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Research Article

Larvicidal, Ovicidal and repellent effect of *Citrus hystrix* DC (Kaffir lime) fruit, peel and internal materials extracts on *Aedes aegypti* mosquitoes

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Abstract: The present study aimed to evaluate the larvicidal, ovicidal and repellent activity of ethanol extracts of dry fruit, peels and internal fruit materials of *Citrus hystrix* DC against *Aedes aegypti*. *Aedes aegypti* larvae were collected from Than Byu Zayat Township Mon State and 50 each 3rd and 4th instar larvae were exposed for 24 hours in various concentrations of ethanol extracts of different parts of the *Citrus hystrix* fruit, done 5 replicates. The dry fruit and peels extracts resulted in significantly higher 100% mortality ($P<0.05$) when compared to the mortality (86.8%) caused by internal material of *Citrus hystrix* fruit at the concentration of 0.1gm/100ml against *Aedes* larvae. The dose 0.0125g/100 ml of *Citrus hystrix* fruit extract was found to be 100% protection from oviposition of gravid *Aedes aegypti* mosquitoes in laboratory. The LC_{50} and LC_{90} values were 0.0138, 0.0142 and 0.0276, and 0.0515, 0.0522 and 0.1045 g for fruit extract, peel and internal material. The highest repellency activity of complete protection time of *Citrus hystrix* DC dose 0.0002g/cm^2 was found dry fruit extract followed by peel extract and lowest activity was found internal fruit materials extracts. These three extracts provided 100%, 97.52% and 92.15% protection from bite for 30min and 96.72%, 86.25% and 80.25% protection for 60 min and 88.52%, 80.1% and 73.52% protection for 90min, against adult *Aedes aegypti.* These extracts did not cause dermal irritation when applied to animal skins. The findings of the present study revealed that the ethanol extract of the fruit of *Citrus hystrix* DC has strong larvicidal, ovicidal and repellent properties on *Aedes* mosquitoes as a good source of preparations for mosquito control.

INTRODUCTION

Dengue is the most important mosquito-borne arboviral disease. The four dengue virus (DENV) serotypes (genus Flavivirus, family Flaviviridae) now circulate pan-tropically, DENV1–4 and new genotypes associated with increased virulence have expanded from endemic areas of Asia into the Americas [1, 2]. The diseases are carried by *Aedes* mosquitoes. *Aedes aegypti* is a main vector of dengue virus in urban areas and *Aedes albopictus* is rural area vector. The main vector of dengue virus in Myanmar is *Aedes aegypti* and secondary vector is *Aedes albopictus*. Myanmar is a developing country and planning urbanization in many parts of the country including Yangon environ. Rapid and poorly planned urbanization in association with weak regulatory policies for discharge of solid waste has resulted in the accumulation of solid waste which in turn results in the accumulation of discarded containers in most developing countries. These accumulations have favored the establishment and geographic spread of this *Aedes aegypti*. It discarded largely because of it anthropophilic feeding behavior, resting behavior inside houses and its capability to exploit most water holding containers for breeding [3].

The number of dengue cases reported annually to World Health Organization (WHO) has increased from 0.4 to 1.3 million in the decade 1996–2005, reaching 2.2 million in 2010 and 3.2 million in 2015 [4,5] . There is substantial under-reporting of dengue within health systems and to WHO [6]. Based on mathematical modeling, the global annual incidence has been estimated at about 50 million – 100 million symptomatic cases in recent years, predominantly in Asia, followed by Latin America and Africa, with clinical cases likely to represent about 25% of all dengue virus infections [7, 8]. In 2013 dengue was estimated to be responsible for approximately 3.2 million severe cases and 9000 deaths, the majority occurring in lower middle income countries, and for 1.1 million disability adjusted life years (DALYs) globally [9]. Southeast Asia Region and Western Pacific Region in the world which bear nearly 75% of the current global disease

burden due to dengue [7]. Dengue fever (DF) and Dengue Hemorrhagic Fever (DHF) are increasingly becoming serious public health problems in Myanmar especially among the 5-10 and 11-15 years old age groups and now noted 15 years above, a vast majority of the cases occur in 5-8 years old age group [10,11]. The highest number of cases and deaths recorded were 9149 DHF cases and 55 deaths recorded across Myanmar [12] A severe outbreak of DHF occurred for the first time in Yangon in 1970 [13]. DHF morbidity has been increased from 2006 to 2010 year. In 2011 CFR reduce to 0.33% and 0.36% in 2012 and 0.41% in 2013 respectively. Regarding DHF mortality, highest deaths (445) were found in 1994 outbreak. After that mortality was decreasing throughout the year. But there were more than 200 deaths up to 2001. Although increasing the reported cases, CFR decrease from 4% to less than 1 % (0.41%) in 2013[14]. This epidemic had an affected mostly school going are groups. Generally more DHF cases predominate during the raining season especially in July and August. Highest number of cases was recorded in July [13].

