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Punica Granatum-Based Green Ethanolic Extract as Highly Effective and Eco-Friendly Larvicide, Repellent against Medically Important Mosquito Vectors

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ABSTRACT

Background & objective: Mosquito vectors are living organisms that can transmit infectious diseases between human from animals to humans. It is bloodsucking insect that ingest disease-producing microorganisms during a blood meal from an infected host. The present investigation discovered that the larvicidal activity of ethanol extract of P. granatum showed most mortality among the opposite crude extracts. Methods: The chemical composition of P. granatum ethanolic extract was analyzed by gas chromatography-mass spectroscopy. A total of twenty five III instar larvae of An. stephensi and Cx. quinquefasciatus were exposed to various concentrations (50-250 ppm) in the laboratory by using the standard protocol described by WHO (2005). The repellent activity of P. granatum chemical compositions tested against 100 blood starved female mosquitoes of An. stephensi and Cx. quinquefasciatus using the protocol of WHO (1996). Results: In GC-MS analysis, a total of seven compounds were identified in the ethanolic extract composition, the main component was Methyl 4-piperidineacetate. Further, the LC_{50} and LC_{90} values were found to be 110.36 and 212.28 mg/L against Cx. quinquefasciatus. The repellent activity to be best and therefore the most activity was ascertained at 3.5 mg/cm^2 concentration provided 100% protection up to 240 min against Cx. quinquefasciatus. Conclusion: The results clearly show confirmed that the presence of active compounds in leaf of P. granatum.

Keywords: *Punica granatum, Anopheles stephensi, Culex quinquefasciatus, FT-IR, GC-MS.*

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INTRODUCTION

Malaria is one in all the grave scourges inflicted upon human beings and causes human mortality alongside giant economic loss [1-6]. In line with the newest estimates, there have been regarding 198 million cases of malaria in 2013 and a calculable 584,000 deaths. Most deaths occur among youngsters living in continent, wherever a baby dies each minute from malaria [7] and killed an expected 3,06,000 under-fives widely, including 2,92,000 children in African countries [8]. Death rates have fallen by 61 per cent for 2000 and 2015, with a more 13 countries "approaching elimination" reported WHO, 2016. Presence of the report, India statement for 6 per cent of all malaria cases in the world, 6 per cent deaths, and 51 per cent of the cases in world. The statement estimates the total cases in India found in 1.31 million and deaths at 194 reported WHO, 2017. *Culex quinquefasciatus* is a crucial feature inflicting filariasis, St. Louis encephalitis, Avion malaria and West nile virus. It's extensive-

ly studied, because it transmits crucial diseases [3, 9, 10]. In 2014, estimate is impure with lymphatic filariasis parasites and over 20 per cent of the planet population is at hazard of getting roundworm infection. In Asian nation, it's calculable that regarding 554.2 million folks area unit at hazard of humor disease unhealthiness in a pair of 43 districts [11, 12]. Around the world, 25 million men clumsy person with sex organ sickness and over 15 million folks are afflicted with lymphedema [13].

About 40% of the world's population is at risk from mosquito-borne diseases. In 2015, 2.35 million cases of dengue were reported in the Americas, of which 10 200 cases were identified as severe dengue causing 1181 deaths [14-17]. The year 2015 was characterized by abundant global dengue outbreak, then Philippines reporting more than 169 000 cases and Malaysia exceeding 111 000 doubtful cases of dengue, defining a 59.5% and 16% increase in case numbers to the previous year, respectively. Brazil separately documented over 1.5 million cases in 2015, approximately 3 times higher than in 2014 [18-23].

Punica granatum is one in every of the oldest cultivated plants within the world [24]. It's a crucial crop proverbial by its style and organic process and medicinal properties [25-31]. Many studies have reported the antimicrobial and antifungal [32, 33], molluscicidal [34] and isecticidal [35] activities of extracts from completely different tree components, like bark, leaves, fruit and fruit peel. Biopesticides are alternative to synthetic pesticides because of their generally low environmental pollution, low toxicity to humans and other applications [36]. The chemicals constituents had been isolated and identified from flowers and fruits of pomegranate. The bark and stem contain a number of alkaloids. This research would be helpful to foster research aimed at the identification of novel and safer plant-borne mosquitocides. Further, present study investigated larvicidal and repellent activity of *P. granatum* ethanol extract on important mosquitoes.

