicidal activity of *R. nasutus* extract against *Ae. aegypti* has been reported (Kamaraj *et al*., 2008; Komalamisra *et al*., 2005). The plant predominantly comprises of naphthoquinone esters, namely rhinacanthins; some of rhinacanthins comprise cytotoxicity against *Spodoptera frugiperda* (Sf9) insect cells and inhibitory activities against the mosquito cytochrome P450 detoxification enzymes

(Kotewong *et al*., 2015; Pethuan *et al*., 2012). *Andrographis paniculata* (Acantaceae) is a traditional medicinal plant used in many countries that possesses diverse therapeutic properties (Chao and Lin, 2010). The plant extract has been reported toxicity against *Ae. aegypti* larvae and adults (Govindarajan, 2011; Govindarajan and Sivakumar, 2012). It has been reported that andrographolide diterpenoids and flavones are major bioactive components of *A. paniculata* ethyl acetate and hexane fractions, respectively (Chao and Lin, 2010; Kotewong *et al*., 2014). Extract of flavonoids from *A. paniculata* has shown larvicidal activity against the *Anopheles stephensi* mosquitoes (Gautum *et al*., 2013). Moreover, using Sf9 insect cell-based assays, the *A. paniculata* flavonoids displayed synergistic activity with cypermethrin pyrethroids (Kotewong *et al*., 2014). *Vernonia cinerea* (Asteraceae), a perennial herbaceous medicinal plant, contains flavonoids and sesquiterpene lactones as its active components (Toyang and Verpoorte, 2013). Flavonoids luteolin, quercetin and apigenin have been identified as predominant constituents in *V. cinerea* ethyl acetate fraction (Prasopthum *et al*., 2015). Larvicidal activity against *Culex quinquefasciatus* mosquito and antifeedant effect against lepidopterous insects of *V. cinerea* extract have been reported (Arivoli *et al*., 2011; Tandon *et al*., 1998).

In this study, we aimed to investigate toxicity effects individually or of the binary mixtures of the extracts or their major compounds of three medicinal plant species, including *R. nasutus*, *A. paniculata*, and *V. cinerea* against *Ae. aegypti* mosquitoes. In the present study, the mosquitoes were collected from Nakhon Pathom (NP) province in central Thailand and were found tolerant to cypermethrin and deltamethrin insecticides compared to the susceptible Bora strain. The inhibition effects on mosquito detoxification enzymes of possible active compounds were evaluated along with the extracts. The results of this study should be valuably implicated on eco-friendly strategy for vector control and the resistance management of *Ae. aegypti*.

Materials and methods

Mosquitoes

The *A*e. *aegypti* mosquito larvae collected from Mueang Nakhon Pathom District, Nakhon Pathom province, Thailand during 2013 (NP larvae) were reared under laboratory-controlled conditions at 28±2 ℃, 70-80% RH to the adult stage. The Bora (French Polynesia) *Ae. aegypti* strain, obtained from Laboratoirede Lutte Contreles Insectes Nuisibles (LIN/IRD), WHO Collaborating Center for Vector Control, Montpellier, France, was used as a

reference for susceptible population (Jirakanjanakit *et al*., 2007a). All mosquitoes were maintained in the insectarium at the Institute of Molecular Biosciences, Mahidol University, Nakhon Pathom province, Thailand.

Chemicals

Deltamethrin, cypermethrin, temephos, piperonyl butoxide (PBO), apigenin, luteolin, quercetin, and andrographolide were purchased from Sigma-Aldrich (USA). L-glutathione (reduced form) and 3,3'-5,5'-tetramethyl-benzidine (TMBZ) were purchased from SERVA Electrophoresis GmbH (Heidelberg, Germany). The compounds including α-naphthyl acetate, β-naphthyl acetate, *o*-dianisidinebis (diazotized) zinc double salt (Fast Blue B salt) and 1-chloro-2,4-dinitrobenzene (CDNB) were obtained from Fluka (USA). Commercial grade 95% ethanol (EtOH) and analytical grade *n*-hexane (Hex), ethyl acetate (EA) and absolute EtOH were supplied by RCI Labscan (Bangkok, Thailand).