Synthetic chemicals, chemicals and compound chemical insecticides used for control of vectors are causing irreversible damage to the eco-system and human being, and some of the chemical insecticides are non-degradable in nature. Some repellents of synthetic origin may cause skin irritation and affect the dermis [15, 16]. Majority of commercial repellents are prepared by using chemicals. These chemical repellents are not safe for public use [17]. Because of these are performed unpleasant smell, oily feeling [18] and potential toxicity to human and animals and environments [19]. Resistance to insecticides is a serious problem threatening insect borne diseases control efforts in all regions where insecticides are used to kill mosquitoes and insects. Pyrethroid insecticides were first used for malaria control in 1992, and have since been constantly used in Myanmar. This intensive use may explain the strong selection pressure toward *Aedes aegypti* and found development of pyrethroid resistance in *Aedes aegypti* in Myanmar [20]. *Aedes aegypti* is generally thought to be the vector of dengue in more urban areas, so *Aedes aegypti* is the more important to treat.

Repellents of plant origin do not pose hazards of toxicity to human and domestic animals and are easily biodegradable. The study of biologically active materials derived from plant sources can act as larvicides, insect growth regulators, repellents and ovipositional attractants and have deterrent activities as observed by many researchers [21-23]. Essential oils have received much attention as potentially useful bioactive compounds against insects [24]. Larvicide, adulticide and repellency are known to play an important role in preventing the vector borne diseases by reducing mosquito density and man-vector-contact. Natural plant products are safe for human being when compared to that of synthetic or chemical compounds insecticides and repellents. Therefore Novak [25] emphasized the urgent need for the investigation of phytochemicals as repellents for mosquito control. Many active insecticidal materials have been derived from plant sources, i.e. nicotine's, pyrethrins and rotenones. Kaffir lime (*Citrus hystrix* DC) leave extract has been used as pest control against rice weevil *Sitophilus oryzae* infestation in stored rice and mosquito repellent [26]. Other researchers revealed that ethanol extract of *Citrus hystrix* DC leaves against 3rd and 4th instar *Aedes* larvae and polar and non-polar extract fraction from kaffir lime (*Citrus hystrix*) leaves against 3rd instar larvae were found very effective larvicides [27]. They have not report the larvicidal activity of *Citrus hystrix* DC fruit extract on *Aedes* larvae. And also there was no derivative of Kaffir lime (*Citrus hystrix* DC) fruit on *Aedes aegypti* mosquitoes has been mentioned as an insecticidal and repellent agent in Myanmar. Therefore, investigation on larvicidal, ovicidal and repellent action of dried Kaffir lime fruit, peels and internal materials extracts against *Aedes larvae* and adult mosquitoes from Than Byu Zayat Township, Mon State was done in laboratory. It may be useful for future plant base larvicide or repellent in vector control programme.

MATERIALS AND METHODS

Mosquito larvae collection

Aedes mosquito larvae were collected from different water storage containers from Than Byu Zayat Townships, Mon State from May 2015 to December 2016. All collected larvae were carried to Department of Medical Research laboratory for larvicidal and repellency tests with dried *Citrus hystrix* DC fruit, peel and internal material extracts. *Aedes aegypti* mosquito larvae were reared in tap water in laboratory.

Ethical consideration

The study was done according to the approvable of ethical review committee, Department of Medical Research, Myanmar, ERC Number 007816, Approval No.-Ethics/DMR/2016/079.

Mosquito's species identification

Larvae and adult mosquitoes emerged from larva survey were identified by morphological methods [28].

Collection and preparation of Kaffir lime (*Citrus hystrix* DC) fruit extraction

The Kaffir lime (*Citrus hystrix* DC) fruit was collected from Hnitkine village, Than Byu Zayat Township, Mon State. A total of 5 Kilo grams of Kaffir lime (*Citrus hystrix* DC) fruits were cleaned and different parts of the Kaffir lime fruits as fruit, peel and internal materials were cut into small pieces and put into separate trays and dried at room temperature in shade. 100 gram each of dried Kaffir lime fruit, peel and internal materials were extracted with 95% ethanol 1:5 wt/v by refluxing in a Soxhlet extractor at 70ºC for 6 hour. Complete removal of the solvent from the extract was accomplished in glass rotary evaporator. The resulting 8gm each of viscous materials were obtained from 100 gram each of dried fruit, peel, and internal materials. The viscous materials were stored at 4ºC until use. The extractions of dried *Citrus hystrix* DC fruit, peels and internal materials were done in Pharmacology Research Division, Department of Medical Research.

Larvicidal testing procedure

Based on preliminary tests, further dilutions were prepared with same type of test water. Different emulsified concentration as 0.1, 0.05, 0.025, 0.0125, 0.00625g dried *Citrus hystrix* fruit, peel and internal materials were prepared freshly by dissolving in 100ml each of purified water in 250ml plastic cups. Third and fourth instar *Aedes aegypti* larvae from Than Byu Zayat were inside each 250ml plastic cups and also negative control test was done simultaneously. Fifty (50) each *Aedes aegypti* larvae were put into different concentrations of ethanolic crude extracts of Kaffir lime fruit, peel and internal materials solutions. Detail testing was done according to standard method [29,30]. The exposure period of larvae were exposed 24 hrs for each replication and concentration in laboratory at 26-30ºC and 70 to 90% relative humidity. In the experiments, five replicates were carried out and mortality was checked and recorded after 24 hrs of exposure periods. Dead larvae were identified when the larvae failed to move after probing with a needle in the cervical region.

