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RESEARCH ARTICLE

EFFECTS OF OIL EXTRACTS OF *CYPERUS PAPYRUS* L. AND *TYPHA LATIFOLIA* L. ON SURVIVAL OF LARVAE OF THE MALARIA MOSQUITO, *ANOPHELES ARABIENSIS* PATTON

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ABSTRACT: Around Lake Tana, larvae of the malaria mosquito *Anopheles arabiensis* Patton were rare to be found in lakeshore breeding habitats with *Cyperus papyrus* L. and could only be recovered with low probability from breeding habitats with *Typha latifolia* L. This study aimed to investigate oil contents of the grasses and effect of natural oils extracted from rhizome and aerial parts of these grasses on the survival of *An. arabiensis* mosquito larvae under laboratory conditions. Samples of *C. papyrus* and *T. latifolia* were collected from different mosquito breeding habitats along the shoreline of Lake Tana, northwest Ethiopia. Natural oils from rhizome and aerial parts of these grass species were extracted with n-hexane using Soxhlet extractor apparatus and their contents were quantified. Different doses of the oil extracts were dissolved in ethanol and were tested to determine their toxicity to laboratory reared 4th stage larvae of *An. arabiensis*. The rhizome of *Cyperus papyrus* has significantly higher oil content (2.09 ± 0.23) than that of *T. latifolia* (1.455 ± 0.011) ($P<0.05$). The toxicity of the oil extracts from both *T. latifolia* and *C. papyrus* grasses increased in a dose dependent manner to cause significant larval mortality on *An. arabiensis*. Accordingly, the present study confirmed that the rhizome of *C. papyrus* has substantial amount of natural oils that makes this grass species as a candidate to reduce populations of *An. arabiensis* in the area. The effect of natural oils from these grass species in regulating mosquito populations in potential breeding habitats need to be further evaluated. The ecological significance of the high amount of natural oil production by rhizome of *C. papyrus* in breeding habitats needs to be studied.

Key words/phrases: *Anopheles arabiensis*, Bahir Dar, Breeding habitats, *Cyperus papyrus*, Grass natural oils, Larval mortality, *Typha latifolia*.

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INTRODUCTION

Emergent grasses in permanent and semi-permanent mosquito breeding habitats are important biological characteristics which determine larval productivity and abundance (Fillinger *et al*., 2004; Mekonnen Yohannes *et al*., 2005; Minakawa *et al*., 2012; 2004; Mwangangi *et al*., 2008; Ndenga *et al*., 2012; Rejmánková *et al*., 2013), and oviposition site selection of *Anopheles* mosquitoes (Betelehem Wondwosen *et al*., 2016; 2017; Yelfwagash Asmare *et al*., 2017a). Grasses such as cattails, *Typha* spp.(Typhaceae) and dallis grasses (*Paspalum* spp.; Poaceae) in mosquito breeding sites support the breeding of *Anopheles arabiensis* Patton and *An*. *coluzzii* Coetzee, Hunt & Wilkerson (Bøgh *et al*., 2003; Gouagna *et al*., 2012; Khater *et al*., 2013). On the other hand, grasses such as *Cyperus* spp. (Cyperaceae) and *Phragmites* spp. (Poaceae) have negative effect and reduce occurrence of *Anopheles* larvae in potential breeding habitats (Fillinger et al., 2004; Goma, 1960a; 1960b; Mwangangi *et al*., 2008). This is attributed to the natural oils produced by these grass species which are implicated to have larvicidal effects (Lindsay and Martens, 1998; Mouchet *et al*., 1998).

In line with the above findings, recent larval surveys revealed that *Typha latifolia* L. (Typhaceae) dominated mosquito breeding sites associated with the littoral zone of southern edge of Lake Tana, northwestern Ethiopia, produced less *Anopheles* mosquito larvae than breeding sites with grasses including *Echinochloa pyramidalis* (Lam.) Hitchc. & Chase.) and *E*. *stagnina* (Retz.) P.Beauv. (Poaceae). Interestingly and in contrast, breeding habitats dominated by *Cyperus papyrus* L. (Cyperaceae) failed to produce any *Anophele*s mosquito larvae. Moreover, the gravid females of *Anopheles arabiensis* Patton and *An*. *coluzzii* Coetzee, Hunt & Wilkerson showed relatively lower attraction and oviposition preferences to volatiles of *T*. *latifolia* L., and showed least preferences to that of *C*. *papyrus* L. compared to volatiles of *E*. *pyramidalis* and *E*. *stagnina* (Yelfwagash Asmare *et al*., 2017a).

