



## *Trial of Neem Oil (Azadirachta indica) as Basic Compound of Electric Liquid Vaporizer against Aedes aegypti Mortality*

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**KEYWORDS** *Aedes aegypti; electric; vaporizer; neem; azadirachta indica*

**ABSTRACT** *Dengue Hemorrhagic Fever (DHF), commonly caused by Aedes aegypti mosquito bites, has been one of the world's major concerns for its progressively increasing incidence. To prevent further increase in DHF incidence, an effective yet safe vector control method is needed. One of the most common methods of vector control in Indonesia is using electric liquid vaporizer. Neem trees (Azadirachta indica) could be easily found in many areas in Indonesia, mainly functioning as shading trees. Leaves and seed of neem tree contain active compound used as natural insecticides, azadirachtin. The research aims to analyze killing effect of neem oil as basic compound of electric liquid vaporizer against Aedes aegypti. Research objects were 275 Aedes aegypti mosquitoes, aged 2-5 days. Objects were divided into 3 groups: group A (negative control), group B (100% neem oil), and group C (0.001% d-allethrin as positive control). Knockdown time (KT<sub>1</sub>, KT<sub>50</sub>, KT<sub>95</sub>, KT<sub>100</sub>) and 24-hours mortality were observed. Test replications were done 3 times. The results showed that pure neem oil (Azadirachta indica) has no direct killing effect against Aedes aegypti. Further research is encouraged regarding identification of adulticide characteristics of azadirachtin and other active compounds of neem oil, such as nimbin and nimbidine.*

### **INTRODUCTION**

Dengue Haemorrhagic Fever (DHF) has been one of leading public health problems in South-East Asia, including Indonesia (WHO, 2011; Brady et al., 2012; Caballero-anthony et al., 2015). The disease is commonly found in tropical and subtropical areas,

commonly transmitted to humans by mosquito bites (WHO, 2015). The virus brought by its mechanical vectors, *Aedes aegypti* and *Aedes albopictus*, will cause DHF to occur (CDC, 2010).

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The disease itself may cause negative impacts to human beings, mainly hospital expenses, lost of productivity, even death (Lloyd, 2003; Brady et al., 2012; Caballero-anthony et al., 2015). Since 2011, patients with DHF found in Yogyakarta, Indonesia were 460 people, and 2 people among those patients were dead. In 2012, patients suffering from DHF found in Yogyakarta were 971 people with CFR of 0.21, being the highest number until present (Yogyakarta Health District Office, 2013). If the vector control method is not aggressively applied, further increase in casualties may not be avoided.

Methods of DHF vector control need several aspects to put into consideration before the application: patient data, house location and surrounding area, physical and biological environment, biological agent, the method itself, as well as the expenses (Sugito, 1989; Bhatt et al., 2013). In determining what method of vector control to apply, one should focus on perspectives to cut in disease transmission cycle, either the host, the agent, or the environment (Soedarto, 1990). These approaches could be done by avoiding direct contact with mosquitoes, reducing the source of mosquito breeding place, killing the larvae, or killing adult mosquitoes (Soedarto, 1990; Bhatt et al., 2013). Personal protection from mosquito bites has become major approach for vector control, since it is practical and an economical means of preventing disease transmission to humans (Kiplang'at and Mwangi, 2013).

Common vector control methods used in Indonesia are mosquito bednets, fogging, application of mosquito repellents, mosquito coils, and electric liquid vaporizer (Sutanto and Purnomo, 1990). In order to obtain

a less toxic effect to humans and for a less resistance-producing effect against mosquitoes, one alternative approach is to apply neem oil as a basic compound in mosquito repellent which could be obtained from various parts of neem tree (*Azadirachta indica*) (Valecha et al., 1996; Shivakumar and Kataria, 2011; Mandal, 2011). Neem trees are mostly seen in moderately arid areas in Indonesia as shading trees (Mardiswojo and Rajakmangunsudarso, 1985).

Leaves and seed of neem tree may contain active compound used as natural insecticides, azadirachtin (Aremu et al., 2009; Maia and Moore, 2010). Neem leaves or seed can be simply processed to obtain neem oil, which is commonly used as plant pesticides and insect repellents in African countries (Asogwa et al., 2009). Neem oil is found to be effectively used as a potent larvicide of *Aedes aegypti* (Ndione et al., 2007) and *Culex pipiens* (Alouani et al., 2009), as well as adult *Aedes aegypti* mosquito repellent in cream formulation (Aremu et al., 2009; Kiplang'at and Mwangi, 2013).