Oviposition test

Ovipocition test was done at the concentration of 0.1, 0.05, 0.025, 0.0125, 0.00625 g/100ml purified water in plastic cups were put into 50 gravid *Aedes aegypti* mosquitoes released cage in laboratory at 3:00pm to next day 3:00 pm (24hours). Number of eggs laid was counted under magnification of 10X dissection microscope.

Toxicity test

Acute toxicity test of the samples on albino mice model

Theory

To determine the symptomatology consequent to injection of the plant and to determine the nature and degree of toxicity produced by these extracts and to find out the medium lethal doses (LD_{50}) of the extracts, acute toxicity test was done. Usually the acute lethality a compound is determined on the basic of deaths occurring in 24 h but the survivors should be observed for at least seven days in order to detect delayed effects. In this study, acute toxicity effect of *Citrus hystrix* fruit, peel and internal materials (two doses) were determined on albino mice, at Laboratory Animal Services Division, Department of Medical Research (DMR), Yangon.

Procedure

Acute toxicity of different doses *Citrus hystrix* fruit, peel and internal materials extracts of that samples were evaluated by the methods of OECD Guidelines for the testing of Chemicals 425 [31,32]. According to the test description, total number of 18 adult female albino mice, weighting (25-30g) were selected and divided into three groups. Each group contained six animals. They were fasted for 18 h before giving the extracts. Group (1) mice were orally administrated with 2000 mg/kg dose of *Citrus hystrix* fruit extract. Group (2) mice were given orally with *Citrus hystrix* fruit extracts 5000 mg/kg dose. Group (3) mice performed as a control group and they were treated with clean water and normal animal food. All groups of mice were kept in the three mouse cages in the separated room at the room temperature of 26 ± 1º C. After administration of extracts on each group of animals were observed first 6 h continuously for mortality and behavior changes. Then check the animals each 24 h for fourteen days. The mortality during this period was noted (Nil or percent death). The results obtained from acute toxicity were recorded. Same toxicity test procedure for *Citrus hystrix* fruit peels and internal materials extracts were followed as above mention procedure.

Experiment of irritation test

Primary skin irritation is the production of reversible inflammatory changes in the skin following the application of a test substance as it involves the interaction of chemicals with the sensory receptors in the skin at the site of application. Skin irritation test was done according to 'DBT, Guidelines for toxicity and allergenicity [33]. In the present study three young adult rabbits of the Myanmar white strain were taken from animal service division, two samples for test and one sample for control. All animals were housed in metal cages fitted with perforated floors. Water and standard rabbits feed were given. The room temperature was maintained at 22 ± 3 ° C with 30 - 70 % relative humidity. The light conditions were controlled to give 12 hours artificial light (8 a.m. - 8 p.m.) each day. A minimum of 7 days acclimatization was allowed before the commencement of the study. Each rabbit cage was attached with a tag marked with the animal number, the test and the product name.

Twenty-four hours before the test (dose application), hair on the back of each rabbit was shaped by blade approximately 9 cm^2 area of skin. (DOSE: 0.0036 gm/9cm^2 area of rabbit = double dose of human 0.0002g/cm^2), 0.0072gm of extract was desorbed in 2ml of ethanol and this 1ml each mixture dose form was evenly applied to a small area (approximately 9 cm square) of the shaped skin of each test rabbit. The site of application was not covered with a cotton gauze patch. Similarly control rabbit was treated with only 1ml of ethanol. Skin reaction at the site of application was subjectively assessed and scored once daily at 1, 24, 48, 72 hours, 7 and 14 days after treatment of skin (post-test observation period) according. The reaction at the site of application was assessed and scored according to the following numerical system. : Skin reaction: (A) Erythema and Escher formation (B) Edema formation. Irritation was followed by evaluation of primary skin irritation index: Non Irritant 0.0, Negligible Irritant 0.1- 0.4, Slight Irritant 0.41-1.9, Moderate Irritant 2.0 - 4.9, Severe Irritant 5.0 - 8.0. Same above procedure of irritation test was followed for irritation testing of peel and internal material extracts on other rabbits.

Repellent activity testing

The repellent study was following the method of World Health Organization [34]. Three to five day old blood staved 50 female *Aedes aegypti* mosquitoes were kept in a steel net cage (59 x 59 x 59 cm). The volunteer had no contact with lotions, perfumes, or perfumed soaps on the day of the assay. The arms of volunteer, one ml of ethyl alcohol (95%) diluent

used in the preparation of the test repellent in applied evenly using a pipette to average 343.5 cm^2 of forearm skin between the wrist and elbow and allowed to dry 1 minute. Before insertion of arm into the cage containing 50 *Aedes* female mosquitoes, the hands are protected by plastic gloves to protect mosquitoes bite. The first step, ethyl alcohol applied forearm was inserted into the cage and counted the number of mosquitoes that land on the skin during 30-second period. The control forearm was carefully withdrawn and this arm was then treated with one ml of 0.1g/10ml of *Citrus hystrix* fruit extract solutions and allowed to dry. The treated arm was placed in the cage for another 30 second period and observed for mosquito landing. This procedure was repeated for each additional incremental of *Citrus hystrix* fruit extract dose. The tests were carried out one after the other without delay *Citrus hystrix* fruit extract dose at each test was calculated as the sum of the doses applied to arrive at the cumulative dose for each test. Test was preceding when the mosquito landing rate on the exposed forearm was less than 10 female in 30 second. Two train technicians were recorded the number of landings. At the conclusion of the dose response experiment, 1 ml of ethyl alcohol was applied on the other forearm and allowed to dry. This forearm was inserted in the cage for 30 seconds to verify that the number of landings was more than 10 per 30 seconds as was observed at the beginning of the experiment. Protection (P) was expressed as a proportion of the number of mosquito landing on treated arm (T) in relation to the number of landings on the control arm (C) of the same individual. C is the average landing on two untreated arms. Same above procedure was followed for peels and internal material extracts for repellency testing.

