

# Susceptibility of *Anopheles gambiae* s.l., and *Anopheles funestus* s.l. to seven insecticides in southern Ethiopia

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## Abstract

**Background:** In Ethiopia, as the primary malaria vector control tools rely on the efficacy of insecticides, the susceptibility of malaria vectors should be monitored regularly.

**Methods:** A standard WHO susceptibility test was used to test *Anopheles gambiae* s.l. (order Diptera, family Culicidae, Giles) from four sites and *Anopheles funestus* (order Diptera, family Culicidae, Giles) from one site in southern Ethiopia.

**Results:** Exposure to DDT, permethrin, and deltamethrin resulted in less than 95% knock down of mosquitoes one hour after exposure in all sites. Both species were resistant to DDT, permethrin, malathion, and deltamethrin in all sites tested. Conversely, both species were susceptible to fenitrothion in all sites tested. *An. gambiae* s.l. was resistant to bendiocarb in the four sites, with mortalities between 92 and 97%, whereas *An. funestus* was susceptible. Similarly, *An. funestus* was susceptible to propoxur, and *An. gambiae* s.l. mortalities after exposure to propoxur were between 97 and 100%.

**Conclusions:** The widespread resistance to pyrethroids is a concern for the efficacy of long-lasting insecticidal nets (LLINs), whereas the resistance to bendiocarb and incomplete mortality after exposure to propoxur present worrying signs for the efficacy of indoor residual spraying (IRS). There is a need for implementation of resistance management strategies to ensure continued gains in reducing malaria.

**Key words:** Ethiopia, malaria, *Anopheles gambiae* s.l., *Anopheles funestus*, insecticide, resistance

## Introduction

The contribution of currently available vector control options to the recent decrease in the global malaria burden has been well-known. For instance, the scale-up of chemical-based vector control interventions including indoor residual spraying (IRS) and long-lasting insecticidal net (LLIN) is worth recognizing (Maxmen 2012; WHO 2006). There are four classes of insecticide recommended by World Health Organization (WHO) for use in the fight against malaria transmitting mosquitoes especially in public health program. However, malaria vector control mainly relied on pyrethroids in the recent years; in fact, pyrethroids offer several advantages in terms of cost, less toxicity to mammals and duration of residual action. At present these insecticides are also widely applied as agricultural and household pesticides (WHO 2011a). This implies that the wider application of a class of insecticides has consequently brought a serious concern to malaria control through its effect on insecticide resistance of principal malaria vectors.

In recent years, sufficient data have been collected following the wide-scale use of insecticide-based malaria control strategies in the last decade. The development of resistance in several important vector species including *Anopheles gambiae* s.l., *An. funestus* and *An. arabiensis* has been reported. Pyrethroid resistance has been reported in malaria vector mosquito populations in 27 countries in sub-Saharan Africa (WHO

2011b; WHO 2011c). So far, high variability in the magnitude of insecticide resistance that covered most of the tropical and subtropical countries has been documented. For example, widespread resistance of insecticide resistance has been reported in many malaria endemic countries. Studies showed resistance to pyrethroids and carbamates in Indonesia and Myanmar, on the other hand resistance to pyrethroids, carbamates and organophosphates was documented in Peru (WHO 2012). In addition, resistance has been reported to all four classes of insecticides in Turkey; to DDT in Azerbaijan; and to carbamates and organophosphates in Uzbekistan (WHO 2012).

In Africa, Benin, Burkina Faso, Cameroon, Cote d'Ivoire and Ghana have also reported high frequencies of resistance and encountered acute problem. These countries have widespread resistance to pyrethroids and Dichlorodiphenyltrichloroethane (DDT); Cote d'Ivoire has also reported resistance to carbamates and organophosphates (WHO 2012). In addition, from southern Africa, Mozambique and South Africa have reported a broad spectrum of resistance over the past decade. A high frequency of metabolic resistance to pyrethroids has also been reported in Malawi and Zambia (WHO 2012). Similarly, resistance to all four classes of insecticide was also documented in Ethiopia and Sudan (Balkew et al. 2012; Seidahmed et al. 2012; Balkew et al. 2006).