Based on its various use in insect control methods worldwide, research on killing effect of neem oil against adult mosquitoes then should be encouraged, especially for *Aedes aegypti* causing DHF. As one of the most common methods of vector control in Indonesia is using electric liquid vaporizer, the electric liquid vaporizer would be a suitable tool to put the trial on, since the preparation of pure neem oil is simple and could be used right away. The research aims to analyze killing effect of 100% neem oil as basic compound of electric liquid vaporizer against *Aedes aegypti* using data of mosquito knockdown time. After the results and the killing effect of neem oil against *Aedes aegypti* is pronounced, the

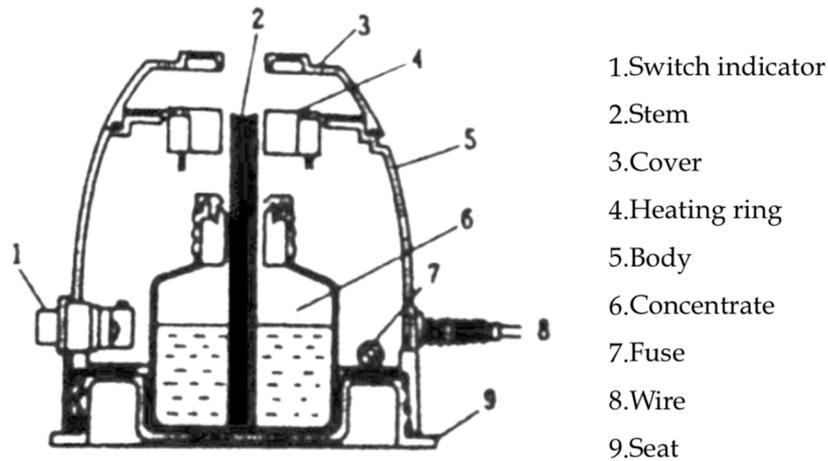
research may also be used as a point to put into consideration in determining future DHF vector control method.

## MATERIALS AND METHODS

The research was a quasi-experimental study with posttest-only control group design and was conducted in Laboratory of Entomology, Department of Parasitology Faculty of Medicine Universitas Gadjah Mada Indonesia on January 2013. The research objects were *Aedes aegypti* mosquitoes aged 2-5 days, divided into 3 groups: group A for negative control group (aquadest), group B for neem oil group (pure, 100% neem oil), and group C for positive control group (synthetic pyrethroid, 0.001% d-allethrin). Neem seeds are obtained from neem fruits harvested during dry monsoon in Bali, Indonesia. The neem seeds are woven dried and then feed into the oil-extracting machine, as in mechanical pressing method. The neem oil is obtained by pressing it mechanically and collected in a drum. Thus filtration is done to remove the various unwanted particles left in the extracted oil in order to obtain pure neem oil. More than 70% of neem oil content is azadirachtin, a bioactive compound in vector control and can also be utilized as natural insecticide (Quarles, 1994; Thacker, 2002; Aremu et al., 2009; Maia and

Moore, 2011). Using Federer formula (Federer, 2013) and previous research regarding use of mosquito repellents (Kesetyaningsih, 2008), 25 mosquitoes were used for each group.

The research procedure was performed according to Guomin, et al. (Guomin et al., 2003). Suitable objects were moved into mosquito cage sized 20 cm<sup>3</sup> with mosquito aspirator and were put in test room previously exposed with vapor produced from standardized electric liquid vaporizer (Fig. 1) for 20 minutes. The test room used in this research was an airtight, small, dark room sized 18 m<sup>3</sup> to enhance the mosquitoes to be actively flying in the utmost suitable condition. A medium-sized electric fan was turned on during the 20-minutes electric liquid vaporizer exposure so that the vapor would be dispersed evenly throughout the test room. In each group, as many as 25 *Aedes aegypti* mosquitoes underwent the trial and the knockdown time (KT<sub>1</sub>, KT<sub>50</sub>, KT<sub>95</sub>, KT<sub>100</sub>) were obtained. Mosquito mortality was confirmed by observing the 24-hours mortality the next day. Test replication was done 3 times. D-allethrin at a concentration of 0.001% was used as both gold standard and positive control in this research. Objects died after 24 hours were noted in each group.



