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Mosquito larvicidal and brine shrimp activities of *Commiphora merkeri* Engl. (Burseraceae) exudate

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Abstract
The current study evaluated *Commiphora merkeri* Engl. exudate and its fractions for brine shrimp lethality and mosquito larvicidal activity against *Aedes aegypti* L, *Anopheles gambiae s.s* and *Culex quinquefasciatus* Say. The exudate exhibited larvicidal activity with LC_{50} of 34.59, 36.54 and 41.07 µg/mL at 24 h against *Ae. aegypti, Cx. quinquefasciatus* and *An. gambiae* larvae, respectively. Petroleum ether extract showed activity which was significant different (≥ 95%) when mosquitoes were exposed for 24 and 72 h. Larvae of *Ae. aegypti* was most susceptible to dichloromethane fraction compared to other species with LC_{50} of 10.39, 8.82 and 4.31 µg/mL at 24, 48 and 72 hrs, respectively. The exudate showed cytotoxicity to brine shrimps with LC_{50} of 3.85 µg/mL while petroleum ether fraction exhibited 3.34 µg/mL, followed by dichloromethane (LC_{50} of 41.52 µg/mL) and aqueous fraction had LC_{50} >100 µg/mL. The exudate and petroleum ether fraction possess high cytotoxicity to brine shrimps. Further study to identify compounds in dichloromethane fraction which is having moderate cytotoxicity may yield safe insecticidal compounds.

Keywords: Cytotoxicity, *Commiphora merkeri*, *Aedes aegypti*, *Anopheles gambiae* and *Culex quinquefasciatus*, brine shrimps

1. Introduction
Mosquitoes are vectors of most life threatening diseases such as malaria, dengue fever, yellow fever, zika, chikungunya, filariasis and encephalitis [1]. In order to prevent increase of mosquito borne diseases, mosquito control is important [2]. Mosquito control methods have been targeting both larval and adult stages of the lifecycle by using insecticides in the form of indoor residual spraying (IRS) long lasting insecticide treated nets (LLINs), insect repellents, using larvicides including biological control such as fish and bio-pesticides such as fungi [3-6]. Most of the mosquito insecticides are synthetic insecticides which are facing challenge of resistance and non-selective to non-target organism. Further search of alternative and simple method for mosquito control including botanical insecticides is warranted.
Plants remain a crucial source of medicines for a large percentage of the world's population, mostly in the developing countries [7, 8]. They produce various natural chemicals (phytochemicals), many of which show insecticidal properties. Previously, some groups of phytochemicals such as steroids, essential oils, terpenoids, alkaloids and phenolics from different plants species have been reported for their insecticidal activities [9].
The genus *Commiphora* (Burseraceae) consists of over 150 species. They are characterized as small trees or shrubs with spinescent branches, pale-gray bark and reddish-brown resinous exudates. *Commiphora* species has been reported to exhibit a wide range of biological activities including anticancer, analgesic, antifungal, acaricidal, mosquito larvicidal activities [10–12]. *Commiphora merkeri* Engl. is a dioecious tree 2-5 m tall with a single trunk having grey bark with dark patches, peeling around the stem in yellowish papery strips. The young branch lets are glabrous, smooth, purplish, and often spine-tipped. The plant is native to Northern part of Tanzania and Southern part of Kenya [13, 14]. Its latex is ethno-botanically used for controlling ticks and to treat tuberculosis, Chest pains and asthma [15]. The root has been reported to exhibits anti-inflammatory and analgesic activity [16]. The present study was conducted to evaluate *C. merkeri* exudate for brine shrimp lethality and mosquito larvicidal activity against *Ae. aegypti, An. opheles gambiae* and *Cx. quinquefasciatus.*

2. Materials and methods

2.1 Collection of sample

The identification of plant was done by Mr. Frank Mbago, a botanist from the Department of Botany, University of Dar es salaam, Tanzania. The plant exudate was collected by incising the bark with a sharp knife and collecting oozing exudate in clean containers. The voucher specimen is deposited at herbarium of the Institute of Traditional Medicine, Muhimbili University of Health and Allied Sciences (voucher specimen No. I12).