In Ethiopia, DDT remained the mainstay for IRS operation since 1959 until it was completely replaced in 2007 (Gebreyesus et al. 2006; Abose et al. 1998; Balkew 2003). However, DDT was replaced with Malathion (organophosphate) in selected areas during the mid-1980s and 1990s. Overall, between 2007 and 2012, Deltamethrin (Pyrethroids) and Propoxur, Bendiocarb and Pirimiphos-methyl uses were recommended as alternative insecticides for IRS operation to overcome challenges of malaria vector control posed due to resistance in Ethiopia. Although these insecticides are the preferable options, increasing resistance remained the main obstacle to malaria control and possibly to elimination strategies. Similarly, WHO also documented resistance of Propoxur and Bendiocarb to malaria vectors in Kenya, Uganda and Tanzania (WHO 2012).

Early studies indicated that *An. arabiensis* is a primary malaria vector while *An. pharoensis*, *An. funestus* and *An. nili* were regarded as secondary vectors in Ethiopia (Jolivet 1959; O'Connor 1967; Krafur 1970; Mekuria et al. 1982). Previous studies showed that the country's longer period application of insecticides for malaria prevention and control is believed to have affected the feeding and resting behaviour of *An. arabiensis* (Geber-Mariam et al. 1988; Tulu 1993; Nigatu et al. 1992; Hargreaves et al. 2000; Abose et al. 1998; Krafur 1977; Fettene et al. 2013). The resurgence of resistant *An. funestus* in certain parts of southern Africa indicates the potential impact of this species (Hargreaves et al. 2000).

Resistance to DDT and pyrethroids were found in survey done in SNNPR (Fettene et al. 2013). Although, Fettene and his colleagues found no bendiocarb resistance in the two sites tested in SNNPR in 2011, due to short residual life on walls, the emergence of resistance to bendiocarb appears to be a recent phenomenon (Yemane et al. 2016). In Ethiopia a resistance mechanism of changes in the sites of insecticidal target in the main malaria vector was identified, thus, some of these resistant vector genes have the potential to threaten the effectiveness of current malaria vector control interventions in the country (Ameneshewa and Service 1996; Balkew et al. 2010; Yewhalaw et al. 2011). Therefore, the observed resistance to most of the insecticides used for malaria vector control joined with the incidence of high knockdown resistance (kdr) frequency in populations of *An. arabiensis* could profoundly challenge the malaria vector control program in Ethiopia (Massebo et al. 2013; PMI/Africa 2015).

Insecticide resistance management can only be done effectively if accurate data is available in order to assess the status of insecticide resistance in a country. In Ethiopia, various institutions have been engaged in generating insecticide resistance status of insecticide(s) for IRS. The resistance status of *An. arabiensis* to insecticides widely used for IRS is worrying in Ethiopia.

For instance, resistance to DDT and pyrethroids is widespread, and resistance to malathion and carbamates has been detected as well (Yewhalaw et al. 2011; Massebo et al. 2013; PMI/Africa 2015). Therefore, the aim of the present study was to generate reliable information on resistance status of the main vector and to propose alternative insecticides to be used for indoor residual spray in selected sites as well as the entire areas of Ethiopia.

## Material and methods

**Study design and area:** A prospective cohort study design and a multistage sampling method were used. A standard World Health Organization procedure was applied and bioassay test kit was used (WHO 1998). The present study was conducted in Meskan (Gogete), Sodo (Jolie), Hawasa Zuria (Tkure Weha), Arba Minch Zuria (Shelie) and Boloso Sore (Areka) districts administratively found in Sothorn Nations Nationalities Peoples' Region (SNNPR), Ethiopia (Figure 1). Most of the areas in the region were malaria endemic in which about two-thirds of the population was at risk of malaria infection.



Figure 1: Map of study areas in Ethiopia, 2017

**Characteristics of the study sites:** The present study was conducted in five selected sites located in lowland and highland areas with elevation between 1137 m and 1846 m. The detailed characteristics of the study sites are presented below (Table 1).

**Study area selection procedure:** Currently, Ethiopia is administratively structured into nine regional states (Tigray, Affar, Amhara, Oromiya, Somali, Benshangul-Gumuz, Southern Nations, Nationalities and Peoples: SNNP, Gambela, and Harari), and two city administrations (Addis Ababa and Dire Dawa). The SNNP regional state was purposively selected based on the local knowledge from the respective regional health bureau. The study districts are among malaria endemic areas selected from various eco-epidemiological settings, which are administratively located in Sidama,

Gurage, Gamo Gofa and Wolayita zones. Within each zone multi-stage sampling method was used to select districts, and a *Kebele* from each district was randomly selected from malarious ones. Thus, collections of mosquitoes` larvae were conducted in a variety of

aquatic habitats of the study area, from pools of temporary and permanent breeding sites (ponds, pools of rain water, water from hand dug well, river pocket, stagnant water in irrigation canals and marsh) in the study areas using dippers, pipettes and containers.