Preparation of plant extracts

Aerial parts of *R. nasutus* (RN), *A. paniculata* (AP) and *V. cinerea* (VC) were obtained from the traditional medicine market, Bangkok, Thailand, and plant materials were identified and prepared to obtain crude EtOH extracts, Hex and EA fractionated extracts, as described (Kotewong *et al*., 2014; Pouyfung *et al*., 2014; Prasopthum *et al*., 2015). Briefly, dried aerial parts of each plant were macerated three times in 95% EtOH (1 kg in 10 liters) for 3 days and filtrated through a Buchner funnel with Whatman number 1 filter paper. The filtrate was subjected to rotary vacuum evaporator for removal of solvent. Crude EtOH extract obtained after solvent removing was fractionated based on polarity using Hex and EA solvent, yielding Hex and EA fractions, respectively. Compound purification to obtain rhinacanthin-C from RN Hex fraction and 5-hydroxy-7,8-dimethoxyflavone from AP Hex fraction, was performed according to procedures described previously (Kotewong *et al*., 2014; Kotewong *et al*., 2015; Pethuan *et al*., 2012). Purification of the two compounds employed silica gel column chromatography (silica gel 60; 0.063-0.200 mm, Merck, Germany), with successive elution using Hex/EA solvent system, thin layer chromatography (TLC), followed by HPLC analysis using Symmetry RP-18 column (3.9 x 150 mm, Waters, Ireland). All extracts and purified compounds were kept at -20 ℃ until use.

Larvicidal bioassay

Larvicidal bioassay was conducted according to WHO standard procedure with slight modification (WHO, 2005). Individual insecticides, plant extracts, or compounds were dissolved in absolute EtOH. Twenty healthy fourth-instar *Ae. aegypti* larvae were placed in disposable plastic cup containing 100 ml dechlorinated tap water. After an observation for mortality or any abnormalities of test larvae for 1 h, small, unhealthy or damaged larvae were removed and replaced. A desire amount of test substance was subsequently dispensed to each treatment cup. Five cups were set up for each concentration and vehicle control with equal number was tested in parallel for larvicidal bioefficacy. Each test was done in triplicates on different days, no food was fed to larvae during experiments. After 24 h, the observed mortality was recorded. Larvae with no sign of movement after mild touch with a glass rod, incapable of rising to the surface or not showing the characteristic diving reaction when the water was disturbed were counted as dead as described (WHO, 2005). Data from all replicates were pooled for analysis of 50% lethal concentration (LC_{50}) values by a log dosage–probit mortality analysis using StatPlus program version 5.9.8 (AnalystSoft Inc., USA).

In vivo synergistic larvicidal activity

Larvicidal activities of binary mixtures of plant extracts were evaluated for synergistic or additive toxicity effects. The RN Hex fraction (RN-Hex) was set as the main extract for combining with AP or VC extracts since it produced the most effective larvicidal activity among test extracts. Plant extract binary mixtures (RN-Hex+AP-Hex, RN-Hex+AP-EA, RN-Hex+VC-Hex, RN-Hex+VC-EA) in the ratios of 1:1, 1:2 and 2:1 were prepared and mixed thoroughly until dissolved in absolute EtOH. Larvicidal bioassay were performed using various concentrations of the mixtures (0-400 ppm) and LC_{50} values of each mixture were determined. Vehicle controls without extracts were set up in parallel. To determine synergistic potency of each of the binary mixture, the Loewe additivity equation (Loewe, 1953, Nelson and Kursar, 1999) was used. The equation expressed as $(d_x/D_x) + (d_y/D_y) = 1$, where d_x and d_y were concentrations of extracts X and Y, respectively, in the mixture that gave 50% larval mortality, while D_x and D_y were the concentrations of each independent extract that gave 50% larval mortality. The sum of the two ratios represents as potency ratio (PR), where PR=1 indicates additive effect, PR<1 indicates synergism, and PR>1 indicates antagonism.

