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# PHYTOCHEMICAL SCREENING AND INSECTICIDAL ACTIVITY OF ENDOPHYTIC FUNGAL EXTRACTS FROM STEM BARK OF *Azadirachta indica* AGAINST *Anopheles gambiae*

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***ABSTRACT***
***Mosquitoes are the vectors of several life-threatening diseases in humans such as malaria, yellow fever, chikungunya fever, e.t.c. The present study explored the insecticidal effect of three endophytic fungal extracts isolated from the stem bark of Azadirachta indica on larvicidal, pupicidal and adulticidal activity against Anopheles gambiae and their phytochemical constituents. Aspergillus specie (AI-1), Mucor specie (AI-2) and Penicillium specie (AI-3) were isolated from the stem bark of Azadirachta indica and identified based on the morphological characteristics of the colonies and microscopically using lacto phenol cotton blue staining technique. The ethyl acetate extract was obtained by liquid-liquid extraction of the fermented fungus in potato dextrose broth medium. Results of qualitative phytochemical screening of the extracts revealed the presence of saponins in all the extracts, cardiac glycosides and phenols are present in AI-1 and AI-3. Alkaloids, Tannins and terpenoids were detected only in AI-1, steroids and anthraquinones were found only in AI-2 and AI-3 respectively. The extract of AI-1 was also observed to record the highest insecticidal activity against Anopheles gambiae with $LC_{50}$ values of 143.88, 272.70 and 418.23 ppm for larvicidal, pupicidal and adulticidal respectively, AI-3 showed significant activity with $LC_{50}$ values of 155.14, 336.21 and 612.62 ppm accordingly while AI-2 proved to be less active. These results suggest that ethyl acetate extract of Aspergillus specie (AI-1) have the potential to be used as an eco-friendly approach for vector control program.***
***Keywords: Phytochemicals, Insecticidal, Endophytic fungi, Azadirachta indica.***

## INTRODUCTION

Mosquitoes are the vectors of various diseases in human beings, among which are; malaria, dengue, filariasis and yellow fever, thereby causing millions of deaths every year (WHO, 2010). *Anopheles* mosquitoes are distributed worldwide except in cold temperate regions, over 400 species of Anopheles that are pathogenic to humans are known. Almost 30 species transmit Plasmodium significantly in nature, Vinayagam *et al*. (2008). *Anopheles gambiae* is the known vector of malaria in Nigeria. Malaria is one of the most prevalent diseases in the tropical world with 200 million to 450 million infections annually worldwide, it causes up to 2.7 million deaths (WHO, 2010). Malaria is a public health problem most especially in the tropical countries where majority bear the burden of the disease. It has been estimated that 40% of the world's population is at risk and 500 million people suffer from the disease annually (WHO, 2010). About two million children, mostly less than five years old, and pregnant women die from the malaria related illness each year and nine out of ten cases are found in Sub-Saharan Africa (WHO, 2001).

Vector control is the most successful method for reducing the incidences of mosquito-borne diseases; the widespread use of insecticide has resulted in the development of resistance, undesirable effects on non-target organisms, and fostered environmental and human health concern (Thomas *et al*., 2004). The search for herbal preparations that do not produce any adverse effects in the non-target organisms and are easily biodegradable remains a top research issue for scientists associated with alternative vector control (Chowdhury *et al*., 2008).

Endophytes are microbes that colonize the internal plant tissues and organs without causing any apparent harm to their host. Fungi are the most frequently recovered endophytes from plant tissues. Due to the relationship between endophytes and their host plants, they become a source of promising biologically active metabolites for pharmaceutical and agricultural applications. It has been reported that fungal endophytes residing within part of plants could also produce metabolites similar to or with more activity than that of their respective hosts (Strobel, 2002). Many plants contain, as yet, uncharacterized species and strains of endophytes (Arnold *et al*., 2000; Stone *et al*., 2004). Although the potential of endophytic fungi are known, there are limited experimental data available as references (Zikmundová *et al*., 2002).

Larvicidal activity of extracts from different parts of Neem (*Azadirachta indica*) against *Aedes Aegypti* mosquitoes' larvae showed that leaf acetone and root chloroform extracts were more toxic against larvae and causes 100% mortality at concentration of 1000ppm in 24h and the remaining extracts achieved 100% mortality at 1000 ppm in 48h (Azhari *et al*., 2012).