**Table 1: Geo-location, elevation and epidemiological description of the selected sites, SNNP Regional State, Ethiopia, 2017.**

| Study site  | District          | Latitude/Longitude               | Elevation | Description                                                                                                                  |
|-------------|-------------------|----------------------------------|-----------|------------------------------------------------------------------------------------------------------------------------------|
| Shelie      | Arba Minch Zuriya | N 05° 52. 007'<br>E 037° 28.655' | 1137 m    | Lowland area experiencing high malaria endemicity                                                                            |
| Tikure Weha | Hawassa Zuriya    | N 07° 05.453'<br>E 038° 28. 818' | 1677 m    | Highland area with seasonal transmission and epidemic risk                                                                   |
| Gogete      | Sodo              | N 08° 10.371'<br>E 038° 30. 449' | 1846 m    | Highland-fringe area with low malaria prevalence in which risk of outbreaks is high during abnormal weather conditions occur |
| Jolie       | Meskan            | N 08° 10.371'<br>E 038° 30. 449' | 1846 m    | Highland-fringe area with low malaria prevalence in which risk of outbreaks is high during abnormal weather conditions occur |
| Areka       | Boloso Sore       | N 07° 08. 388'<br>E 032° 41.409' | 1691 m    | Highland area with seasonal transmission and epidemic risk                                                                   |

**Types of insecticides and their classes tested:** A total of seven insecticides recommended for indoor residual spray purpose categorized into four classes were

evaluated in the present study. These insecticides are enlisted as follows in the table shown below (Table 2).

**Table 2: List of tested classes, insecticides and year of implementation for indoor residual spray, Ethiopia, 2017**

| S.N | Class                | Insecticides | Concentration (%) | Implementation year |
|-----|----------------------|--------------|-------------------|---------------------|
| 1   | Organochlorine (OC)  | DDT          | 4                 | 1959                |
| 2   | Organophosphate (OP) | Malathion    | 5                 | 1986                |
| 3   | Pyrethroid (PY)      | Deltamethrin | 0.05              | 2008                |
| 4   | Carbamates (OP)      | Fenitrothion | 1                 | Not implemented     |
| 5   | Carbamates (C)       | Bendiocarb   | 1                 | 2010                |
| 6   | Carbamates (C)       | Propoxure    | 0.1               | 2012                |

#### **Mosquito collection and processing Procedure:**

Aquatic forms of *Anophelienes* were collected from common breeding sites using standard procedures applied elsewhere (WHO, 1998). Then, mosquitoes were reared to adults and identified at species level using keys provided by (Verrone 1962) and (Gilles and Coetzee 1987), which use morphological characteristics.

**Insecticide susceptibility tests procedure:** Adult mosquitoes obtained from the specific study sites were exposed to the standard test with specific diagnostic doses for determining insecticide resistance (WHO 1998). The procedure for exposing mosquitoes to the standard test for both experimental and control groups is presented briefly as follows. First, adult of known species and 20-25 unfed as well as 2-3 days old *Anopheline* mosquitoes were transferred to plastic tube with insecticide-impregnated filter paper. The filter papers were held in position by special rings prepared for this purpose for the experimental group. Similarly, the same number of *Anophelines* were transferred to a holding tube lined with insecticide-free paper for the control group (a paper impregnated with olive oil for Organophosphate (OP) insecticides and DDT, and silicon oil for pyrethroids). Second, the investigator closed the slides and allows the exposure and control tube to remain for an hour long. Next, mosquitoes were

transferred back to the holding tube, which should stand upright for 24 hours. A piece of moist cotton wool on the gauze end and a wooden box with large holes (for ventilation) covered with a damp towel was used in order to maintain the ideal test conditions of temperature between 25°C and 30°C ( $\pm 2^\circ\text{C}$ ) and relative humidity of between 70 and 80%. Finally, mosquitoes were held in cages for 24 hours duration with access to sugar solution. The knockdown effect (KD) of insecticides was also recorded after 60 minutes with an interval of 10 minutes after exposure and mortality after 24 hours. The test was repeated four times for the experimental group and two replicas were used for the control.