**Fig. 1.** Typical structure of electric liquid vaporizer used in the study.

Data analysis was conducted in a descriptive manner by calculating mosquito knockdown time ( $KT_1$ ,  $KT_{50}$ ,  $KT_{95}$ ,  $KT_{100}$ ) and percentage of dead mosquitoes after 24 hours in 3 groups and 3 test replications. Neem oil group results hence were compared with the positive control group to identify its killing ability on *Aedes aegypti* mosquitoes.

## RESULTS AND DISCUSSION

Based on the research carried out in January 2013, the following results are obtained: no data of Knockdown Time obtained in group A (aquadest) because there were no mosquitoes knocked down during the vapor exposure, and the 24-hours mortality was 0% (Table 1; Table 2). Group A hence is used as a negative control. In group B (100% neem oil), mean Knockdown Time was 235 minutes for  $KT_1$ , but further increasing number of knocked down mosquitoes was unable to be observed,

thus  $KT_{50}$ ,  $KT_{95}$ , dan  $KT_{100}$  data were not obtained. In this group, mean 24-hours mortality was 33.3% in 3 test replications. Meanwhile, in group C, mean Knockdown Time observed were 2 minutes 26 seconds for  $KT_1$ , 37 minutes 1 second for  $KT_{50}$ , 90 minutes 49 seconds for  $KT_{95}$  and 92 minutes 50 seconds for  $KT_{100}$ . Mean 24-hours mortality for group C in 3 test replications was 100%. Group C is then used as positive control.

The results showed us that mean Knockdown Time for  $KT_1$ ,  $KT_{50}$ ,  $KT_{95}$ ,  $KT_{100}$  in group C (0.001% d-allothrin) were faster than group A (aquadest) and group B (100% neem oil), which was 2 minutes 26 seconds for  $KT_1$ , 37 minutes 1 second for  $KT_{50}$ , 90 minutes 49 seconds for  $KT_{95}$  and 92 minutes 50 seconds for  $KT_{100}$ . Mean 24-hours mortality for group C in 3 test replications was 100%, which was in larger percentage than those of group A and group B.

**Table 1.** Efficacy test results in each group against *Aedes aegypti*

Group	Mean Knockdown Time (min)			
	KT <sub>1</sub>	KT <sub>50</sub>	KT <sub>95</sub>	KT <sub>100</sub>
A (aquadest; negative control)	-	-	-	-
B (100% neem oil)	235 min	-	-	-
C (0.001% d-allethrin; positive control)	2 min 26 s	37 min 01 s	90 min 49 s	92 min 50 s

KT<sub>1</sub> = knockdown time 1% of total mosquitoes  
 KT<sub>50</sub> = knockdown time 50% of total mosquitoes  
 KT<sub>95</sub> = knockdown time 95% of total mosquitoes  
 KT<sub>100</sub> = knockdown time 100% of total mosquitoes

**Table 2.** Results of 24-hours mortality in each group against *Aedes aegypti*

Group	24-hours Mortality (%)			
	I	II	III	Mean
A (aquadest; negative control)	0	0	0	0
B (100% neem oil)	52	36	12	33.3
C (0.001% d-allethrin; positive control)	100	100	100	100

Mosquitoes undergo knockdown state may be caused by active compounds contained in the vapor produced by electric liquid vaporizer which act as toxic gases to mosquitoes. The impact on mosquitoes will vary from merely repelling mosquitoes or even killing

them (Supargiyono, 1988). One of active compounds contained in neem oil is azadirachtin, which is presumably has the ability to act as a natural insecticide (Quarles, 1994; Thacker, 2002; Aremu et al., 2009; Maia and Moore, 2011). Since there were limitations due to laboratory

working hour, we could not observe when and how precisely the mosquitoes died in group B (100% neem oil). We discussed the possibilities that may cause different research outcomes, nevertheless. There are several factors, which have the potential to affect the research outcomes, such as mosquito factor, device factor, active compound factor and environmental factor.

Mosquitoes are able to produce cross-resistance against different insecticides (Guomin et al., 2003). *Aedes aegypti* mosquitoes adapt easily to their surrounding environment, including seasonal changes, thus bionomical shift will more likely to happen in this kind of mosquito (Sugito, 1989). Under starvation in outdoor neighborhood, *Aedes aegypti* would last for 3-4 days before it is suffocated and then died (Costero et al., 1999). Resistance to certain insecticides will develop slowly (Guomin et al., 2003), so will with neem oil (Biswas et al., 2002). We concluded that the possibility of mosquito resistance to neem oil was not the cause of live mosquitoes despite the exposure of neem oil. Dead mosquitoes may result from suffocation due to airtight test room.