Identification of aquatic grass species which have negative effect on breeding success of *Anopheles* mosquitoes is crucial to address the limitations in malaria vector interventions (Walton, 2005). Larval control markedly contributes to suppress populations of *An*. *gambiae* and *An*. *arabiensis*, and reduce malaria transmission in Africa (Walker and Lynch, 2007), and incorporates source reduction and utilization of chemical and bio-larvicides as components of integrated malaria vector management

(Bukhari *et al*., 2013; Mekonnen Yohannes *et al*., 2005; WHO, 2006). However, these larval control options have limitations including slow activities with entomopathogens, and development of insecticide resistance in malaria mosquitoes, as well as development of resistance to the toxins of *Bacillus thuringiensis israelensis* and *Bacillus sphaericus* (Cory and Franklin, 2012; Kamareddine, 2012; Lacey, 2007; Wirth *et al*., 2010). Moreover, the use of conventional insecticides to target larval stage of mosquitoes introduces risks of toxicity to humans and other non-target organisms in the environment (Amer and Mehlhorn, 2006). The use of plants and their extracts for mosquito larval control is more ecologically sound and sustainable than the conventional insecticides and the bacterial toxins, and therefore, can be integrated in vector management to reduce malaria transmission.

This study investigated the oil contents of *T. latifolia* and *C. papyrus*, and concentration gradient effects of natural oil extracts from rhizome and aerial parts of these aquatic grass species on survival of *An. arabiensis* larvae under laboratory conditions, and correlated their toxicity with the amount of natural oils extracted from the rhizome and aerial parts of the grasses. The potential of these grass species in producing substantial yield of natural oils and implications for their ecological role in regulating populations of *Anopheles* mosquitoes are discussed.

MATERIALS AND METHODS

Description of the study area

The rhizome and aerial parts of *Typha latifolia* and *Cyperus papyrus* were collected from their natural habitats at the southern edge of Lake Tana, Bahir Dar, northwestern Ethiopia (11°37′N, 37°21′E; 1,830 m above sea level). The area is characterized by the climate of semi-arid regions close to the equator. The daily temperature varies between diurnal extremes of 30°C to night time lows of 6°C, with mean temperatures between 20 and 27°C. The average annual rainfall is about 1,440 mm with the long rainy season extending from May to October, with a peak in July-August (Vijverberg *et al*., 2009).

Test plant oil extraction procedure

The rhizome and aerial parts of *T. latifolia* and *C. papyrus* were brought to the laboratory where the plant parts were processed for oil extraction (Fig. 1A, 1B, 1C, & 1D). Natural oils from these plant species were separately extracted using solvent extraction methods (Bream *et al*., 2009). The rhizome and areal parts of the test plant species were manually sliced to small sizes separately (Fig. 1E & 1F) and allowed to dry at room temperature 25ºC for about 10 days under shade (Bream *et al*., 2009). The dried plant materials were then crushed into fine powder (Fig. 1G & 1H) using locally available coffee grinder.

Fig. 1. Plant material collection and preparation for oil extraction procedure: plant material samples including aerial (A) and rhizome (B) parts of *Cyperus papyrus*, and aerial (C) and rhizome (D) parts of *Typha latifolia*; the aerial parts and rhizome of both grass species were sliced (E, F), dried (G), and powdered (H) separately.

Extraction of crude natural oils from the plant materials were made in Bahir Dar University, School of Chemical and Food Engineering, Ethiopia, using Soxhlet extractor with an organic solvent, n-hexane (Fig. 2). About 20 g of finely powdered test plant material was added to the Soxhlet extractor apparatus in one run and subjected to solvent extraction with n-hexane for about 4 hrs. The oil extracts were placed in an oven at 40ºC for 2 hrs to remove excess solvent (n-hexane) from the extracted oil samples. The oil extracts were weighted and kept in refrigerator (+4ºC) until use for experiments. The oil content of the test plant materials was determined as percentage of the weight of extracted oil (g) to dry weight of the plant materials (g).