$$P=1-(T/C) =(C-T)/C$$

Estimation of complete protection time

The complete protection time of *Citrus hystrix* fruit, peel and internal materials extract were determined 100% protection dose (0.08g/ml, 0.08g/ml and 0.16g/ml) were using on 341.75cm^2 area of forearm skin between the wrist and elbow. The protection test was followed by the procedure described as above for each extract.

Before testing four mosquito cages (size 59x59x59cm) each containing 50 non blood fed 5days old *Aedes aegypti* female mosquitoes were normally used. Two cages were used for testing two female volunteers and another two cages were used for testing two male volunteers. Before testing the arms of volunteer, one ml of ethyl alcohol (95%) diluent used in the preparation of the test *Citrus hystrix* fruit, peel and internal materials extract repellent in applied evenly using a pipette to average 341.75 cm^2 of forearm skin between the wrist and elbow and allowed to dry 1 minute. Before insertion of arm into the cage containing 50 *Aedes* female mosquitoes, the hands are protected by plastic gloves to protect mosquitoes bite. The first step, ethyl alcohol applied forearm was inserted into the cage and counted the number of mosquitoes that land on the skin during 3 minute period. The control forearm was carefully withdrawn from the cage. Then 0.08g of *Citrus hystrix* fruit was prepared in one ml of ethyl alcohol solution was applied evenly on 341.75 cm^2 of another forearm skin between the wrist and elbow. The treated arm was placed in the cage for 3minute period and observed for mosquito landing.

After 30minutes, the *Citrus hystrix* fruit extract repellent treated arm was inserted again into the cage and exposed for 3 minutes to determine landing activity. This procedure was repeated at 30 minute intervals for 180 minutes and the procedure was used consistently throughout the experiment. The mosquitoes that landed on the hand were recorded and then shaken off before imbibing any blood. Complete protection time was estimated after experiment. Same repellency test procedure was done other three volunteers. *Aedes aegypti* is a day time biter therefore tests was done between 08.00hrs and 16:00hrs. Tests were done in 15x10x10 fits room, at 25 - 27ºC and relative humidity of 60-80%. The repellency tests for *Citrus hystrix* fruit peel and internal materials extract repellents were done as above procedure with same volunteers in same conditions for other days.

Data analysis plan

Data entry and processing was made using micro soft Excel software. LC_{50} and LC_{90} values were calculated by using dose-effect probit calculations [35, 36].

RESULTS

Fig. 1 showed that 0.1g/100ml dose found 100% mortality against 3rd and 4th instar *Aedes* larvae. Followes by 0.05gm of concentration found 87.2% and 86.4 % mortalities respectivily. The lowest mortality was found 6.8% at 0.00625 concentration of *Citrux hyatrix* internal material. Fig. 2. Showed that 0.0125g/100 ml dose of *Citrus hystrix* fruit extract was found to be 100% protection from oviposition of *Aedes aegypti* mosquitoes in laboratory. Although *Citrus hystrix* peels and internal material extracts were found to be 100% and 98.92% protection at the dose of 0.025g/100ml of tap water.

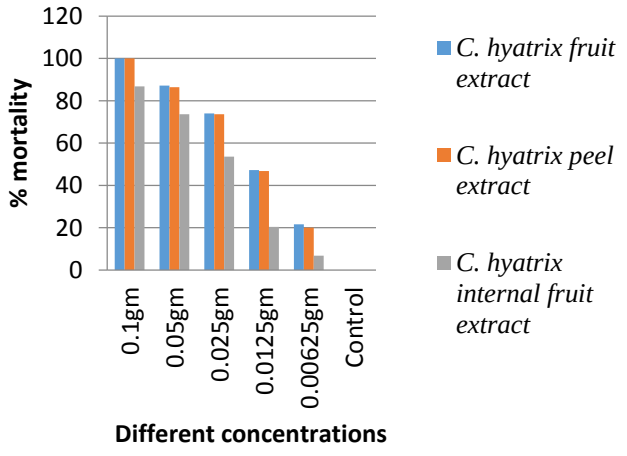


Fig.1. Larvicidal activity of *Citrux hyatrix* fruit , peel and internal materials extraxts