Evaluation of the synergistic effect of representative test compounds including andrographolide (found in AP-EA), luteolin and quercetin (found in VC-EA) with RN-Hex or the purified rhinacanthin-C (the major compound of RN-Hex) was conducted. Mosquito larvae were treated with binary mixtures of RN-Hex or rhinacanthin-C with each of test compounds at approximately the concentration of LC_5 values against NP larvae in each treatment to obtain countable mortality that would be less than 100%.

Biochemical enzymatic assays

Biochemical enzymatic assays were done according to the protocol reported previously (Brogdon, 1984; Pethuan *et al*., 2007; Polson *et al*., 2011). Fifty larvae of NP or Bora mosquitoes were homogenized in ice-cold 100 mM potassium phosphate buffer pH 7.2. The homogenates were centrifuged at 10,000 x g for 15 min at 4 ℃ to obtain the supernatant which were made up to 50 ml total volume and were used as the enzyme source. Total protein was determined using Bradford dye with bovine serum albumin as a standard. Enzymatic assays were performed in the total 300 µl reaction mixture in 96-well plate at room temperature and absorption was measured using Multiskan EX microtiter plate reader (Thermo Labsystems, Finland). For MFOs assay, larval homogenate was incubated with 200 µl of 2 mM TMBZ and 25 µl of 3% hydrogen peroxide for 5 min and the absorption was measured at 620 nm. Nonspecific α- and β-esterases activities were similarly assayed by addition of 100 µl of 3 mM α- or β-naphthyl acetate into the homogenate and incubated for 10 min, then 100 µl of 2 mM fast blue B salt was added. The reaction was further incubated for 2 min before the absorption at 540 nm was measured. For GSTs, 100 µl of 2 mM reduced glutathione and 100 µl of 1 mM CDNB were mixed with larval homogenate and absorption at 340 nm was read at time 0 and 5 min.

In order to determine the effect of plant extracts and test compounds against larval detoxification enzymes, reagents for each enzymatic assay were placed into each well prior to addition of each test plant extract or compound at desire concentration. Larval homogenate was introduced into each well to start the reaction. The absorption of each reaction was subtracted with the absorption of reaction without homogenate for normalization of non-enzymatic changes. Reaction without any test extract or compound was performed as control enzymatic reaction and was set as 100 percent activity. All tests were done in triplicates.

Statistical analysis

Significant differences of data were evaluated by *t*-test or one-way analysis of variance (ANOVA) with Dunnett's Multiple Comparison Test using GraphPad Prism 5 program (GraphPad Co. Ltd., USA).

Results

Larvicidal bioassay performed on the fourth-instar NP larvae showed that the mosquitoes were tolerant to cypermethrin pyrethroids compared to the susceptible Bora reference strain, as shown by higher LC_{50} values of NP compared to Bora (Table 1). The LC_{50} values of cypermethrin were approximately 3 folds higher in NP compared to Bora (RR=2.85). Activities of MFOs, α- and β-esterases, and GSTs enzymes of NP larvae were significantly higher (approximately 20-60%) than those of Bora ($P<0.05$; *t*-test) (Figure 1).

Toxicities of RN, AP and VC extracts against NP larvae are shown in Table 2. The RN-Hex displayed highest toxicity with LC_{50} value of 68.52 µg/ml, while EA fractions of RN and AP, as well as VC extract and fractions were not toxic against NP larvae.