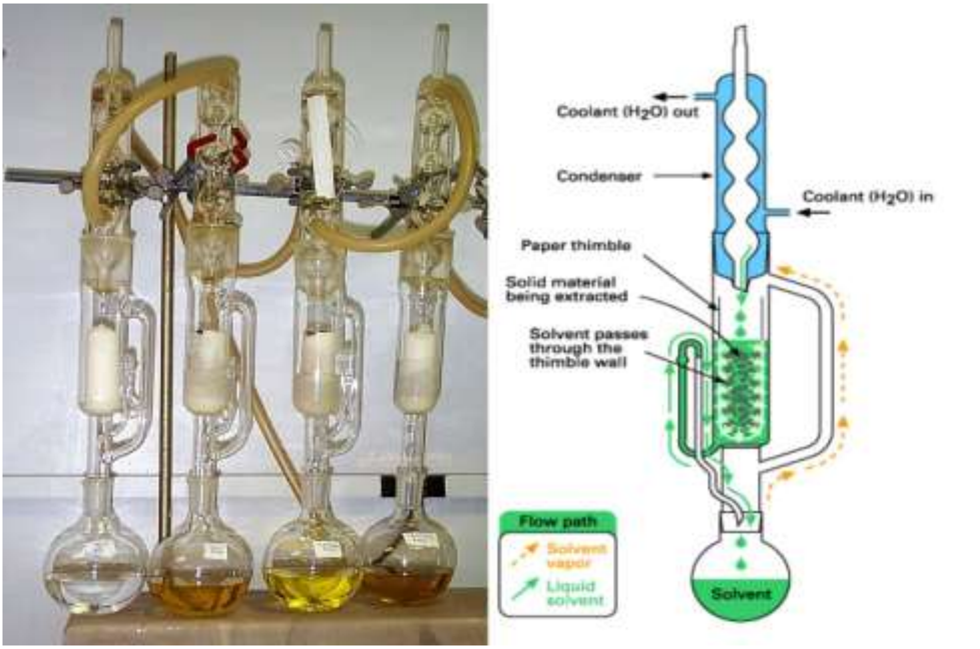


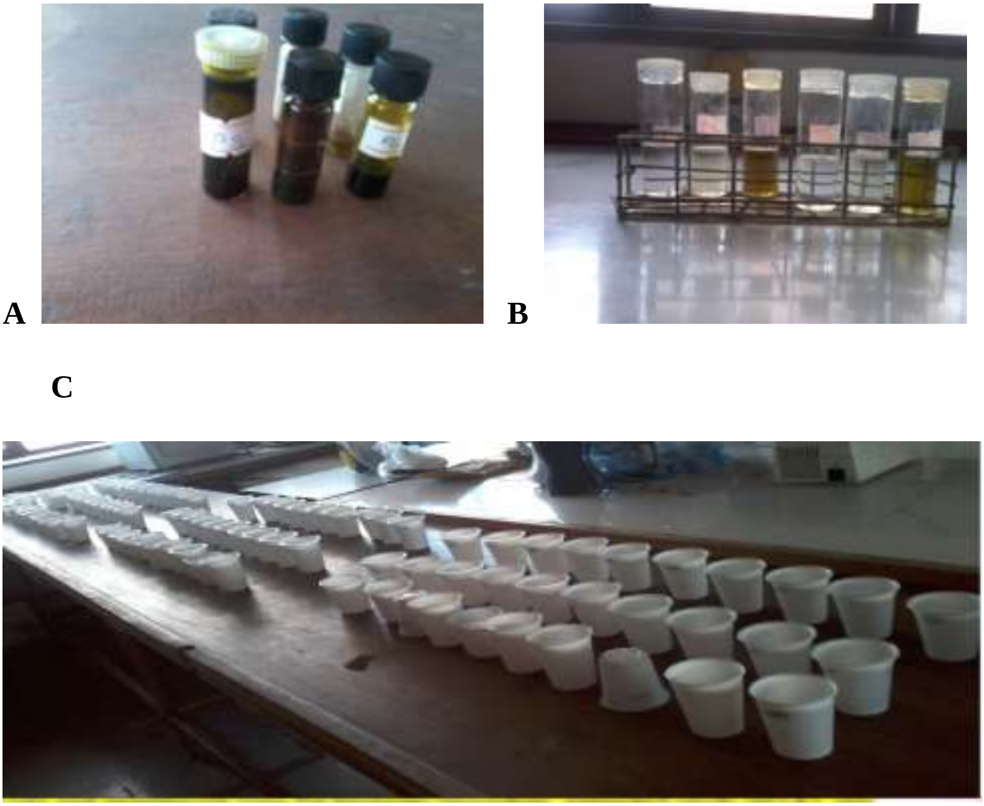
Fig. 2. Schematic representation of oil extraction apparatus and procedures using Soxhlet extractor (Source: Rassem *et al*., 2016).