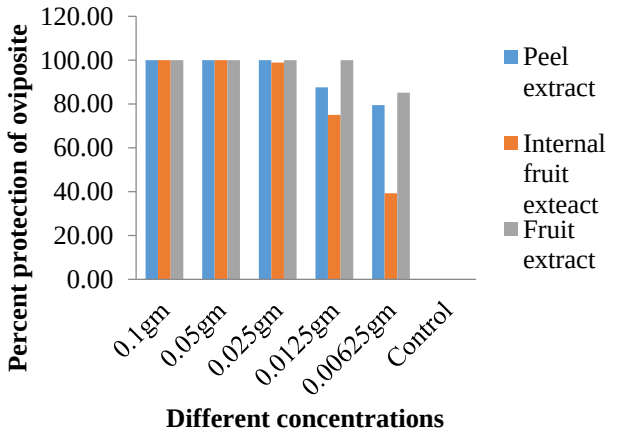


Fig. 2. Effect of different dilution of *Citrus hystrix* fruit, peels and internal materials extracts on oviposition of gravid *Aedes aegypti* females

Dose effect analysis of LC_{50} and LC_{90} value for *Citrux hyatrix* fruit , peel and internal materials extraxts were found 0.0138,0.0142, 0.0276 for LC_{50} and 0.0515, 0.0136, 0.1045 g/100ml dose for LC_{90}.

Toxicity test

The results obtained from acute toxicity are described in the table 1. Table 2 shows that acute toxicity screening of *Citrus hyatrix* fruit , peel and internal materials extraxts were done with the dosage of 2000 mg/kg and 5000 mg/kg body weight in albino mice. The condition of mice was recorded after fourteen days administration. The results show no lethality of the mice was observed up to fourteen days administration. Each group of animals were also observed still alive and did not show any visible symptoms of toxicity like restlessness, respiratory disorders, convulsion, aggressive activities, coma and death. Even with the dose up to 5000 mg/kg body weight administration, there is no lethality at the day of fourteen.

Table 1. LC_{50} and LC_{90} value *of Citrux hyatrix* fruit , peel and internal materials extraxts

Extrects	Lethal concentration LC_{50}	Lethal concentration LC_{90}	Chi squire ,P value
Fruit extract	0.0138	0.0515	0.0084 , p<0.05, *df=4*
Peel extract	0.0142	0.0522	0.0136, p<0.05, *df=4*
Fruit material extract	0.0276	0.1045	0.0332, p<0.05, *df=4*

Table 2. Acute Toxicity effect of *Citrux hyatrix* fruit , peel and internal materials extraxts on albino mice model after two weeks administration

No	Groups	Extracts Administration	Dosage	No. of death	% of death
1	Group 1	*Citrux hyatrix* fruit , Peel extract internal materials extraxts	2000 mg/kg 2000mg/kg 2000mg/kg	Nil Nil Nil	0 % 0% 0%
2	Group 2	*Citrux hyatrix* fruit Peel extract Internal materials extraxts	5000 mg/kg 5000mg/kg 5000mg/kg	Nil Nil Nil	0 % 0% 0%
3	Group 3	No administration	Nil (clean water)	Nil	0 %

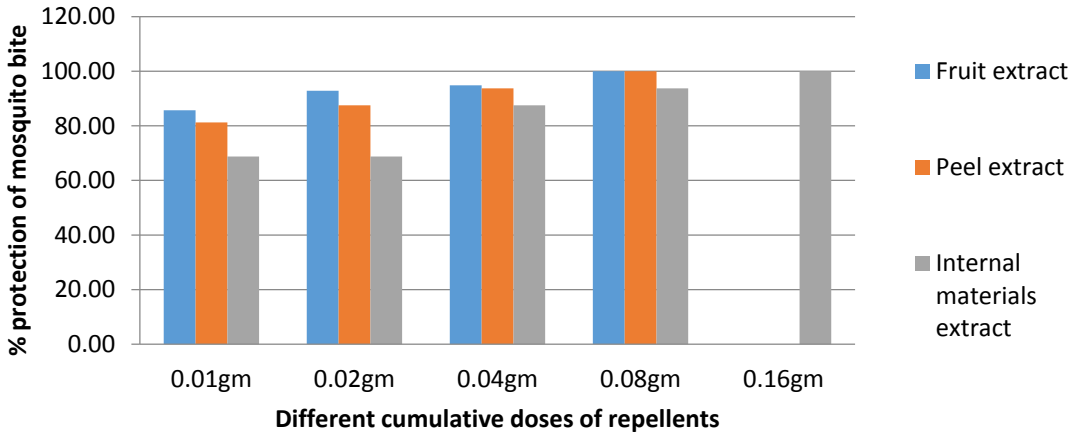


Fig. 3. Experiment of successive doses of *Citrus hystrix* fruit, peel and internal materials extracts applied to arrive at a cumulative dose for *Aedes aegypti*