Table 1. Insecticidal toxicity against 4th instar *Ae. aegypti* upon 24 h exposure

Insecticides	**Mosquito**	**LC_{50} (LCL–UCL)[1/]**	**χ^2 (df=4)**	**RR[2/]**
Cypermethrin	NP	0.97 (0.82–1.16)	1.61	2.85
	Bora	0.34 (0.30–0.39)	2.53	–
Deltamethrin	NP	0.65 (0.59–0.72)	1.20	1.35
	Bora	0.48 (0.43–0.53)	2.54	–
Temephos	NP	14.55 (13.80–15.36)	1.73	1.06
	Bora	13.69 (12.73–14.79)	1.98	–

[1/]LC_{50} represents lethal concentration that kills 50% of the exposed larvae, expressed in ng/ml, LCL means lower confidence level, UCL means upper confidence level
[2/]Resistance Ratio (RR): LC_{50} of NP larvae/ LC_{50} of susceptible Bora larvae

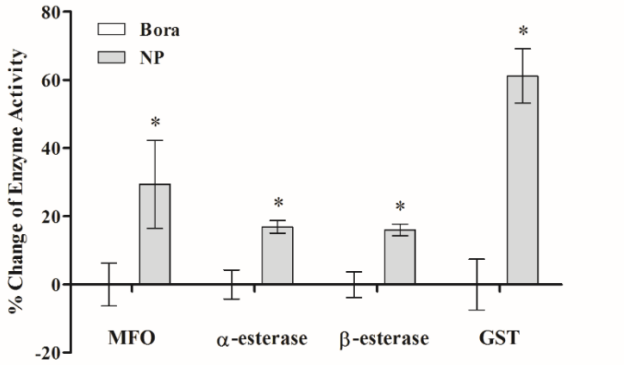

Figure 1. Percent change in activity of detoxification enzymes, including MFOs, α-esterase, β-esterase and GSTs, of untreated fourth-instar NP larvae relative to larval enzyme activities of susceptible Bora which were set as zero baselines. Asterisk (*) indicates significant difference compared to the Bora enzyme activity (*t*-test, $P<0.05$). Data are means ± SDs of triplicate experiments.

Table 2. Toxicity of plant extracts against NP larvae upon 24 h exposure

Plant	Extract	LC_{50} (LCL–UCL) [1/]	χ^2 (df=4)
R. nasutus	Crude EtOH	116.75 (109.49–123.67)	2.07
	Hex	68.52 (65.65–71.34)	1.39
	EA	> 400	–
A. paniculata	Crude EtOH	364.86 (350.98–379.91)	2.66
	Hex	365.20 (341.56–393.55)	2.27
	EA	> 400	–
V. cinerea	Crude EtOH	> 400	–
	Hex	> 400	–
	EA	> 400	–

[1/] LC_{50} represents lethal concentration that kills 50% of the exposed larvae, expressed in μg/ml, LCL means lower confidence level, UCL means upper confidence level

Binary mixtures containing RN-Hex extract combined with AP or VC fractions were further investigated their larvicidal activities and synergistic toxicities toward NP larvae. As shown in Table 3, AP-Hex and VC-EA synergized in toxicity with RN-Hex. Synergism of binary mixtures of the fractions at different ratios was also observed (Table 3). However, AP-EA and VC-Hex showed no synergism effect at all ratios tested.

Upon treatment of NP larvae with mixtures of RN-Hex and selected major compounds found in VC-EA including luteolin and quercetin, at approximately concentrations of LC_5 values, percent observed mortality of the combination was approximately 8.2 and 5.5 folds higher than those expected, respectively (Table 4). In contrast, this effect was not found when andrographolide (found in AP-EA) was in the mixture with RN-Hex (Table 4). Since rhinacanthin-C was reported as major component in RN-Hex, we thus purified and tested for its larvicidal activity against NP larvae. The results showed that rhinacanthin-C had LC_{50} values of 33.48 and 8.64 µg/ml against NP and Bora larvae, respectively (data not shown). When using rhinacanthin-C at about LC_5 value, in combination with luteolin or quercetin, the percent observed mortality was increased up to 88.0 ± 2.8 and 65.0 ± 9.4, respectively (Table 4).

Table 3. Synergistic larvicidal effects of binary mixtures of RN, AP and VC extracts against NP larvae upon 24 h exposure.