Endophytic fungal derived products are highly toxic to mosquitoes, and reported to have low toxicity against non-target organisms (Govindarajan *et al.*, 2005). Extracellular secondary metabolites from many fungi have been screened for larvicidal activity against mosquitoes, (Zimmerman and Vitousek, 2012). However, endophytic fungi are considered a good candidate for bio-control of mosquitoes because of their safety, limited host range and large scale production of secondary metabolites (Fillinger *et al.*, 2003). In line with the development, the present study was aimed at screening the phytochemicals and determination of mosquito larvicidal, pupicidal and adulticidal activity of endophytic fungal extracts from *Azadirachta indica* stem-bark against *Anopheles gambiae*.

## MATERIALS AND METHODS

### Collection of plant material

The fresh and healthy stem barks of *Azadirachta indica* were collected from Botanical Garden of Department of Plant Biology, Bayero University, Kano, Nigeria in a sterile polythene bag and transported to Centre for Biotechnology Research Laboratory, Bayero University, Kano, Nigeria for fungal culture within 24 hours.

### Isolation and Culture of Endophytic Fungi

The plant samples were washed several times under running tap water to remove soil and other debris. The sample sterilization and the culture of endophytic fungi utilized the methods described by Strobel *et al.* (1996). The plant sample was dipped in 70% ethanol for one minute, 0.5 % NaOCl for five minutes, and 96 % ethanol for 30 seconds. The surface sterilized sample was then washed with sterile distilled water thrice and allowed to surface dry under aseptic conditions. After washing, sterile blades were used to excise 0.5 cm × 0.5cm pieces of the plant part. In each Petri dish, two segments were plated on Potato Dextrose Agar (PDA) medium supplemented with chloramphenicol (250 mg) to inhibit bacterial growth. The Petri dishes were sealed with Paraffin, incubated for 7 days at 27 ± 3°C, and examined periodically. Fungal hyphae were transferred to PDA plates through hyphal tipping for purification.

### Endophytic fungi identification

The fungi were identified macroscopically by observing the morphological characteristics such as colony topography, color, textures and growth pattern. Microscopic identification was done by preparing slides from cultures by hyphal tipping, stained with lactophenol aniline blue and viewed under microscope. The isolates were identified through their reproductive structures. Mycological reference books were used as guides for the morphological identification (Webster and Weber 2007; Sarah, 2016).

### Fermentation and Extraction of the Endophytes

Endophytic fungi isolates were subjected to liquid surface fermentation and ethyl acetate extraction following the methodology described by Radji *et al.* (2011). Three pieces of mycelia agar plugs (0.5 × 0.5cm$^2$) were inoculated into 100mL Potato Dextrose Broth (PDB). They were incubated at room temperature for three weeks. After the incubation period, ethyl acetate 100 ml was added to the broth culture and the formed mycelial mat was manually macerated using mortar and pestle. Afterwards, the mycelial mat was extracted with methanol, filtered using Whatman number 1 filter paper and then concentrated using rotary evaporator. The methanol extract was added to the ethyl acetate-broth culture. After 24 hours, it was extracted with ethyl acetate three times. The extract was concentrated using a rotary evaporator at 40$^0$C.

### Phytochemical screening

All the endophytic fungal extracts obtained from the stem bark of *Azadirachta indica* were screened for the presence or absence of secondary metabolites using standard procedure as described by Trease and Evans (2007).

### Collection of Larvae

The larvae of *Anopheles gambiae* were collected in a plastic bucket from Auyo Local Government Area Jigawa State. Nigeria

### Culture of test organism

The larvae were fed with tetramine in a plastic tray and kept in a 90 × 90 × 90cm mosquito cage for pupae and adult emergence. The pupae were collected from the culture trays using plastic pasteur pipette and transferred to a 1L beaker containing 500ml tap water. The Adult mosquitoes were fed with 10% glucose solution by soaking a cotton pad and placing it on the cage. Mosquitoes were held at 28 ± 2°C, 85% relative humidity, and a photoperiod of 12:12 (Light: Dark) hour.

### Larvicidal/pupicidal assay

The larvicidal/pupicidal activity of fungal extracts were assessed by using the standard method as prescribed by World Health Organization (2005). From the stock solution, four different test concentrations (1000, 500, 250 and 125ppm) were prepared. Twenty-five number of third instars larvae and pupae were exposed to small disposable test cups each containing 100ml of distilled water and 1ml of prepared test concentrations or acetone as control. Each experiment was in triplicate. The control experiments were also run parallel with each replicate. The larval and pupal mortality were calculated after 24 hours of the exposure period.