2.2 Solvents, Reagents and Test orgasms

Methanol (absolute) was bought from FlukaChemie GmbH (Sigma-Aldrich®, Zwijndrecht, Netherlands), dichloromethane was purchased from UNILAB (UNILAB®, Nairobi, Kenya), petroleum ether was purchased from (Loba Chemie Pvt Ltd, Mumbai, INDIA), dimethyl sulfoxide (DMSO) was purchased from Sigma® (Poole, Dorset, UK). Larvae of *Cx. quinquefasciatus*, *An. gambiae* and *Ae. aegypti* (Culicidae) were obtained from the insectarium at Muhimbili University of Health and Allied Sciences, Tanzania. The Brine Shrimps eggs were purchased from Aquaculture innovations (Grahamstown 6140, South Africa) and sea salt was prepared locally by evaporating water collected from the Indian Ocean, along the Dar es Salaam Coast.

2.3 Fractionation of exudate

About 30 g of *C. merkeri* exudates was dissolved in 250 mL of 80% MeOH/distilled water and fully extracted by consecutive liquid-liquid partition with 250 mL petroleum ether and then 250 mL dichloromethane using a separating funnel. The fractions was then concentrated under reduced pressure and kept at 4ºC till analysis.

2.4 Mosquito Larvicidal Assay

The larvicidal test was performed according to World Health Organisation (WHO) protocol with minor modification [17]. The stock solutions (50 mg/mL) of stem bark extract were prepared by first dissolving them in DMSO. The dilution of stock solutions was made with distilled water to make 100, 50, 25, 10 µg/mL solutions of exudate and 50, 25, 10, 5, and 5µg/mL solution of petroleum ether and dichloromethane fractions. Ten late third instar laboratory reared *An. gambiae*, *Cx. quinquefasciatus*, and *Ae. aegypti* larvae were then introduced in the test solutions and mortality was observed after 24 h, 48 h and 72 h. Negative control tests contained mosquito larvae, DMSO (0.5%) and water only. All tests were carried out in triplicate under controlled temperature (26 ± 2 °C) and relative humidity of 75-85%. The number of dead larvae was recorded after 24 h, 48 h, and 72 h, and the mean percentage mortalities were calculated for each concentration. The mean results of the percentage mortality were plotted against the logarithms of concentrations using the Fig P computer program. The concentrations killing fifty percent of the larvae (LC_{50}) were calculated from the regression equations obtained from the graphs.

2.5 Brine shrimps lethality test (BST)

The brine shrimp lethality assay was done as per Meyer [18], whereas the tested stock concentrations were prepared by dissolving 40 mg of respective compounds in DMSO. Then 5, 10, 15 and 30 µl of the test extract were pippeted into vials containing 10 larvae in saline water to make 5 ml of the final mixture. Thus, final concentrations were 240, 120, 80, 40 and 24 (µg/ml). Larvae were left for 24 hours after which the number of survivors in each concentration was counted and percentage mortality determined. DMSO treated larvae were left as the control.

2.6 Statistical analysis

Statistical analysis of the experimental data was done by using the Fig P computer program. The regression equations were used to determine LC_{16}, LC_{50}, LC_{84} and 95% C.I values [19].