**Data management and analysis:** Mosquito mortality was calculated using a formula derived by (Abott 1925):

$$\text{Control mortality (C)} = \frac{\text{No. of dead mosquitoes}}{\text{Total No. of mosquitoes in control tube}}$$

$$\text{Exposure mortality (E)} = \frac{\text{No. of dead mosquitoes}}{\text{Total No. of mosquitoes in experimental tube}}$$

If control mortality is greater than or equal to 5% and less than or equal to 20%, the value for exposure mortality (E) will be corrected by using the following formula (Abbott's formula) (Abott, 1925):

$$\text{Corrected exposure mortality (\%)} = E - C \times 100/100 - C$$

The percentages of Knock Down were also calculated. Interpretation was done by using the World Health Organization (WHO) cut-off point [WHO, 1998], therefore, the value from 98-100% mortality indicates susceptibility (S), 90-97% mortality implies the possibility of resistance that needs to be confirmed (PR) and < 90% mortality suggests resistance (R).

## Result

Overall, a total of 5,250 *Anopheles* mosquitoes were

used for the test in the five sites. In Shelie, Tikure Weha, Gogete and Jolie a total of 4,200 *An. gambiae s.l.*, were used that is, 1,050 *An. gambiae s.l.*, were tested in each study site and only 1,050 *An. funestus s.l.*, were tested in Areka. Knockdown was monitored only for the three insecticides with known excito-repellent activity (DDT, permethrin, and deltamethrin). The results are shown in Table 3. None of the three insecticides resulted in more than 95% knockdown, the cut-off value recommended by WHO.

**Table 3: Percentage of knockdown (and 95% CI) in *Anopheles* mosquitoes 60 minutes after exposure to insecticide in WHO susceptibility tests (at each does 100 mosquitoes were used), Ethiopia, 2017**

| Study site  | Species                  | DDT            | Permethrin     | Deltamethrin   |
|-------------|--------------------------|----------------|----------------|----------------|
| Shelie      | <i>An. gambiae s.l.</i>  | 54 (43.7–64.0) | 67 (56.9–76.1) | 73 (63.2–81.4) |
| Tikure Weha | <i>An. gambiae s.l.</i>  | 52 (41.8–62.1) | 64 (53.8–73.3) | 67 (56.9–76.1) |
| Gogete      | <i>An. gambiae s.l.</i>  | 51 (40.8–61.1) | 62 (51.7–71.5) | 65 (54.8–74.3) |
| Jolie       | <i>An. gambiae s.l.</i>  | 49 (38.9–59.2) | 61 (50.7–70.6) | 64 (53.8–73.3) |
| Areka       | <i>An. funestus s.l.</i> | 57 (46.7–66.9) | 71 (61.1–79.6) | 63 (52.8–72.4) |

Similar results were found for mortality in bioassays with these insecticides (Table 4). Mortality after exposure to DDT and permethrin was never higher than 50% in any of the sites. Mortality after exposure to deltamethrin ranged between 54 and 61% for *An. gambiae s.l.*, and was similar (61%) for *An. funestus*. The mortality after exposure to organophosphates differed by insecticide, with completely susceptibility to

fenitrothion in all sites, but resistance to malathion in all sites (mortality ranging between 64 and 79%). *Anopheles funestus* were completely susceptible to both carbamates tested (bendiocarb and propoxur). *Anopheles gambiae s.l.* had a range of mortality between 97 and 100% for propoxur, and displayed more resistance to bendiocarb, with mortalities between 92 and 97%.