### Adulticidal assay

Toxicity assays was performed following the method in WHO (2006) using an experimental WHO kit consisting of two cylindrical plastic tubes. One tube serves to expose the mosquitoes to the extracts and another tube was used to hold the mosquitoes before and after the exposure periods. Filter papers were impregnated with various concentrations of the fungal extracts and a blank paper consisting of only acetone was used as control, the papers were left to dry at room temperature to evaporate off the solvent. The impregnated papers were rolled and placed in the exposure tube. Glucose fed and blood starved 2-5 days old twenty-five mosquitoes were exposed during 1 hour to a treated paper (with extracts or with the acetone only) in the exposure tube used for the contact irritancy assay. Mosquitoes were then transferred into an untreated tube (holding tube) with a cotton pad soaked in 10% glucose solution and maintained at 27$^0$C and 80% RH.

Mortality of mosquitoes was recorded after 24 hours. The assay was considered valid whenever there were less than 10% of dead mosquitoes in the control (treated paper with acetone) after 24 hours. The toxic effect of each extract was expressed as the proportion of dead mosquitoes.

**STATISTICAL ANALYSIS**

The average larval, pupal and adult mortality data were subjected to Probit analysis to estimate the $LC_{50}$ values with the statistical package SPSS.

**RESULTS**

**Isolation of Endophytic Fungi**

The endophytic fungi isolated from the stem bark of *Azadirachta indica* were identified on the basis of their microscopic and macroscopic features. A total number of three endophytic fungi were isolated and coded, *Aspergillus* sp., *Mucor* sp. and *Penicillium* sp. (AI-1, AI-2 & AI-3 respectively) as shown in plates 1 - 3.

The isolated endophytic fungi were identified based on the colony morphology and microscopic features;

AI-1 (*Aspergillus* sp.)

Culture: The colony has whitish colour that rapidly turn black with creamy reverse.

Microscopy: long conidiophores with large conidia, globose black colour and rough walled are observed.

AI-2 (*Mucor* sp.)

Culture: Colony is cottony, grow rapidly, white at first later become greyish, reverse is colourless.

Microscopy: Sporangiophores are long, unbranched with terminal round sporangia are found.

AI-3 (*Penicillium* sp.)

Culture: Bluish grey-green colony.

Microscopy: Broom-like phialides, consisting of 3-5 metulate and chains of spherical to oval conidia are present.

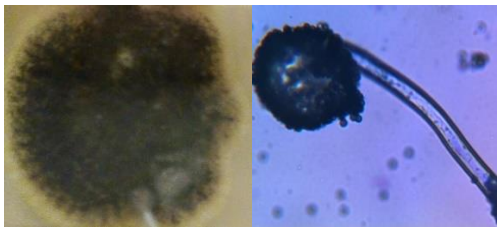

Plate 1: AI-1 (*Aspergillus* sp.)

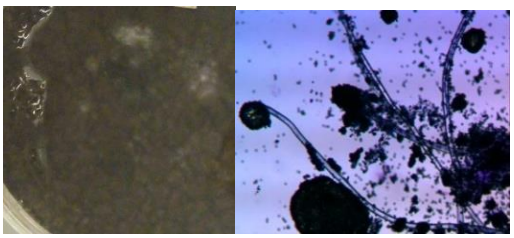

Plate 2: AI-2 (*Mucor* sp.)

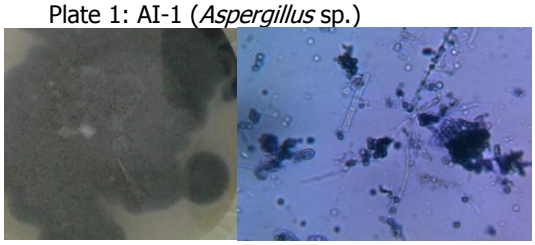

Plate 3: AI-3 (*Penicillium* sp.)

Among all the tested endophytic fungal extracts on larvicidal activity against *Anopheles gambiae* from Table 2, the highest percentage mortality was noticed in extract of AI-1 (*Aspergillus* sp.) with 92% and 85% at 1000 µg/ml and 500 µg/ml respectively of the third instar larval stage with $LC_{50}$ of 143.88 and $LC_{90}$ of 745.20. AI-3 (*penicillium* sp.) also showed a remarkable activity of 88% and 79% mortality at above mentioned concentrations respectively with $LC_{50}$ of 155.14 and $LC_{90}$ of 1053.92. Extract of AI-2 (*mucor* sp.) is the less active among all the extracts.