Mixtures	**Ratio**	**LC_{50} (LCL–UCL), (µg/ml)**	**Potency ratio (PR) [1/]**	**Effects**
RN-Hex	–	68.52 (65.65–71.34)	–	–
+ AP-Hex	1:1	66.68 (61.84–72.04)	0.58	Synergism
	1:2	81.91 (79.37–84.74)	0.55	Synergism
	2:1	63.29 (61.40–65.20)	0.67	Synergism
+ AP-EA	1:1	149.45 (142.69–156.26)	1.28	Additivity
	1:2	172.20 (166.11–178.48)	1.12	Additivity
	2:1	93.11 (89.56–96.91)	0.98	Additivity
+ VC-Hex	1:1	124.73 (118.66–131.11)	1.07	Additivity
	1:2	184.39 (176.46–192.75)	1.20	Additivity
	2:1	105.38 (101.11–109.95)	1.11	Additivity
+ VC-EA	1:1	65.22 (62.61–67.82)	0.56	Synergism
	1:2	77.53 (74.19–80.90)	0.51	Synergism
	2:1	63.36 (61.58–65.17)	0.67	Synergism

[1/] Potency ratio (PR) is determined from Loewe additivity equation (see Materials and Methods section). D_y for AP-EA, VC-Hex, and VC-EA is the value of 400 µg/ml)

Table 4. Combined larvicidal effects of RN-Hex or rhinacanthin-C with andrographolide, luteolin or quercetin against NP larvae upon 24 h exposure.

Treatment [1/]		% Observed mortality [2/]
RN-Hex		5.0 ± 3.6
Rhinacanthin-C		2.0 ± 2.7
Andrographolide		1.0 ± 2.2
Luteolin		6.0 ± 4.2
Quercetin		7.0 ± 2.7
RN-Hex	+ Andrographolide	7.0 ± 2.7
	+ Luteolin	90.0 ± 3.6
	+ Quercetin	66.0 ± 10.8
Rhinacanthin-C	+ Andrographolide	3.0 ± 2.7
	+ Luteolin	88.0 ± 2.8
	+ Quercetin	65.0 ± 9.4

[1/]Concentrations used for RN-Hex, rhinacanthin-C, luteolin, quercetin, and andrographolide were 50, 16, 10, 60, and 35 µg/ml, respectively.
[2/]Data are means ± SD of triplicates

We further tested whether the observed synergism of larvicidal toxicity was a result of inhibition on mosquito detoxification enzyme system. As displayed in Figure 2a, RN-Hex, AP-Hex, VC-EA and their compounds including rhinacanthin-C of RN-Hex, 5-hydroxy-7,8-dimethoxyflavone of AP-Hex, apigenin, luteolin, and quercetin of VC-EA exhibited inhibition activity toward MFOs of NP larvae *in vitro*. However, andrographolide from AP-EA did not show inhibition effect against MFOs, similar to that of AP-EA. The VC-EA and its flavonoid luteolin slightly inhibited α-esterase activity by approximately 20% while other test fractions and compounds showed less inhibition (Figure 2b). For β-esterase activity, luteolin and quercetin flavonoids exerted strongest inhibition effect, while rhinacanthin-C showed slight inhibition effect, in accordance to the results of their respective plant sources (Figure 2c). Reduction of GSTs activity was generally observed by all plant extracts and most compounds tested except PBO, 5-hydroxy-7,8-dimethoxyflavone and apigenin which showed less than 20% inhibition (Figure 2d).

Taken together, the major flavonoids of VC-EA including luteolin and quercetin potentially inhibited NP larval detoxification enzymes tested in this study, especially MFOs and β-esterase, while andrographolide generally showed less inhibition effect. These results suggested that synergism of VC-EA with RN-Hex observed could be partly due to luteolin and quercetin in VC-EA. Thus both compounds might also be able to synergize the toxicity of RN-Hex.