**Table 4: Percentage of mortality (and 95% CI) in *Anopheles* mosquitoes 60 minutes after exposure to insecticide in WHO susceptibility tests (at each does 100 mosquitoes were used), Ethiopia, 2017**

| Study sites | Insecticides      |                   |                   |                   |     |                   |                   |
|-------------|-------------------|-------------------|-------------------|-------------------|-----|-------------------|-------------------|
|             | DDT               | Per               | Del               | Mal               | Fen | Pro               | Ben               |
| Shelie      | 15<br>(8.6-23.5)  | 41<br>(31.3-51.3) | 58<br>(47.7-67.8) | 76<br>(66.4-83.9) | 100 | 99<br>(94.4-99.9) | 97<br>(91.5-99.4) |
| Tikure Weha | 29<br>(20.3-36.9) | 41<br>(31.3-51.3) | 61<br>(50.7-70.6) | 68<br>(57.9-76.9) | 100 | 100               | 95<br>(88.7-98.3) |
| Gogete      | 15<br>(8.6-23.5)  | 39<br>(29.4-42.3) | 56<br>(45.7-65.9) | 64<br>(53.8-73.3) | 100 | 97<br>(91.5-99.4) | 93<br>(86.1-97.1) |
| Jolie       | 12<br>(6.3-20.0)  | 37<br>(27.5-47.2) | 54<br>(43.7-64.0) | 67<br>(56.9-76.1) | 100 | 98<br>(93.0-99.7) | 92<br>(84.8-96.5) |
| Areka*      | 29<br>(20.3-38.9) | 44<br>(34.1-54.3) | 61<br>(50.7-70.6) | 79<br>(69.7-86.5) | 100 | 100               | 100               |

\* *An. funestus* tested.

## Discussion

The present study was conducted to assess the resistance status of *An. gambiae s.l.*, and *An. funestus s.l.*, to insecticides used for malaria vector control in southern Ethiopia. Propoxur was recently applied in pockets of areas in the country for IRS use. However, other insecticides from Pyrethroid class were applied for impregnation of mosquito nets and IRS operation. The present finding indicated that both *An. gambiae s.l.* and *An. funestus s.l.* were susceptible to Fenitrothion and Propoxur. Otherwise, *An. gambiae s.l.* has shown possibility of resistance for Bendiocarb that needs confirmation. But, *An. funestus s.l.*, depicted susceptibility to Bendiocarb. While, the KD and mortality of DDT, Malathion, Permethrin and Deltamethrin to *An. gambiae s.l.* and *An. funestus s.l.*,

were found not satisfactory. From the present finding it appears knockdown resistance mechanism could be operating in this mosquito population. The result of knock down were comparable to those published for *Anopheles* strains classified as resistant to DDT, permethrin and Deltamethrin (Balkew et al. 2006; Balkew et al. 2010; Yewhalaw et al. 2011; Massebo et al. 2013). Besides, the resistance to DDT and pyrethroids found in this survey are consistent with other susceptibility tests done in SNNPR. Fettene and his colleagues (Fettene et al. 2013) found resistance to deltamethrin in Mirab Abaya, and resistance to deltamethrin and DDT in Boloso Sore, both near to the sites studied here. Besides, Massebo and his associates (Massebo et al. 2013) found low levels of mortality with lambdacyhalothrin, alphacypermethrin, cyfluthrin,

deltamethrin, and DDT (10-56%), but found acceptable levels of knockdown for cyfluthrin (96%) and deltamethrin (100%). In the most recent report (2016) on insecticide susceptibility by the AIRS project (Abt Associates), *An. gambiae s.l.* was resistant to DDT, deltamethrin, and permethrin in Arba Minch (PMI/Africa IRS 2015). While DDT and pyrethroids are not used for IRS in Ethiopia, LLINs continue to be distributed and the effects of pyrethroid resistance on the effectiveness of LLINs should be further studied.

In our present finding *An. gambiae s.l.* was completely susceptible to Fenithrithion and Propoxur in three sites such as Shelie, Tikure Weha, and Jolie while *An. funestus* in Areka area which was in line with studies conducted in eastern and south-western Ethiopia (Balkew et al. 2012; Yewhalaw et al. 2011). A study in eastern Ethiopia illustrated that *An. gambiae s.l.* was found to be highly susceptible to propoxur (mortality rate of 99.5%) in 'low risk' and in south-western (100% mortality) in 'high risk' villages (Yewhalaw et al. 2011). In addition, Balkew and his colleagues (Balkew et al. 2012) reported susceptibility of *An. gambiae s.l.* to Fenithrithion and Propoxur with a result of 98.2% and 99.5%, respectively. However, the result of propoxur in Gogete was 97%, which indicates potential resistance of *An. gambiae s.l.* that needs to be confirmed (WHO 1998). The difference between our present finding and earlier studies is not surprising because our study was done two years later of introduction compared to the earlier studies that were done before the introduction which was five years ago.