Similar trend was observed in the pupicidal activity against *Anopheles gambiae* with AI-1 extract displaying the highest percentage mortality of 80% and 69% at the concentrations of 1000 µg/ml and 500 µg/ml respectively with $LC_{50}$ of 272.70 and $LC_{90}$ of 1895.72 followed by extract of AI-3 which showed a significant percentage mortality of 75% and 65% at above mentioned concentrations respectively with $LC_{50}$ of 336.21 and $LC_{90}$ of 2046.92 while AI-2 extract with the least activity was found to be moderately active as shown in Table 3.

The adulticidal activity against *Anopheles gambiae* (Table 4) also followed the same order as that of the larval and pupal stages. The best percentage mortality was recorded in the extract of AI-1 with 71% and 52% at 1000 µg/ml and 500 µg/ml respectively followed by extract of AI-3 which revealed moderate activity with percentage mortality of 68% and 40% at above mentioned concentrations respectively with $LC_{50}$ of 612.62 and $LC_{90}$ of 2291.12. Whereas extract of AI-2 proved to be inactive.

**Table 1: Phytochemical Screening Results of endophytic fungal extracts**

| | Endophytic fungal extracts code | | |
|---|---|---|---|
| **Phytochemical Test** | AI-1 | AI-2 | AI-3 |
| Alkaloids | + | - | - |
| Anthraquinones | - | - | + |
| Cardiac glycosides | + | - | + |
| Flavonoids | - | - | - |
| Saponins | + | + | + |
| Steroids | - | + | - |
| Tannins | + | - | - |
| Terpenoids | + | - | - |
| Phenols | + | - | + |

Key: AI-1; *Aspergillus* sp., AI-2; *Mucor* sp., AI-3; *Penicillium* sp. + = present; - = absent

**Table 2: Larvicidal activity of endophytic fungal extracts against *Anopheles gambiae***

| | % Mortality ±SD Concentration (µg/ml) | | | | 95% confidence limit | |
|---|---|---|---|---|---|---|
| **Fungal Extracts** | 125 | 250 | 500 | 1000 | $LC_{50}$ (LCL-UCL) | $LC_{90}$ (LCL-UCL) |
| **AI-1** | 44±1.16 | 68±1.00 | 85±1.53 | 92±1.73 | 143.88 (99.66-183.56) | 745.20 (559.79-1182.27) |
| **AI-2** | 12±0.00 | 28±1.73 | 53±1.53 | 64±1.00 | 541.97 (440.93-702.49) | 2997.27 (1862.51-6749.20) |
| **AI-3** | 41±0.58 | 67±1.53 | 79±0.58 | 88±1.73 | 155.14 (102.54-202.45) | 1053.92 (734.05-1986.53) |

Key: Nil mortality in control; LCL: lower confidence limit, UCL; upper confidence limit.
AI-1; *Aspergillus* sp., AI-2; *Mucor* sp., AI-3; *Penicillium* sp.

**Table 3: Pupicidal activity of endophytic fungal extracts against *Anopheles gambiae***

| | % Mortality ±SD Concentration (µg/ml) | | | | 95% confidence limit | |
|---|---|---|---|---|---|---|
| **Fungal Extracts** | 125 | 250 | 500 | 1000 | $LC_{50}$ (LCL-UCL) | $LC_{90}$ (LCL-UCL) |
| **AI-1** | 28±1.73 | 48±1.73 | 69±0.58 | 80±1.00 | 272.70 (211.99-337.86) | 1695.72 (1128.29-3402.89) |
| **AI-2** | 9±0.58 | 25±1.53 | 48±1.00 | 56±1.73 | 678.78 (536.54-949.62) | 4236.49 (2387.32-11928.76) |
| **AI-3** | 21±1.16 | 44±1.73 | 65±1.16 | 75±1.583 | 336.21 (265.59-417.39) | 2046.92 (1331.34-4266.35) |

Key: Nil mortality in control; LCL: lower confidence limit, UCL; upper confidence limit.
AI-1; *Aspergillus* sp., AI-2; *Mucor* sp., AI-3; *Penicillium* sp.