Allergenicity (Irritation) test

Allergenicity effect of *Citrux hyatrix* fruit , peel and internal materials extraxts reactions were performed on rabbit model. The sample has been tested as per 'DBT, Guidelines for toxicity and allergenicity Evaluation of Transgenic Seeds, Plants and Plant parts' for non- clinical laboratory studies and the result of the sample of *Citrux hyatrix* fruit , peel and internal materials extraxts dose 0.0036 g/9cm^2 was found to be 'nonirritant' to the skin of rabbits when compared with the sample of control rabbits (only ethanol alcohol treated rabbits). Fig. (3) shows that successive cumulative dose of *Citrux hyatrix* fruit , peel and internal materials extraxts applied on 343.5 cm^2 area of arm for 100% protection of *Aedes aegypti* mosquito landing to probe the skin were found 0.08g/ml or 0.0002g/cm^2, 0.08g/ml or 0.0002g/cm^2, and 0.16g/ml or 0.0004g/cm^2 respectively. Fig. 4. Shows that repellency activity of complete protection time of *Citrus hystrix* fruit, extracts dose 0.0002g/cm^2 provided 83.33% protection for 180 min and 96.72% protection for 60min and 100% protection for 30min were observed against *Aedes aegypti* adult mosquitoes. Repellency effect of peel and internal materials extracts were found over 90% protection i.e. 97.52% and 92.15% protection for 30min and 86.25% and 80.25% protection for 60min. *Citrus hystrix* fruit extract was found to be most effective and long term protection of mosquito bite than peel and internal material extracts.

DISCUSSION

Mosquitoes alone transmit disease as malaria, filaria, dengue haemoragic fever(DHF), dengue fever(DF), japanis enseplaitis, zika and yellow fiver to more than 700 million people annually [37]. Therefore, the control of mosquitoes is an important public health problem around the world. *Aedes aegypti* (Culicidae) occurs in tropical countries as Asia, Africa and Central and South America and transmits four different types of Flavivirus viruses (as DNV1 to DNV4), etiologic agents of human diseases like DF and DHF dengue, Zika and Yellow fever. Chemicals and synthetic insecticides used for control of vectors are causing irreversible damage to the eco-system and human being and animals.

According to Bowers et al., [33], the screening of locally available medicinal plants for mosquito control would generate local employment, reduce dependence on expensive imported products and stimulate local efforts to enhance public health. Different parts of the plants contain a complex of chemicals with unique biological activity [38,39] which is thought to be due to toxins and secondary metabolites which act as mosquitocidal agent [23]. Natural products are safe for humans when compared to that of synthetic compounds and chemical insecticides.

Citrus hystrix is a valuable medicinal plant used widely in traditional medicine [40]. In the present study the *Citrus hystrix* plant tested is known to be ecofriendly and is not toxic to man and environments and also used as a medicinal plant in Asian countries as in pain release herbal medicines in Myanmar [27]. This plant is grown widely as herbal plant in costal and mountain region in Myanmar whereas Mon State and Bago Region. Moreover, it is clearly proved that ethanol crude extracts of dried *Citrus hystrix* fruit, peels and internal materials provided highly efficacious for the control of 3rd and 4th instar *Aedes* larvae within 24 hours. Although other researcher revealed that crude extract or partially purified plant extracts are less expensive and highly efficacious for the control of mosquitoes rather than the purified compounds or extracts [41-45]. *Aedes aegypti* 3rd &4th instar larvae against 0.1g/100 ml concentration of *Citrus hystrix* fruit from Than Byu Zayat were more susceptible 100% mortality at 0.1g than the *Aedes* larvae from Hlaing Thayar and Dagon Myothit North 95-100% mortality at 0.15g/100ml to *Citrus hystrix* fruit and peel ethanol extracts [46]. The *Citrus hystrix* fruit extracts were found to be larvicidal in nature and 90% mortality of *Aedes* larvae at 41.9ppm steam distilled extract *of Citrus hystrix* [47].

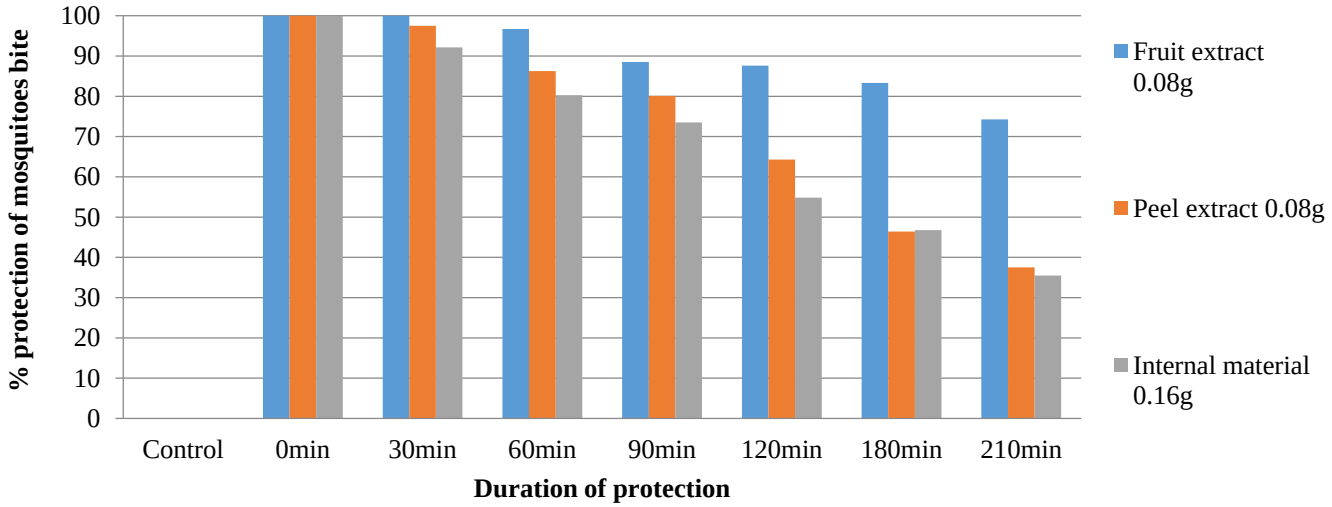


Fig. 4. Percentage of complete protection time of *Aedes* mosquito bite against different parts of *Citrus hystrix* fruit, peel and internal material extracts.