3. Results

3.1 Mosquito larvicidal activity

The mosquito larvicidal activities of exudate and fractions are presented in Table 1. At 24 h, exudate exhibited larvicidal activity with LC_{50} value of 34.59, 36.54 and 41.07 µg/mL against *Ae. aegypti*, *Cx. quinquefasciatus* and *An. gambiae*, respectively. The activity at 72 h was however not significantly different from activity at 24 and 48 h exposure time for all species (Table 1). After liquid-liquid partition of exudate, dichloromethane (CMDCM) and petroleum ether (CMPE) fractions demonstrated higher larvicidal activity on *Ae. aegypti*, *Cx. quinquefasciatus* and *An. gambiae*. At 24 h, Petroleum ether and dichloromethane fractions exhibited larvicidal activity with LC_{50} value of 31.04, 35.24, 38.43 µg/mL and 10.39, 46.56, 48.01 µg/mL against *Ae. aegypti*, *Cx. quinquefasciatus* and *An. gambiae*, respectively. The mortalities for all mosquito species were significantly different for both dichloromethane and petroleum ether fractions at 24 h and 72 h. However, much higher activity demonstrated by dichloromethane at 72 h against *Ae. aegypti* (LC_{50}=4.31 µg/mL) and *Cx. quinquefasciatus* (LC_{50}=17.10 µg/mL). Larvae of *Ae. aegypti* was most susceptible to dichloromethane fraction compared to other species with LC_{50} of 10.39, 8.82 and 4.31 µg/mL at 24, 48 and 72 hrs, respectively. On the other hand, aqueous fraction (CMAQ) showed weak activity (LC_{50} =599.79 µg/mL) against all mosquito larvae (Table 1).

Table 1: Larvicidal activity of *C. merkeri* exudates and fractions against mosquito larvae

Sample Name	Exposure time (h)	LC_{50} in µg/mL (95% CI)		
		Ae. Aegypti	***Cx. quinquefasciatus***	***An. gambiae***
CMEX	24	34.59 (30.04-39.82)	36.54 (27.89-47.88)	41.07 (31.83-53.01)
	48	26.51 (26.00-27.03)	31.56 (24.35-40.89)	37.87 (30.44-47.12)
	72	24.01 (16.24-35.50)	27.98 (21.15-37.00)	27.98 (21.15-37.00)
CMPE	24	31.04 (25.44-37.87)	35.24 (29.76-41.74)	38.43 (32.16-45.93)
	48	18.07 (13.28-24.59)	25.35 (22.14-29.02)	27.66 (23.87-32.01)
	72	14.89 (10.95-20.25)	22.22 (18.76-26.31)	22.89 (19.16-27.36)
CMDCM	24	10.39 (5.64-19.13)	46.56 (28.81-75.23)	48.01 (30.35-75.95)
	48	8.82 (4.79-16.24)	21.39 (11.15-41.03)	26.88 (14.47-49.91)

	72	4.31 (2.12-8.76)	17.10 (9.73-30.05)	25.14 (15.14-41.74)
CMAQ	24	>1000	>1000	>1000
	48	792.28(420.56-1492.56)	>1000	>1000
	72	599.79 (372.27-966.36)	599.79 (341.35-1053.90)	599.79 (341.35-1053.90)

Key: CMEX= *C. merkeri* exudates crude; CMPE= *C. merkeri* Petroleum ether; CMDCM= *C. merkeri* Dichloromethane; CMAQ= *C. merkeri* aqueous fraction; LC_{50}= Lethal concentration; CI= Confidence Interval

3.2 Brine shrimps lethality test (BST)

The obtained LC_{50} values of BST for exudate and fractions are presented in Table 2. Exudate was lethal to brine shrimps with LC_{50} value of 3.85 µg/mL. For fractions, petroleum ether had LC_{50} value of 3.34 µg/mL followed by dichloromethane (LC_{50} of 41.52 µg/mL) and aqueous fraction (LC_{50} value 1896 µg/mL). Cytotoxic activity of petroleum ether fraction was comparable to that of exudate.

Table 2: Cytotoxicity of *C. merkeri* exudates and fractions against brine shrimps larvae

Sample Name	LC_{50} (µg/mL)	95% CI		R^2	Regression equation
		LCI	UCI		
CMEX	3.85	1.92	7.71	0.919	y = 64.77x + 16.10
CMPE	3.34	2.30	4.86	0.919	y = 64.77x + 16.10
CMDCM	41.52	31.85	54.13	0.927	y = 91.51x - 98.09
CMAQ	1896.00	677.12	5309.03	0.943	y = 27.21x - 39.19

Key: CMEX= *C. merkeri* exudates crude; CMPE= *C. merkeri* Petroleum ether; CMDCM= *C. merkeri* Dichloromethane; CMAQ= *C. merkeri* aqueous fraction; LC_{50}= Lethal concentration; CI= Confidence Interval; R^2= Regression coefficient

4. Discussion

Plants especially higher plants are well known for diverse secondary metabolites. They produce various natural chemicals some of which show activities against mosquito larvae [9, 20]. Cytotoxicity and larvicidal activity assay was done to predict the presence of bioactive secondary metabolites that are able to kill mosquito larvae. In the current study, the findings suggested the presence of bioactive compounds which are responsible for mosquito larvicidal and cytotoxicity properties in *C. merkeri* exudate.