Experimental mosquito rearing procedures

Laboratory colonies of *Anopheles arabiensis* Patton were maintained at 27±2 °C, 80% relative humidity and at a 12 h: 12 h light: dark photoperiod in the insectary of the Ethiopian Public Health Institute (EPHI), Addis Ababa. Larvae were reared in plastic trays (22 cm x 34 cm x 10 cm) filled with 1 l distilled water and fed powdered Phoenix Koy Pellets (Armitage Pet Products Ltd., Nottingham, UK) daily. Pupae were removed from the rearing trays and placed in insect cages (30 cm x 30 cm x 30 cm) for adult emergence. Adult males and females were kept together and provided *ad libitum* access to 10% sucrose solution. For colony maintenance, female mosquitoes were blood fed on a live rabbit at 3-4 day intervals. Feeding mosquitoes on live rabbits was administered following the Ethiopian Public Health Institute (EPHI) rearing procedures (EPHI, 2017; FMST, 2014). Gravid females were allowed to lay eggs in 30 ml plastic cups filled with distilled water, and eggs were transferred to larval trays for hatching. Subsequently, 4th stage of *An. arabiensis* larvae from rearing trays were transferred to experimental bioassay cups and were used for experiments to evaluate the bioactivities of the natural oils extracts.

Bioassays on *Anopheles arabiensis* larval survival with grass oils

Ten mg of the crude natural oil extract from aerial and rhizome parts of *Cyperus papyrus* L. and *Typha latifolia* L. and the same amount of control oil (refined seed oil of sunflower, *Helianthus annuus*) was dissolved in separate vials in 10 ml of 97% ethanol solvent (Fig. 3A). This stock solution of 1 mg oil/ml of solvent (Fig. 3B) was subjected to a ten-fold serial dilution resulting 0.1 mg/ml, 0.01 mg/ml concentration gradients with a logarithmic scale and used for experiments. Fourth stage of *Anopheles arabiensis* were placed individually in 150 ml size plastic cups (Dongyang City Plastics Co., Shanghai, China) filled with 49 ml distilled water and 1 ml from each concentration gradient of treatment and control oils as well as 1 ml solvent ethanol (the oil negative control) was directly applied into each plastic cup in the experiment setup (Fig. 3C) to investigate larval survival with oils over dose series. Each concentration gradient of treatment and control oil were tested on individual 4th instar larvae with 10 replicates set on the same day in parallel to buffer day effects. The experiment was repeated on three separate days. Larval mortality was recorded after 12 h, 24 h, 48 h, 72 h and 84 h exposure time series and the larvae were maintained without nutritional supplement for this time series during the experiment.



Fig, 3. Preparation of oils and experiment cups for bioassay procedure: extracted crude oil (A), oils dissolved in solvent ethanol (B), and set up of experiment cups (C).

Statistical analyses

The General Linear Model (GLM) Univariate procedure was applied to analyze the oil content of the grass species and their parts using the statistical software IBM SPSS Statistics for Windows, Version 21.0. Significant differences between means were determined at α=0.05 and post hoc multiple comparisons among the grasses were made using the Tukey's HSD test.

A fully parametric form of survival time to event analysis was used to model the data and to produce a measure of the probability of larval death that occurred over time series in *An*. *arabiensis* larvae treated with oils over dose series using SAS (JMP®) Version 10.0.0. (SAS Institute, University of UK). The model was based on the Weibull distribution for natural oils of the macrophyte species (*Typha latifolia* and *Cyperus papyrus*) and the control refined seed oil of *Helianthus annuus*. Overall model of effects of both type of oils and dose series of oils on larval survival of *An. arabiensis* was run for the time intervals between start of the experiment, i.e., 4th stage larval death as the response (Y) variables; censoring those that emerged before the end of the experiment. Likelihood ratio test was used to determine significant differences between oils at different doses by their effect on the larval mosquito survival based on χ^2 and P-value. Then, analysis of time series was made to determine the particular time at which the peak (maximum) larval death resulted by overall effect of oils. Finally, GLM Univariate procedure was applied to analyze overall larval death by the effect of oils over dose series at the particular time of peak (maximum) larval death using the statistical software IBM SPSS Statistics for Windows, Version 21.0. Significant differences between means were determined at $\alpha=0.05$ and post hoc multiple comparisons among the grasses were made using the Tukey's HSD test.