Very high ovicidal property was 0.0125g/100 ml dose of *Citrus hystrix* fruit extract was found to be 100% protection from oviposition of *Aedes aegypti* mosquitoes in laboratory. Although *Citrus hystrix* peels and internal material extracts were found to be 100% and 98.92% protection at the dose of 0.025g/100ml of tap water. Pushpanathan and his associated revealed that hundred percent ovicidal activities was observed at higher concentration of 300 ppm of *Cymbopogan citratus* Stapf (Graminae) essential oil against the adult female mosquito *Cx. quinquefasciatus* [48]. The essential oil extracted from the grapefruit (*Citrus paradisi*) peel by steam distillation against *Aedes aegypti* gravid female showed that egg hatching was completely inhibited at 400 ppm, while further development of 1st to 2nd larval stage was inhibited at 100 ppm and the peel oil could be a potent persistent larvicide [49].

From the LC_{50} it was evident that higher concentration of *Citrus hystrix* fruit internal materials extract was required for 3rd and 4th instars, it required 2 folds higher dose than of *Citrus hystrix* fruit, and peel extracts. Same result has been found in LC_{90} values of *Citrus hystrix* fruit, peel and internal material extracts against *Aedes* larvae. LC_{50} for 50% mortality and LC_{90} for 90% produced 1.73% and 2.08% mortality with ethanol leaf extract of *Citrus hystrix* [27]. Non-polar extract fraction from *Citrus hystrix* leaf is more toxic and is an effective biolarvicide with LC_{90} = 2,885 ppm compared with polar extract fraction from *Citrus hystrix* leaf which has an LC_{90} = 3,180 ppm [50]. The essential oil of *Citrus hystrix* peel contained 24 identified components, amounting to 99.52% of the whole oil with β-pinene (22.54%) and d-limonene (22.03%) as the principal constituents, followed by terpinene-4-ol (17.37%), together with trace amounts of α-terpineol (6.29%) and sabinene (5.49%). According to LC_{50} and LC_{90} values, Dagon Myothit North bred *Aedes* larvae had high tolerability to *Citrus hystrix* fruit extracts than Thanbyuzayat *Aedes* larvae [46]. The bioactive component, β-thujaplicin, derived from *Chamaecyparis obtusa* leaf extract demonstrated strong larvicidal potential against *Ae. aegypti*, *Ae. togoi*, and *Culex pipiens* pallens, with LC_{50} of 2.91, 2.60, and 1.33 ppm, respectively[42]. Larvicidal investigation of *Eucalyptus grandis* essential oil and its major components on *Ae. aegypti* revealed that the most effective was β-pinene, followed by α-pinene, and 1,8-cineole with the LC_{50} of 12.1, 15.4 ppm, and 57.2 ppm, respectively [51]. Other researcher revealed that the principal constituents found in peel essential oil of *Citrus hystrix* were β-pinene (22.54%) and d-limonene (22.03%), followed by terpinene-4-ol (17.37%) and it was toxic against both pyrethroid-susceptible and resistant *Aedes aegypti* laboratory strains at LC_{50}, LC_{95}, and LC_{99} levels [47].

In the present study no toxicity effect and allergenicity were found in animal model and no irritation was found on human when testing repellency test. Similar result has been found by Govindarajan et al., [52], they revealed that the plant crude extracts gave protection against mosquito bites without any allergic reaction. The repellent activity was dependent on the strength of the plant extracts. However some study reported that, a few volunteers suffered mild and short-lived skin itching and sneezing reactions arising from extracts from peels of *Citrux sinensis*, *Citrus limonum*, *Citrus reticulata* and *Citrus aurantifolia* compared to individuals without topical application used as control in the three demonstration areas Mbukpa in Calabar, Ekori in Yakurr and Mbube in Ogoja of Nigeria [53]. Repellent and attractant properties of phytochemicals from plants other than citrus plant species have been investigated by various researchers [54-58].

Santyaand and Hendri [59] observed that protection capacity of *Citrus hystrix* extract for 6 hours on average gave 34.82% of protection against *Aedes aegypti* and 41.44% of *Aedes albopictus*. The *Citrus hystrix* (Kaffir lime) extract has been able to reject *Aedes aegypti* and *Aedes albopictus* it is agreed with the present study. The thrust of the Kaffir lime is not as good as chemical repellents, but can be used as alternative mosquitoes repellent. Although present study found 0.08g or 0.0002g/cm^2 of *Citrus hystrix* fruit ethanol extract was very effective, 80-100% protection of *Aedes* mosquitoes bite for 180 minutes. Peel extracts was found 80-100% protection for 90 minutes and and internal material extract found 60 minutes protection respectively. The active ingredients (alkaloids, flavonoids, saponins, phenolics and tannins), present in the phytochemical extracts from the citrus peels might have exerted some inhibitory effect on lactic acid receptor cells by masking or changing the lactic acids that normally attract them thereby confusing or distracting the mosquitoes [55].