**Table 4: Adulticidal activity of endophytic fungal extracts against *Anopheles gambiae***

| | % Mortality ±SD Concentration (µg/ml) | | | | 95% confidence limit | |
|---|---|---|---|---|---|---|
| **Fungal Extracts** | 125 | 250 | 500 | 1000 | $LC_{50}$ (LCL-UCL) | $LC_{90}$ (LCL-UCL) |
| **AI-1** | 21±0.58 | 41±1.53 | 52±1.73 | 71±1.16 | 418.23 (327.89-550.86) | 3378.39 (1889.99-10063.31) |
| **AI-2** | 0±0 | 12±1.00 | 27±0.58 | 47±1.16 | 1016.52 (811.89-1434.84) | 3844.33 (2392.08-8820.17) |
| **AI-3** | 4±1.00 | 24±1.73 | 40±1.73 | 68±1.00 | 612.62 (515.97-758.37) | 2291.12 (1607.02-3998) |

Key: Nil mortality in control; LCL: lower confidence limit, UCL; upper confidence limit.
AI-1; *Aspergillus* sp., AI-2; *Mucor* sp., AI-3; *Penicillium* sp.

**DISCUSSION**

Mahesh *et al.* (2005) recovered 77 endophytic fungi belonging to 15 genera from the inner bark of *A. indica*. Tenguria and Khan (2011) reported diversity of endophytic fungi isolated from leaves of *A. indica* collected from Panchmarhi biosphere reserve. Further, they reported *Trichoderma* as dominant species followed by *Pestalotiopsis* sp. and *Penicillium* species.

The phytochemical screening result from table 1 revealed the presence of saponins in all the extracts, cardiac glycosides and phenols are present in AI-1 and AI-3. Alkaloids, Tannins and terpenoids were detected only in AI-1, steroids and anthraquinones were found only in AI-2 and AI-3 respectively. The phytochemical results of this finding is similar to that of Nwankwo *et al*. (2021) who reported the quantitative phytochemical assessment of the endophytic fungi extract from the leaves of *Azadirachta indica* with abundant alkaloids and flavonoids.

*Aspergillus* sp. and *Fusarium* sp. were observed in leaves of *Azadiarcta indica*, *Fusarium* sp. showed the highest mortality against *Aedes aegypti* with percentage mortality of 83.33 and *Aspergillus* sp. has 66.66 at 20ppm (Sathiyanathan and Umarajan, 2019). The ethyl acetate extract of *Aspergillus* tamarii isolated from the stem of *Opuntia ficus-indica* showed a high larvicidal effect against mosquitoes (*Ae. aegypti* and *Cx. quinquefasciatus*) as reported by Kannan *et al*. (2020). These results of larvicidal activity are in consistent with the results of the present findings.

**CONCLUSION**

The medicinal plant *Azadirachta indica* harbor several endophytic fungi. The three endophytic fungi extracts obtained from the stem bark of *A. indica* showed significant activity against the larvae, pupae and adults of *Anopheles gambiae.* The presence of secondary metabolites is attributed to the activity. These results suggest that ethyl acetate extract of *Aspergillus* specie has the potential to be used as an eco-friendly approach for vector control programme.

**REFERENCES**


Arnold, A.E., Maynard, Z., Gilbert, G.S., Coley, P.D. and Kursar, T.A. 2000. Are tropical fungal endophytes hyperdiverse. Ecology Letters 3: 267-274.

Azhari H. Nour, Jessinta D/O Sandanasamy and Abdurahman H. Nour (2012). Larvicidal activity of extracts from different parts of Neem (*Azadirachta indica*) against *Aedes Aegypti* mosquitoes' larvae. Scientific Research and Essays Vol. 7(31), pp. 2810-2815.

Chowdhury, N., Ghosh, A., Chandra, G. (2008) BMC Complementary and Alternative Medicine. Pp 8-10.

Fillinger U., B.G. Knols, N. Becker (2003). Efficacy and efficiency of new Bacillus thuringiensis var. israelensis and Bacillus sphaericus formulations against Afrotropical anophelines in Western Kenya, Trop. Med. Int. Health 8, 37–47.

Govindarajan M., A. Jebanesan, D. Reetha, (2005). Larvicidal effect of extracellular secondary metabolites of different fungi against the mosquito, *Culex quinquefasciatus* say. *Trop. Biomed*. 22 1–3.