RESULTS

Oil contents of *Typha latifolia* and *Cyperus papyrus*

There were differences in the oil contents of the rhizome and aerial parts of the grasses *Typha latifolia* and *Cyperus papyrus* by percentage of the weight of extracted oil (g) to dry weight of the plant materials (g) (Fig. 4). The results showed rhizome section of *C. papyrus* was found with significantly higher oil content (2.09 ± 0.23) than that of *T. latifolia* (1.455 ± 0.011) ($P<0.05$). Moreover, the oil content of rhizome part of *C. papyrus* was significantly higher than that of its aerial part (1.836 ± 0.001) and that of aerial part of *T. latifolia* (1.8 ± 0.23) ($P<0.05$). *T. latifolia* rhizome yielded the lowest oil content of all the tested plant materials oil contents.

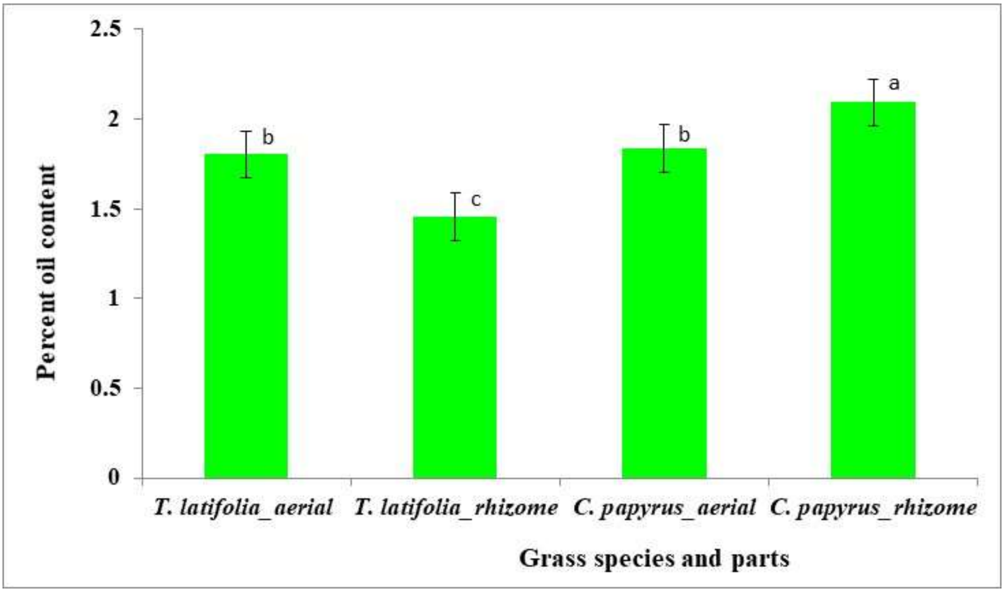


Fig. 4. Oil contents of *Typha latifolia* and *Cyperus papyrus* and their parts determined as percentage yield of oil (percent of the ratio of weight of oil to dry weight of aerial and rhizome parts the plants). The mean oil content with different letters are significantly different from one another (univariate general linear model with a Tukey's post-hoc analysis; $P<0.05$). Error bars represent the standard error of the mean. Doses with different letters are significantly different.

Effects of oil extracts on survival of *Anopheles arabiensis* larvae

There were no overall significant differences in the effect of oil extracts from the rhizome and aerial parts of *Cyperus papyrus* and *Typha latifolia* as well as the control (seed oil from *Helianthus annuus*) on survival time of 4th stage larval *An. arabiensis* (df = 4, $\chi^2 = 2.611$, P = 0.624). No significant differences were observed among the oil extracts in their effect on survival time of larval *An. arabiensis* over increasing dose series (df = 4, $\chi^2 = 0.095$, P = 0.998). Analysis of dose dependent effects of tested oil extracts on survival time of *An. arabiensis* over time series showed that peak (maximum) mortality of 4th stage larvae of *An. arabiensis* occurred at 24 h after application of the tested oils on the larvae (Fig. 5).