The repellent action of the plant extracts tested was varied depending on the solvent used in extraction and the dose of the extract. The most effective plant extract that evoked 100% repellency or biting deterrence was petroleum ether extract of *Tribulus terrestris* L., (Zygophyllaceae) at a dose of 1.5 mg/cm^2 compared with 100% repellency for commercial formulation, N,N-diethyl-3-methylbenzamide (DEET) at the same dose [60]. Present study found *Citrus hystrix* fruit and peel repellency activity of complete protection time were higher than the internal materials extract against *Aedes* mosquitoes.Other researcher observed that among the tested solvents the maximum efficacy was observed in the leaf and seed methanol extracts of *Delonix elata* (*D. elata*) against *Aedes aegypti*. The highest concentrations of 5.0 mg/cm^2 provided over 180 and 150 min protection, respectively. Overall, the crude methanol extract of *Delonix elata* showed an excellent potential to develop newer and safer control tools the dengue vector mosquito *Aedes aegypti*. It is stated that petroleum ether extract of *Vicoa indica*, *Buddleja asiatica*, *Chenopodium ambrosoides*, *Clerodendrum inerme* and methanol extract of *Solanum erinthum* gave three hours protection against mosquitoes at 9% concentration [22]. It is reported that at 1% of garlic extract gave 8 h protection against *Culex fatigans* [61]. Repellency activity of complete protection time of *Artemisia vulgaris* oil dose 0.0002g/cm^2 provided 85.71% protection for 120 min, 97.62% protection for 30min respectively [62].

The plant products have been used traditionally to repel or kill the mosquitoes in many parts of the world. Novak [25] emphasised the urgent need for the investigation of phytochemicals as repellents for mosquito control. Certain

natural products have been investigated for repellent activity against mosquitoes. *Zanthoxylum armatum*, DC. syn. *Z. alatum* Roxb. (Rutaceae); *Azadirachta indica* (Maliaceae) and *Curcuma aromatica* (Zingiberaceae) were among them and have been reported to possess repellent properties against mosquitoes [15]. Effiom et al.,[53] reaveled that efficay of all the extracts from the different species of *Citrus* fruits exhibited repellent activity in their different concentrations though with varying degrees of time duration with the exception of 5% and 10% concentrations that did not produce any repellent effect. In extracts where 15% concentration recorded repellent effect, it was of very short duration (< 1 hour). The repellent effects of the *Citrus* phytochemical extracts were more pronounced in higher concentrations (of 20% and 25%). The observed variability of repellent activity amongst extracts from the different citrus fruit species may suggest that repellent activity is not only dependent on the concentration of a phytochemical extract but also on the source (i.e., the *Citrus* fruit species) from which it was obtained. The mode of action of these phytochemicals can not be unconnected with the suggestions made earlier by Jacobson [63]. *Citrus hystrix* essential oil has good potential for being used as a cockroach repellent [64]. In the present study *Citrus hystrix* fruit extracts 0.08g in 1ml of ethanol applied on 343.5 cm^2 of forearm skin between the wrist and elbow found 100% protection from biting of *Aedes* mosquitoes. Ethanol extract of *Citrus spp*., Chemical compounds of flavonoid, terpenoid, saponin, and essential oil are identified [65]. These compounds are potential as antifeedant to insects, larvicidal, and insect repellent. Terpenoid compounds of group limonoid can cause loss of organ coordination in *Ae.aegypti* larvae [66]. It is generally known that the yield of essential oil depends not only on the plant species and their climatic or geographical areas, but also other variables such as method of extraction and plant-related factors, including parts of plant, rearing condition, maturation of the harvested plant, and plant storage or preservation [26, 67]. In order to achieve the best yield, it is therefore necessary to establish the most appropriate combination of these variable factors. However, in addition to the yield of essential oil, much consideration was given to the quality and quantity of chemical constituents, particularly the major active ingredients.

CONCLUSION

Therefore the study concluded that *Citrus hystrix* fruit, peel and internal material extracts were very effective to control larvae and protection of mosquito bite and they were used as larvicidal, ovicidal and repellent against *Aedes aegypti* mosquitoes. Further studies on identification of active compounds and field trials are needed to recommend the active fraction of *Citrus hystrix* fruit extracts for development of eco-friendly chemicals and indigenous plant base materials were developed for the protection of mosquitoes bite. To avoid mosquito resistance to chemical/ synthetic insecticides and to protect the environment and public health, native larvicidal and insecticidal plant base extracts or essential oils have been suggested as an alternative source of material for larva and mosquitoes control in our enviroment. An insect repellent of plant origin ought to be well-defined and harmless to human and other non-target organisms. Therefore, use of these botanical derivatives in mosquito control instead of synthetic insecticides could reduce the cost and environment effects. The study documented the promising larvicidal , ovicidal and repellency potential of extracts of *Citrus hystrix* DC fruit, peel and internal materials, which could be considered as a potentially alternative source for developing novel larvicides and repellency to be used in controlling vectors of mosquito borne disease.

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