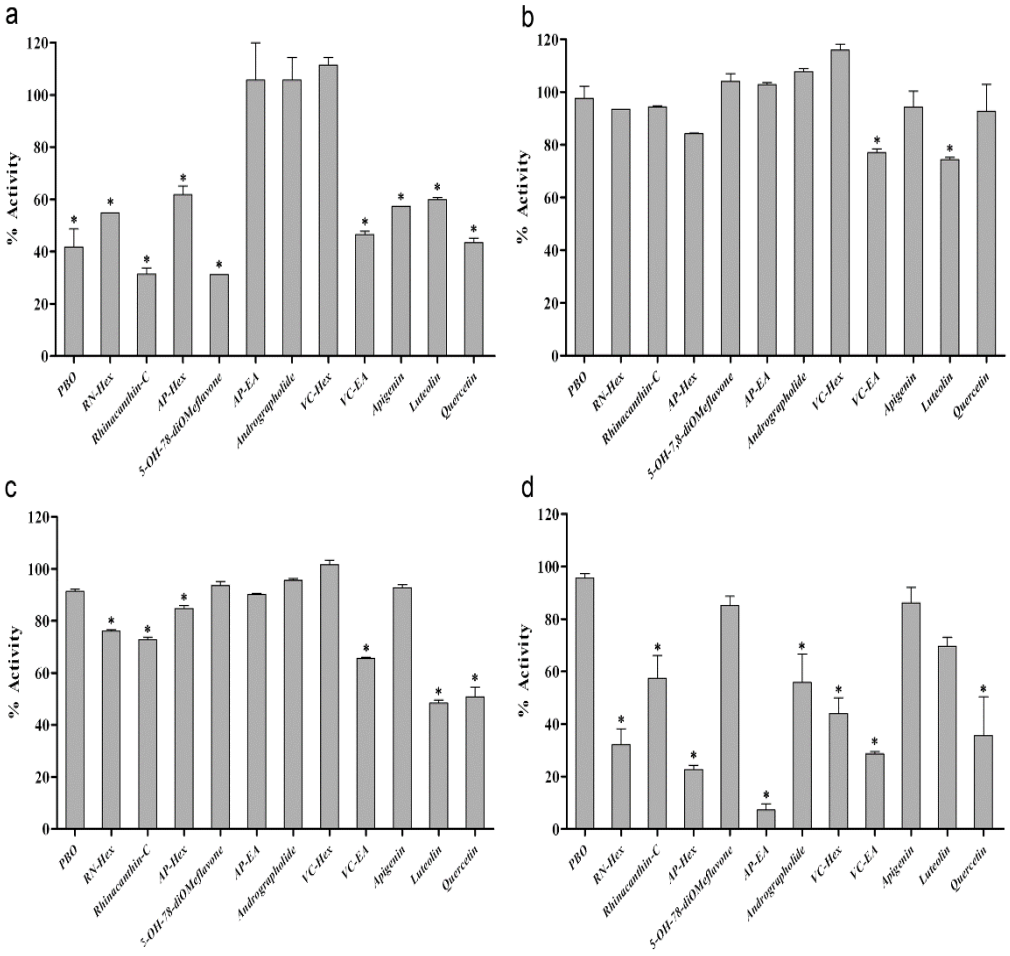

Figure 2. Effect of plant extracts and test compounds on activities of *Ae. aegypti* larval detoxification enzymes *in vitro*, including MFOs (a), α-esterase (b), β-esterase (c) and GSTs (d). Pool of fifty untreated NP larval homogenate was used as enzyme source. Final concentrations of plant extracts and test compounds used in the reaction were 50 and 15 µg/ml, respectively. PBO at final concentration of 5 µg/ml was used as a control inhibitor of MFOs. The bar indicates percentage of enzyme activity relative to the control reaction (vehicle control) which was set as 100% activity. Data are means ± SDs of triplicate experiments. The asterisks (*) refer to significant differences compared to the control reaction of each assay (ANOVA, $P<0.01$).

Discussion

In the present study, we observed that an *Ae. aegypti* mosquito NP sample was tolerant to cypermethrin pyrethroids and expressed higher activities of MFOs, α-esterase, β-esterase and GSTs detoxification enzymes than the Bora reference strain, suggesting that increased activity of the detoxification defense enzymes could be the underlying tolerance mechanism to pyrethroids in this NP larvae.