Kannan Baskar, Ragavendran Chinnasamy, Karthika Pandy, Manigandan Venkatesan, Prakash Joy Sebastian, Murugesan Subban, Adelina Thomas, Eliningaya J. Kweka, Natarajan Devarajan (2020). Larvicidal and histopathology effect of endophytic fungal extracts of Aspergillus tamarii against *Aedes aegypti* and *Culex quinquefasciatus*. *Heliyon* 6, e05331

Mahesh, B., Tejesvi, M.V., Nalini, M.S., Prakash, H.S., Kini, K.R., Ven, S. and Shetty, H.S. (2005) Endophytic mycoflora of inner bark of *Azadirachta indica* A. Juss. *Current Science* 88(2):218-219 [Comprehensive studied the role of endophytes in the healing of bark].

Nwankwo OL, Chukwuebuka OC, Collins OO, Samuel BJ, Obasi JC, Iloh ES, Nwankwo EO (2021). Quantitative phytochemical analysis of the fungus endophytic extracts isolated from Azadirachta indica using gas chromatography- flame ionisation detector. *Journal of Drug Delivery and Therapeutics*. 11(5):80-83.

Radji M, Sumiati A, Rachmayani R, Elya B. (2011). Isolation of fungal endophytes from Garcinia mangostana and their antibacterial activity. *African J Biotechnol*.;10(1):103-7.

Sarah kiddi, Catriona Halliday, Helen Alexiou, David Ellis (2016). Descriptions of medical fungi. Third Edition, Newstyle Printing. 41 Manchester Street Mile End, South Australia 5031

Sathiyanathan M and KM Umarajan (2019). Larvicidal activity of endophytic fungi isolated from selected medicinal plants on *Aedes aegypti*. *Journal of Pharmacognosy and Phytochemistry* 8(2): 247-253.

Stone, J.K., Polishook, J.D. and White, J.F. (2004). Endophytic fungi. In: G.M. Mueller, G.F. Bills and M.S. Foster, (eds.), Biodiversity of Fungi: Inventory and Monitoring Methods. Elsevier Academic Press, New York, USA, pp. 241-270.

Strobel G.A. (2002). Rainforest endophytes and bioactive products. Crit Rev Biotech 22, 315-333.

Strobel, G.A.; Hess, W.M.; Ford, E.; Sidhu, R.S.; Yang, X. (1996). Taxol from fungal endophytes and the issue of biodiversity. *J. Ind. Microbiol. 17*, 417–423.

Tenguria, R.K. and Khan, F.N. (2011) Distribution of endophytic fungi in leaves of *Azadirachta indica* A. Juss. (Neem) of Panchmarhi Biosphere Reserve. *Current Botany,* 2(2): 27-29 [Complete study of diversity of endophytic fungi in one plants]

Thomas TG, Rao S, Lal S (2004) Mosquito larvicidal properties of an indigenous plant, Ipomoea cairica, Linn. *Jpn J Infect Dis,* 57:176– 177.

Trease, G.E. and Evans, W.C. (2007). Text Book of Pharmacognosy (16th edition) WB Saunders Harcourt Publishers Ltd. London, UK. 137-139, 230-240.

Vinayagam A, Senthilkumar N, Umamaheswari A. (2008). Larvicidal Activity of Some Medicinal Plant Extracts Against Malaria Vector *Anopheles stephensi*. *Research journal of Parasitology*, 3(2):50-58.

Webster J. and R. W. S. Weber (2007). Third edition, Published in the United States of America by Cambridge University Press, New York.

World Health Organisation (2010). Malaria. Factsheet No.94. Geneva. http://www.who.int/mediacentre/factsheets/fs094/en/.

World Health Organization (2001). What is malaria? RBM Info sheet Geneva.

World Health Organization (2005). Guidelines for laboratory and field testing of mosquito larvicides. Communicable disease control, prevention and eradication, WHO pesticide evaluation scheme.WHO, Geneva, WHO/CDS/WHOPES/GCDPP/1.3.

World Health Organization (2006). Guidelines for testing mosquito adulticides for indoor residual spraying and treatment of mosquito nets. WHO/CDS/NTD/WHOPES/GCDPP/2006.3

Zikmundová, M., Drandarov, K., Bigler, L., Hesse, M. and Werner, C. (2002). Biotransformation of 2-Benzoxazolinone and 2-Hydroxy-1,4-Benzoxazin-3-one ny endophytic fungi isolated from Aphelandra tetragona. Applied and Environmental Microbiology 68: 4863–4870.

Zimmerman N.B. and P.M. Vitousek (2012). Fungal endophyte communities reflect environmental structuring across a Hawaiian landscape, Proc. Natl. Acad. Sci. Unit. States Am. 109. 13022–13027.