According to WHO guideline results with an average percentage of mortality rate of below 80% are interpreted as potentially resistant that need to be confirmed (WHO 1998). Our findings of below the cut-off in Shelie, Tikure Weha, Gogete and Jolie for *An. gambiae s.l.* against Bendiocarb is in agreement with another study done in Ethiopia (Balkew et al. 2012). In addition, our present finding was in harmony with a study done elsewhere (WHO 2012). Moreover, a sporadic resistance to carbamates and organophosphates has also been reported in Somalia and Sudan (Maxmen 2012; WHO 2012).

While considerably less bendiocarb is being used than in previous years due to short residual life on walls (Yemane et al. 2016), the emergence of resistance to bendiocarb appears to be a recent phenomenon since Fettene and his friends, found no bendiocarb resistance in the two sites tested in SNNPPR in 2011. The AIRS report also did not indicate resistance to bendiocarb in the 2016 results from Arba Minch (PMI/Africa IRS 2015). More concerning are the incomplete mortalities to propoxur, an insecticide which is still widely used for IRS in Ethiopia. While only one site reported less than 98% mortality (Gogete), two other sites had a small

percentage of mosquitoes surviving the bioassay, potentially an early sign of resistance.

The present study found that *An. gambiae s.l.* and *An. funestus* showed a marked resistance to DDT, Permethrin, Malathion and Deltamethrin in all sites. Additionally, in South Ethiopia Massebo et al. (2013) reported that, a high proportion of *An. gambiae s.l.* was resistant to the pyrethroids, with a mortality rate of 56% for lambdacyhalothrin, 50% for cyfluthrin and alphacypermethrin, 47% for Deltamethrin, and 10% for DDT. Similarly, the wide-scale use of insecticide-based malaria control strategies over the past decade has been associated with the development of resistance in several important vector species, including *Anopheles gambiae s.l.*, *An. funestus* and *An. pharoensis* (WHO 2011b; WHO 2011c; Balkew et al. 2006). Likewise, many studies conducted in different parts of Ethiopia repeatedly confirmed the presence of resistance to the above-mentioned insecticides, so our result was not a surprising one (Balkew et al. 2012; Balkew et al. 2003; Ameneshewa and Service 1996; Balkew et al. 2010; Yewhalaw et al. 2011; Massebo et al. 2013).

The less predominance of *An. gambiae s.l.* in Areka study area reflects the fact that larvae were sampled at the ending of the rainy season when conditions tend to be drier and unsuitable. In this study area *An. funestus* was found abundantly in semi-permanent body of fresh water with emergent vegetation such as swamps and large ponds. Similarly, Ameneshewa and Service (1996) noted 37.5 % exophily in an insecticide sprayed village in the Upper Awash Valley. This shows that the species is increasingly exhibiting exophily, as a result, it is becoming more difficult to get rid of by chemical insecticides applied indoors.

More interestingly, the present study reported resistance level of *An. funestus* unlike previous studies. This finding is in harmony with an earlier study by Jolivet (1967) in Gambela which noted the complete disappearance of this species from insecticide sprayed dwellings, while few catches were made from unsprayed dwellings (Jolivet 1967; Nigatu et al. 1992).

The susceptibility results for *An. funestus* are important as few tests have been conducted on this species in Ethiopia. The role of *An. funestus* should be further studied, as it was reported that it has a wide distribution in Ethiopia (Jolivet 1967). Whether the lack of recent reports is due to a decrease due to vector control interventions or systematic underreporting, the resurgence of resistant *An. funestus* in certain parts of southern Africa (Hargreaves et al. 2000) should serve as a reminder of the potential impact of this species.

## Conclusion

The resistance to the pyrethroids used on LLINs is concerning, as is the reduced mortality to insecticides

currently used in Ethiopia for IRS. Susceptibility tests should continue as a part of insecticide resistance monitoring in southern Ethiopia, as well as researches to determine the effects of resistance on control.

**Conflict of interests:** The authors declare that they have no conflict of interests.

**Authors' contributions:** EKW designed the study; analyzed and interpreted data participated in the field and laboratory work and drafted the manuscript. SI participated in data analysis and interpretation; SA revised the manuscript. YA coordinated field activity and revised the manuscript. AH participated in the field activities. YW participated in the field activities and laboratory work. HT designed the study, supervised and revised the manuscript.

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