Among the three plant extracts tested, RN-Hex fraction was superior in toxicity. In *R. nasutus*, naphthoquinone esters were found (Wu *et al*., 1998) and under our compound purification conditions, we obtained rhinacanthin-C as the major *R. nasutus* constituent, with the amount of approximately 6% found in crude EtOH extract (Pouyfung *et al*., 2014). In the present study, the results showed that rhinacanthin-C was active for larvicidal activity. Compounds comprising of the naphthoquinone core structure such as isoshinanolone, plumbagin, hydroxyplumbagin, lapachol, methyldroserone, and maritinone were reported insecticidal activity toward fourth-instar *Ae. aegypti* mosquitoes with LC_{50} ranging from 1 to > 40 ppm (Ribeiro *et al*., 2009; Sreelatha *et al*., 2010).

Due to its naphthoquinone core structure, rhinacanthin-C might participate in inhibition on mitochondrial complex III of the respiratory system, as has been reported for naphthoquinones (Jewess *et al*., 2002). Moreover, rhinacanthin-C and other rhinacanthins purified from *R. nasutus* have shown ability to inhibit the *Anopheles minimus* mosquito P450s (Kotewong *et al*., 2015; Pethuan *et al*., 2012). Extracts of *V. cinerea*, in this study, were non-toxic to NP larvae with LC_{50} values > 400 ppm. Low toxicity of VC-EA extract has been reported in *Culex quinquefasciatus* with LC_{50}> 1500 ppm (Arivoli *et al*., 2011). However, acetone extract of *V. cinerea* showed effective toxicity (LC_{50} of 64.57 ppm) against *Ae. albopictus* in laboratory (Yadav *et al*., 2014).

Synergistic larvicidal activity was found upon treatment of NP larvae with RN-Hex fraction with either AP-Hex or VC-EA fraction. These results suggest that constituents of AP-Hex and of VC- EA play role in synergistic action with RN-Hex. Flavonoids such as apigenin, luteolin and quercetin have been reported as component of AP-Hex and VC- EA. Moreover, apigenin, luteolin, quercetin, and 5-hydroxy-7,8-dimethoxyflavone displayed inhibition effect on *Ae. aegypti* MFOs, while only luteolin and quercetin notably could also potentially inhibit β-esterase enzyme in this study. Previous observation of inhibition effect of apigenin and 5-hydroxy-7,8-dimethoxyflavone on the heterologously expressed P450 enzymes of the *An. minimus* mosquito has been reported (Kotewong *et al*., 2014), suggesting that these flavonoids might

function on modulation of detoxification enzymes of both *Aedes* and *Anopheles* mosquito species. Luteolin and quercetin exhibited broad inhibition activity against detoxification enzymes of *Ae. aegypti* larvae as observed in this study. In addition, inhibition by taxifolin flavonoid on Colorado potato beetle esterases has been documented and the compound could increase insect mortality upon exposure to flavonoid combined with the organophosphorus insecticide (Wang *et al*., 2016).

Purified rhinacanthin-C in combination with either luteolin or quercetin increased the mortalities of NP larvae suggesting that both flavonoids may have synergism in toxicity with rhinacanthin-C. These results indicated that these major constituents of both RN and VC extracts is one of the compound responsible for the observed synergistic toxicity. In contrast, combination of rhinacanthin-C and andrographolide did not show any sign of synergism towards NP larvae, in agreement with low or absence of inhibition on mosquito detoxification enzymes.

In this context, the observed synergistic toxicity of rhinacanthin-C and luteolin or quercetin flavonoids could be due to the toxicity of rhinacanthin-C together with inhibition on detoxification enzymes of such flavonoids thus synergize in killing mosquito larvae. In summary, the active plant compounds reported herein could have an implication and could be considered as an alternative for eco-friendly mosquito control.

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