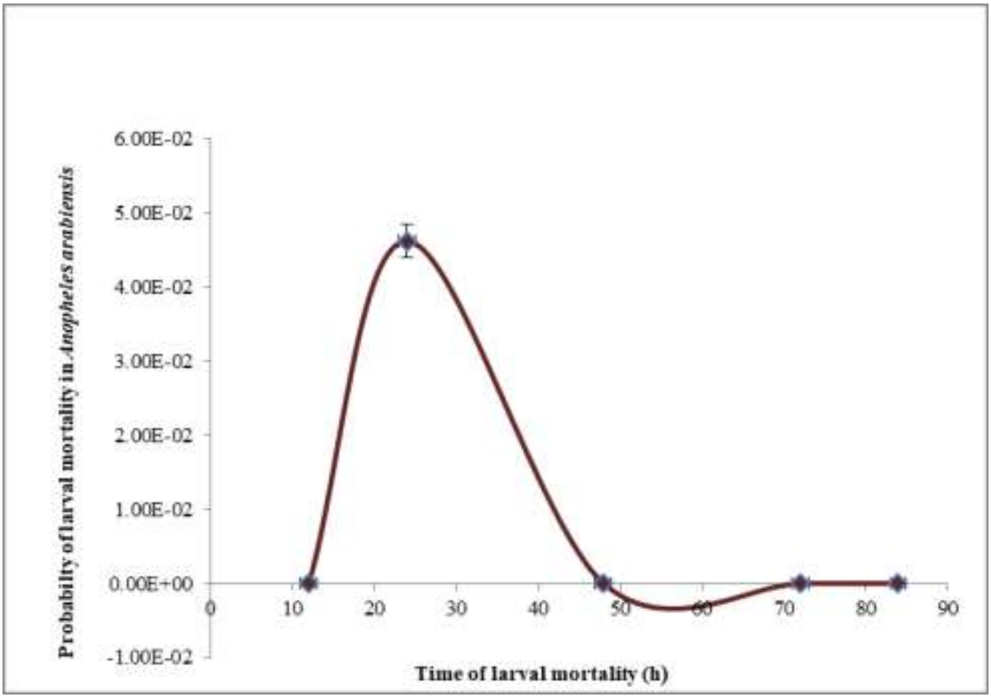


Fig. 5. Probability of 4th stage larval mortality of *Anopheles arabiensis* over time series in response to oil under laboratory conditions: Error bars represent the standard error of the mean.

Analysis of effects of grass oil extracts on *Anopheles arabiensis* larvae over dose series showed that significantly higher percent mortality of the larvae occurred by increasing the dose of oils (df = 1, χ^2 = 32.391, P<0.0001). Further analysis of effect of oil extracts on survival of larvae over dose series showed that the dose of 1 ml oil extract resulted in significantly higher percent mortality of 4th stage *An. arabiensis* larvae than doses 0.1 ml and 0.01 ml oil extracts and the control (ethanol) at the specified 24 h time of maximum larval mortality (df =3, χ^2 = 34.374, P<0.001) (Fig. 6). There were no statistically significant differences between the lower doses of oil extracts (0.1 ml and 0.01 ml) and the control (ethanol=0.00 ml oil) by their effect on survival of *An. arabiensis* larvae (df = 3, χ^2 = 0.097, P = 0.978).