Pure (100%) neem oil used in this research was viscous due to its insoluble macromolecules, thus affecting its capillary action towards the device stem. Capillary action depends on stem diameter, liquid viscosity, surface tension and contact angle between stem and liquid surface (Abbey and Essiah, 1990). The more viscous the liquid is, the harder it gets to the top of the stem because the macromolecules could not resist the gravity force (Guomin

et al., 2003). The compound d-allethrin ( $C_{19}H_{26}O_3$ ) is a synthetic pyrethroid widely used for its fast knock down activity against household pest insects, including mosquitoes (WHO, 2002). With its relatively low molecular weight of 302.41 g/mol, d-allethrin may easily counter the capillary and gravity action during vaporizing process, unlike azadirachtin ( $C_{35}H_{44}O_{16}$ ) with molecular weight of 720.72 g/mol (Abbey and Essiah, 1990; WHO, 2002; National Center for Biotechnology Information, 2017). In the research procedure, we substituted the original stem with kerosene-stove cord, which was smaller in diameter. These factors may have developed an impaired heating of the cord, thus causing an imperfect vaporizing process. While in other studies, application of neem oil in cream formulation is proven as safe and showed remarkable repellent activity (Sharma, et al., 1993; Dua, et al., 1995; Kiplang'at and Mwangi, 2013). In the study performed by Kiplang'at and Mwangi (2013), the extracts were formulated using pure petroleum jelly and neem oil was diluted in melted jelly to certain concentrations (1%, 2%, 3%, 4%, and 5%) prior to being applied to experimental rabbit skin. At a concentration of 5%, there was a marked improvement in the repellent activity against *Aedes aegypti* and the activity was significant when compared to concentrations of 1%, 2%, 3%, and 4%, hence 5% neem oil in cream formulation is the appropriate concentration as a topical application on humans (Kiplang'at and Mwangi, 2013). Similar results were also

reported by Sharma et al. (1993) using 5% neem oil against *Culex quinquefasciatus* and *Anopheles culicifacies* mosquitoes.

When mosquito eats azadirachtin, it actively attacks mosquito's reproductive cycle, its feeding pattern, its bodily development, as well as acting as direct toxin (Schmutterer, 1990). Mosquitoes that were still alive after the exposure to neem oil vapor might be due to active compounds contained in neem oil that is not particularly acting as a direct adulticide. Azadirachtin is the most profound active compound found in neem oil as per gram of neem seeds may contain 2-4 mg azadirachtin, and is believed to have certain role in repelling insects (Quarles, 1994; Isman, et al., 2006; Aremu et al., 2009; Maia and Moore, 2011). This compound may cause follicular cell degeneration in adult mosquitoes and preventing further egg development when in contact (Shivakumar and Kataria, 2011). Azadirachtin may not cause direct killing effect on adult mosquitoes, nevertheless its fertility-suppressing mechanisms will be a long-term use in vector control method (Shivakumar and Kataria, 2011; Sharma et al., 1993).

Azadirachtin is also known to have the properties of insect growth regulator, antifeedant, and may affect mosquito oviposition, while the other remaining major constituents of neem oil (e.g. nimbin, nimbidine, gedunin) are reported to have anti-inflammatory, antipyretic, fungicidal, antiparasitic, antihistamine, and antiseptic properties (Quarles, 1994;

Schmutterer, 1990; Veitch, et al., 2007; Murugan, et al., 2014). Environmental factor plays a significant role in affecting research outcomes. Most insecticides get into mosquito body by direct contact or through the respiratory system, diffusing to neuronal junction, inactivates neurotransmitters, thus causing mosquito to be paralyzed soon after exposure (Kumar, 1986). The higher the surrounding temperature, the faster mosquitoes breathe and the toxic effect of vapor will occur more rapidly (Bond and Monro, 1984). The lower the neighborhood humidity, then water will more likely to evaporate from mosquito's body (Sugito, 1989). If excessive water evaporation occurs inside mosquito, then it will not be able to fly and soon will be dead (Sugito, 1989). In our research, we could not control the temperature and humidity in the test room, which might be the cause of dead mosquitoes is group B (100% neem oil).

## CONCLUSION

From the research results, data analysis and discussions, we conclude that pure neem oil (*Azadirachta indica*) does not have the ability to directly kill adult *Aedes aegypti* mosquitoes, if used as basic compound in electric liquid vaporizer.

## SUGGESTION

Further investigations regarding the use and action mechanisms of azadirachtin and other active compounds of neem oil such as nimbidine and nimbin are highly

encouraged, mainly for its future use in DHF vector control method.