Classification of plant larvicidal activities is considered highly effective when LC_{50} is less than 50 µg/mL, effective when LC_{50} is between 50 to 100 µg/mL, moderate (LC_{50} is between 100 to 200 µg/mL), weakly effective (LC_{50} is between 200 to 750 µg/mL), nontoxic when LC_{50} is greater than 750 µg/mL [21, 22]. Thus, *C. merkeri* exudate and all fractions evaluated, with the exception of aqueous, are highly effective to mosquito larvae. Of all mosquito species tested, the most susceptible mosquito species was *Ae. aegypti* (Table 1). Similarly, other researchers have indicated that *Aedes* larvae are more susceptible to plant extract than other mosquito species. For example, Mkangara *et al.* discovered that *Ae. aegypti* is more susceptible (LC_{50} 3.95 µg/mL) to *Commiphora swynnertonii* ethyl acetate extract than *Cx. quinquefasciatus* (LC_{50} 5.34 µg/mL) and *An. gambiae* (LC_{50} 8.48 µg/mL) [12]. Moreover, *Ae. aegypti* is more susceptible (LC_{50} 169.61 µ g/mL) to *Plumbago zeylanica* L. roots than *An. stephensi* (LC_{50} 222.34 µg/mL) [23].

Dichloromethane fraction revealed better larvicidal activity against all mosquito larvae than exudate especially after long time of exposure (72 h). Similar effect occurs with studies on *Sphaeranthus indicus* extracts. In those studies, ethyl acetate fraction was found to be more effective than crude extract against *Cx. quinquefasciatus* (LC_{50} 32.60 ppm *vs* 130 ppm, respectively) and *Ae. aegypti* (LC_{50} 36.76 ppm *vs* 140 ppm, respectively) [23, 24]. The reason may be contributed to more concentrated active compounds in the dichloromethane fraction. This is further justified by the findings of mosquito larvicidal activity of exudate which showed rather higher activity but not comparable to that of dichloromethane and aqueous (Table 1). Similarly, the current study indicated that the active principles in *C. merkeri* exudate may be present more in dichloromethane fraction, which gave the lowest LC_{50} values against all mosquito species tested.

The brine shrimp lethality test has been used regularly in the primary screening of the extracts and compounds to evaluate bioactive compounds which could also provide an indication of possible cytototoxic properties of the test materials [25]. In the present study, BST results based on LC_{50} value as follow: LC_{50} <1.0 µg/mL- highly toxic; LC_{50} 1-10 µg/mL- toxic; LC_{50} 10-30 µg/mL- moderately toxic; LC_{50} >30 <100 µg/mL- mildly toxic, and LC_{50} >100 µg/mL non-toxic [9, 26]. Thus, the exudate and petroleum ether are cytotoxic while dichloromethane fraction and aqueous were moderate and non cytotoxic, respectively. Lower LC_{50} values exhibited by petroleum ether (3.34 µg/mL) fraction indicate the presence of non-polar cytotoxic compounds which dominated in the exudate. However these cytotoxic compounds were significantly reduced through partitioning in the dichloromethane and aqueous (Table 2).

5. Conclusion

Exudate of *C. merkeri* exhibits mosquito larvicidal activity. The petroleum ether and dichloromethane fractions showed significant difference (≥ 95%) in activity with exposure time against all mosquito larvae with dichloromethane being the best. Furthermore, cytotoxicity of exudate and petroleum ether is highly contributed by non-polar compounds. Hence, further study to isolate and characterize compounds in dichloromethane may result into less cytotoxic insecticide.

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