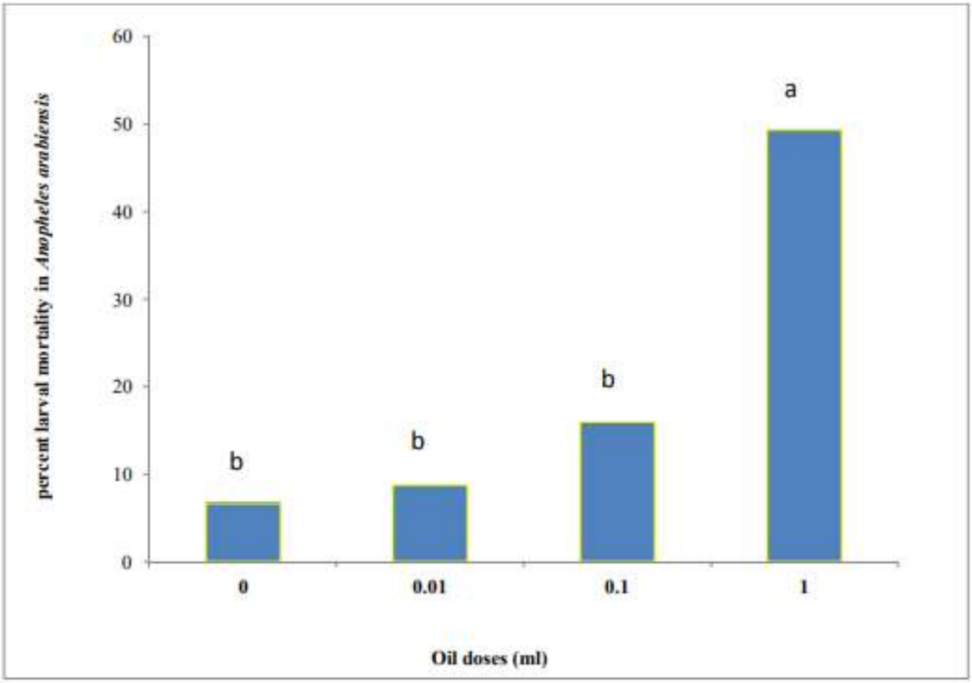


Fig. 6. Mortality of 4th stage larvae of *Anopheles arabiensis* in response to oil dose series at 24 h after application (the time with peak (maximum) larval mortality by overall effect of oils). The mean larval mortality with different letters are significantly different from one another (univariate general linear model with a Tukey's post-hoc analysis; $P<0.05$). Doses with different letters are significantly different.

DISCUSSION

This study showed that natural oil extracts of *Cyperus papyrus* L. and *Typha latifolia* L. significantly reduced the survival of 4th instar *An. arabiensis*. Considerably reduced larval survival time of *An. arabiensis* were recorded by increasing the doses of the oil extracts of *C. papyrus* and *T. latifolia* as well as the refined seed oil of *Helianthus annuus*. *Cyperus papyrus* rhizome yielded significantly higher natural oil content than that of its aerial part and that of aerial parts of *Typha latifolia*. The rhizome of *T. latifolia* yielded the lowest oil content of the plant materials from both grass species. Oils are effective to bring significant reduction of larval survival in Anopheles mosquitoes at specific lethal dose due to their suffocating and/or insect growth regulatory activities. These findings indicate that the amount of oil production by aquatic grasses in natural conditions may be one of the factors which cause differential effects on survival of larval *Anopheles* mosquitoes and therefore results in larval density variation in breeding habitats.

In parallel studies conducted on grass volatiles from the study area, differential variations in preference of gravid female *An. arabiensis* and *An. coluzzii* to volatile extracts from grasses were demonstrated under laboratory conditions with lower preference to volatiles of *T. latifolia,* and least preference to *C. papyrus* volatiles, in contrast to higher preference to volatiles of the Poaceae grasses including *Echinochloa pyramidalis* and *Echinochloa stagnina* for attraction and oviposition (Yelfwagash Asmare *et al*., 2017a). Moreover, volatiles of rice and maize (Poaceae) attract the gravid female *An. arabiensis* (Betelehem Wondwosen *et al*., 2016; 2017), and this is confirmed by the supportive role of agro-ecology related grasses in *Anopheles* mosquitoes breeding such as increasing larval development rate and larval abundance in seasonal pools close to maize cultivation and rice fields, respectively (Mwangangi *et al*., 2008; Yemane Ye-Ebiyo *et al*., 2003a; 2003b). Discriminatory oviposition site selection by the gravid female *Anopheles* is implied as a way of taking care of their offspring; avoiding breeding habitats associated with the oil rich larvicidal grasses such as *C. papyrus* and *T. latifolia*, and accepting habitats associated with the more nutritious Poaceae grasses (Betelehem Wondwosen *et al*., 2016; 2017; Yelfwagash Asmare *et al*., 2017b). These compounded ecological effects of volatiles and natural oils of grasses on survival and abundance of *Anopheles* larvae in natural mosquito breeding habitats require further investigations.

Recent findings indicate that potential breeding habitats of mosquitoes at southern edge of shore area of Lake Tana dominated by different grass species are found with variation in larval densities (Yelfwagash Asmare *et al*., 2017a) which is in line with the previous studies (Bøgh *et al*., 2003; Fillinger *et al*., 2004; Mwangangi *et al*., 2008). The larval density in habitats with Typhaceae is lower than the habitats with Poacea grasses, and *Anopheles* larvae are not found in habitats with Cyperaceae (Yelfwagash Asmare *et al*., 2017a).