## REFERENCES

- Abbey, T. K., and J. W. Essiah. 1990. Physics for senior secondary schools. Macmillan Education, Ltd., London.
- Alouani, A., N. Rehim, and N. Soltani. 2009. Larvicidal Activity of a Neem Tree Extract (Azadirachtin) Against Mosquito Larvae in the Republic of Algeria. *Jordan J. Biol. Sci.* 2: 15-22.
- Aremu, O. I., M. N. Femi-Oyewo, and K. O. K. Popoola. 2009. Repellent Action of Neem (Azadirachta indica) Seed Oil Cream against Anopheles Gambiae Mosquitoes. *African Res. Rev.* 3: 12-22.
- Asogwa, E. U., T. C. N. Ndubuaku, J. A. Ugwu, and O. O. Awe. 2010. Prospects of botanical pesticides from neem, Azadirachta indica for routine protection of cocoa farms against the brown cocoa mirid - Sahlbergella singularis in Nigeria. *J. Med. Plant. Res.* 4: 1-6.
- Bhatt, S., P. W. Gething, O. J. Brady, J. P. Messina, A. W. Farlow, C. L. Moyes, J. M. Drake, J. S. Brownstein, A. G. Hoen, M. F. Myers, D. B. George, T. Jaenisch, and G. R. Williams. 2013. The global distribution and burden of dengue. *Nature.* 496: 504-507.
- Biswas, K., I. Chattopadhyay, and R. K. Banerjee. 2002. Biological activities and medicinal properties of neem (Azadirachta indica). *Curr. Sci.* 82: 1336-1345.
- Bond, E.J., and H. A. U. Monro. 1984. Manual of fumigation for insect control. Food and Agriculture Organization of the United Nations, Rome.
- Brady, O. J., P. W. Gething, S. Bhatt, J. P. Messina, J. S. Brownstein, A. G. Hoen, C. L. Moyes, A. W. Farlow, T. W. Scott, and S. I. Hay. 2012. Refining the Global Spatial Limits of Dengue Virus Transmission by Evidence-Based Consensus. *PLoS Negl Trop Dis.* 6: 1-15.
- Caballero-anthony, M., A. D. B. Cook, G. Gayle, H. Amul, and A. Sharma. 2015. Health governance and dengue in Southeast Asia. Singapore.
- (CDC) Center for Disease Control and Prevention. 2010. Dengue. <http://www.cdc.gov/dengue/> accessed March 28, 2014.
- Costero, A., J. D. Edman, G. G. Clark, P. Kittayapong, and T.W. Scott. 1999. Survival of starved Aedes aegypti (Diptera: Culicidae) in Puerto Rico and Thailand. *Journal of Medical Entomology.* 36(3): 272-6.
- Dua, V. K., B. N. Nagpal, and V. P. Sharma. 1995. Repellent action of neem cream against mosquitoes. *Indian J Malariol.* 32(2): 47-53.
- Federer, W. 1991. Statistics and society: data collection and interpretation, 2nd ed. Marcel Dekker, New York.
- Guomin, J., Y. Xiaolin, and C. Rongchang. 2003. The handbook of insecticide formulations and its technologies for household and public health uses. Jin Tai Printing Ltd., Hong Kong.
- Isman, M. B. 2006. Botanical insecticides, deterrent and repellents in modern agriculture and an increasingly regulated world. *Ann. Rev. Entomol.* 51:45-66.
- Kesetyaningsih, T. W. 2008. Khasiat Obat Nyamuk Bakar Berbahan Aktif Pyrethroid terhadap Culex quinquefasciatus pada Berbagai Kondisi Ruangan. *Mutiara Medika.* 8(2): 67-76.
- Kiplang'at, K. P., and R. W. Mwangi. 2013. Repellent Activities of Ocimum basilicum, Azadirachta indica and Eucalyptus citriodora Extracts on Rabbit Skin against Aedes aegypti. *J. Entomol. Zool. Stud.* 1: 84-91.
- Kumar, R. 1986. Insect pest control with special reference to African agriculture, 1st ed. The Camelot Press Ltd., Southampton, Great Britain.
- Lloyd, L. S. 2003. Best practices for dengue prevention and control in the Americas. Office of Health, Infectious Diseases and Nutrition Bureau for

- Global Health, U.S. Agency for International Development, Washington, DC.
- Maia, M. F., and S. J. Moore. 2011. Plant-based insect repellents: a review of their efficacy, development and testing. *Malar. J.* 10: 1-14.
- Mandal, S. 2011. Repellent activity of Eucalyptus and Azadirachta indica seed oil against the filarial mosquito *Culex quinquefasciatus* Say (Diptera: Culicidae) in India. *Asian Pac. J. Trop. Biomed.* 109-112.
- Mardiswojo, S. and H. Rajakmangunsudarso. 1985. Cabe puyang warisan nenek moyang. Balai Pustaka, Jakarta.
- Murugan, K., G. J. Pitchai, P. Madhiyazhagan, T. Nataraj, A. Nareshkumar, J. S. Hwang, R. Chandrasekar, M. Nicoletti, A. Amsath, and R. Bhagooli. 2014. Larvicidal, repellent and smoke toxicity effect of neem products against malarial vector, *Anopheles stephensi*. *Int. J. Pure Appl. Zool.*, 2(2): 71-83.
- National Center for Biotechnology Information. PubChem Compound Database; CID=5281303, <https://pubchem.ncbi.nlm.nih.gov/compound/5281303> accessed March 16, 2017.
- National Center for Biotechnology Information. PubChem Compound Database; CID=11442, <https://pubchem.ncbi.nlm.nih.gov/compound/11442> accessed March 16, 2017.
- Ndione, R. D., O. Faye, M. Ndiaye, A. Dieye, and J. Marie. 2007. Toxic effects of neem products (*Azadirachta indica* A . Juss) on *Aedes aegypti* Linnaeus 1762 larvae. *Afr. J. Biotechnol.* 6: 2846-2854.
- Quarles, W. 1994. Neem tree pesticides product ornamental plants. *The IPM Practitioner.* 16(10): 1-13.
- Schmutterer, H. 1990. Properties of natural pesticides from the neem tree, *Azadirachta indica*. *Ann. Rev Entomol.*, 35:271-297.
- Sharma, V. P., M. A. Ansari, and R. K. Razdan. 1993. Mosquito Repellent Action of Neem (*Azadirachta indica*) Oil. *J. Am. Mosq. Control Assoc.* 9: 359-360.
- Shivakumar, M. S. 2011. Comparative efficacy of Azadirachtin on the larval population of *Culex quinquefasciatus*, *Anopheles stephensi*, and *Aedes aegypti* (Diptera: Culicidae) in Gujarat, India. *Int. J. Pharma Bio Sci.* 2: 41-47.
- Soedarto. 1990. Entomologi kedokteran. EGC, Jakarta, Indonesia.
- Sugito, R. 1989. Aspek etnologi demam berdarah, pp. 37-43 In B. Haryanto, R. H. Sri, W. Suharyono, and I. M. Djaya, Berbagai aspek DBD dan penanggulangannya. Annual Report, Depok, Indonesia.
- Supargiyono. 1988. Pengaruh Asap Obat Nyamuk Bakar Terhadap Lama Hidup, Kemampuan Bertelur, Daya Tetas Telur, dan Daya Tetas Larva *Aedes aegypti*. *B.I.Ked. Fakultas Kedokteran UGM.* 20: 61-68.
- Sutanto, B.A. and Purnomo. 1990. Pemakaian repelen komersial yang mengandung Toluamide terhadap *Culex quinquefasciatus* di Jakarta. *Medika.* 16: 38-42.
- Valecha, N., M.A. Ansaari, S. Prabhu and R. K. Razdan. 1996. Preliminary evaluation of safety aspects of neem oil in kerosene lamp. *Indian J. Malariol.* 33: 139-143.
- Veitch G. E., E. Beckmann, B. J. Burke, A. Boyer, S. L. Maslen, S. V. Ley. 2007. Synthesis of azadirachtin: a long but successful journey. *Angew. Chem. Int. Ed. Engl.* 46 (40): 7629-32.
- World Health Organization. 2002. WHO Specifications and Evaluations for Public Health Pesticides: d-allothrin. World Health Organization, Geneva.
- World Health Organization. 2011. Comprehensive Guidelines for Prevention and Control of Dengue and Dengue Haemorrhagic Fever, Revised an. ed. World Health Organization, Regional Office for South-East Asia, New Delhi.

World Health Organization. 2015. Dengue and Severe Dengue. <http://www.who.int/mediacentre/factsheets/fs117/en/> accessed February 11, 2016.

Yogyakarta District Health Office. 2013. Yogyakarta health profile year 2013. Yogyakarta District Health Office, Yogyakarta Special Region, Indonesia.