Oils usually cause suffocation which results in mortality in aquatic stages of mosquitoes (Goma, 1960b; Lindsay and Martens, 1998; Mouchet *et al*., 1998), and this is due to physical nature of oils that create thin film on water surface and block the spiracles of mosquito larvae (Shuo *et al*., 2012). The increased larval mortality in *An. arabiensis* through increasing the dose of oils including the non-toxic control in this study, indicated that oils with and/or without toxic nature can result larval mortality due to suffocation. Absence of *Anopheles* larvae in potential breeding habitats dominated by *C. papyrus* can be due to substantial amount of natural oils produced by its rhizome that is released directly to the breeding water which reduce the probability of mosquito larval survival, and needs further investigations.

Cyperaceae grasses have toxic properties in nature with broad spectrum negative impact on microorganisms (Kakarla *et al*., 2014; Lawal *et al*., 2016). Moreover, essential oil of *C*. *papyrus* shows lethal effect on the larvae of the Crustacean brine shrimp *Artemia salina* (Ameen *et al*., 2011). Therefore, the potential of higher natural oil production by *C*. *papyrus* under natural conditions could have cumulative effect of both toxicity and suffocation which reduces survival of *Anopheles* larvae and their populations in the area where it is dominant in permanent breeding habitats. The toxic nature of *C*. *papyrus* oil on microbial populations (Kakarla *et al*., 2014; Lawal *et al*., 2016) may also reduce microbiota which is the natural food resource of *Anopheles* larvae in breeding habitats, and consequently starvation in the larvae which in turn reduces breeding success of the mosquitoes (Merritt *et al*., 1992). The similar effects of extracted oils from *C*. *papyrus* and *T*. *latifolia*, and the non-toxic refined seed oil of *H*. *annuus* on survival of the mosquito larvae under laboratory conditions could be the less effective property of the Soxhlet extractor with the solvent n-hexane to extract the toxic components in the oils of *C*. *papyrus* and *T*. *latifolia* (Rassem *et al*., 2016). This needs further studies using other oil extraction procedures.

From previous studies of larvicidal and toxic effects of essential oils of *Guarea* species (Meliacae) on *Aedes aegypti* (Magalhães *et al*., 2010), the Indian borage *Plectranthus amboinicus* (Lamiaceae) on *An*. *gambiae* (Kweka *et al*., 2012), and Mediterranean aromatic plants on *Ae*. *albopictus* (Conti *et al*., 2010) are well documented. Moreover, extracts from *Phragmites australis* has adult repellent and larvicidal effects against *Culex pipiens* (Bream *et al*., 2009), and extracts from herbal species including *Ferula asafoetida*, *Coriandrum sativum*, and *Trigonella foenum-gracewm* have larvicidal effects on *Ae*. *aegypti* (Harve and Kamath, 2004). The suffocating effect, toxicity, and growth regulatory impact of these essential oils on mosquito species in related genera provides perspectives for area-wide larval control along shorelines and wetlands.

Cyperus papyrus growing in potential breeding habitats can be used as a green insecticide to suppress populations of mosquitoes in integrated malaria vector management. Studies on human safety have reported that grasses in the family Cyperaceae including *C*. *papyrus* are economically and medically important and their uses include utilization against various disorders such as migraine, abdominal pains, as fumigant and to flavour food (Magalhães *et al*., 2010; Sonwa, 2000). Local people in the study area utilize the aerial part of the grass species for manufacturing some local containers and baskets. The rhizome part of the grass species is also utilized for firewood. Recently, this plant species has been recommended to be used for ornamental, waste water treatment and ecological remediation, as well as conservation purposes in the study area, Bahir Dar.

CONCLUSION

Natural oils extracted from *Cyperus papyrus* L. and *Typha latifolia* L. showed marked larvicidal effects and reduced probability of survival time on larvae of malaria mosquitoes at doses used in the present study. The aquatic grass, *C. papyrus* contains higher natural oil content in its rhizome which potentially leaks to the breeding water in the natural larval habitat of *Anopheles* mosquitoes. *Cyperus papyrus* growing in breeding habitats along shorelines of lakes and wetlands produce considerable natural oils and potentially contribute to control mosquito populations. Therefore, this study recommends promoting *C. papyrus* plantation in permanent breeding habitats along water bodies and wetlands in close proximity to villages to suppress *Anopheles* mosquito densities. Further evaluation of impacts of natural oils of these aquatic grasses in reducing malaria transmission in nearby human habitations is required.

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