MATERIALS AND METHODS

Sample collection and preparation

P. granatum leaves were collected from around Velankanni (10º40'N to 11º12'N latitude and 79º50'E to 80º72'E longitude), Nagapattinam District, Tamilnadu in India. The dried leaves (100g) were powdered by electrical stainless-steel liquidizer and extracted with hexane, ethyl acetate, chloroform and ethanol by Soxhlet equipment. The extract was collected by reduced pressure 22–26 mmHg at 45°C by 'Rotavapour' and therefore the residue obtained was hold on at 4°C. The condensed crude leaves extract was hold on in refrigerator till needed for investigation for larvicidal and repellent activities.

Larvicidal activity

The larvicidal activity of crude *P. granatum* extracts were evaluated based on the method described previously [36]. In view of the wide range and thin range tests, all concentrates from 50-250 ppm were readied and were tried against the newly shed (0-6 shrs) third instar hatchlings of *An. stephensi* and *Cx. quinquefasciatus*. The plants concentrates were added to 1 ml DMSO (Dimethyl sulfoxide) and afterward diluted in 249 ml of dechlorinated faucet water. The control was prepared utilizing 1ml of DMSO as a part of 249 ml of dechlorinated water. The hatchlings of test species (25) were placed in 250 ml plastic glass with 250 ml of fluid medium (249 ml of dechlorinated water + 1ml of Dimethyl Sulfoxide) and the required measure of compound syntheses was included. The larval mortality was inspected and recorded after 24 h post treatment. For every examination, five recreates were kept up at once. Percent mortality was rectified for control mortality according to [37].

Repellent activity

The repellency was evaluated by victimization of the minutes of protection in respect to dose technique was utilized by World Health Organization [38]. Three day old blood-starved mosquitoes (100) were unbroken in a very web cage (45cm × 30cm × 45cm). The volunteer had no contact with lotion, perfumes or perfumed soaps on the day of the assay. The arms of volunteer, solely 25 cm^2 dorsal facet of the skin on every arms were exposed and therefore the remaining space lined by rubber gloves. The crude extracts were applied at 3.5 mg/cm^2 on an individual basis within the exposed space of the fore arm. The time of the take a look at obsessed with whether or not are the target mosquitoes day or

night biters. *An. stephensi* and *Cx. quinquefasciatus* were tested in dark from 20:00 to 4:00. The management and treated arm were introduced at the same time in to the experimental cages, and the mosquitoes were activated. Every take a look at concentration was perennial five times. The volunteer conducted their take a look at of every concentration by inserting the treated and management arm in to an equivalent cage for one full minute for each 5 minutes. The mosquitoes that landed on the hand were recorded and so jolted off before uptake of any blood; creating out a five minutes protection. The proportion of repellency was calculated by the subsequent formula.

$$\% \ Repellency = [(T_a\text{-}T_b)/T_a] \times 100$$

Where T_a is the quantity of mosquitoes in the control group and T_b is the quantity of mosquitoes in the treated group.

Statistical analysis

The examination program probit [39] was utilized for the determination of LC_{50}, LC_{90} and different insights at mean, slope, regression, chi-square qualities were figured utilizing the SPSS 16.0 programming.

RESULTS AND DISCUSSION

The larvicidal activity of crude ethanol, ethyl acetate, chloroform and hexane solvent extracts of *P. granatum* against *An. stephensi* and *Cx. quinquefasciatus* were studied. The ethanol extract of *P. granatum* reported in the present study showed the mosquitocidal properties in the plant, suggestive of their use in mosquito population control (Table 1). *Cx. quinquefasciatus* was more vulnerable followed by *An. stephensi*. Plant extracts exhibited the maximum larvicidal activity with LC_{50} and LC_{90} values of 125.78 and 225.98 mg/L against the larvae of *An. stephensi*, followed by, the ethyl acetate, chloroform and hexane extract with LC_{50} and LC_{90} values are 134.71, 171.27, 198.07 and 232.83, 271.49, 299.88 mg/L. Ethanol, ethyl acetate, chloroform and hexane extracts of *P. granatum* against *Cx. quinquefasciatus* with LC_{50} and LC_{90} values are 110.36, 126.68, 151.06, 176.49 and 212.28, 224.34, 256.75, 275.71 mg/L, respectively (Table 2). The repellent action of the *P. granatum* extract showed important repellent against *An. stephensi* and *Cx. quinquefasciatus*. It showed that repellency depends on the potency of the 3.5 mg/cm^2 provided 100% protection up to 200 and 240 min against *An. stephensi* and *Cx. quinquefasciatus,* respectively (Table 3).

The mass spectral analysis of seven compounds, concentration of percentage (%) and retention indices were summarized in Table 4 and the mass chromatogram was shown in Figure 2. Among all, cardanolide ($C_{23}H_{36}O_2$), n-Boc-4-piperidineacetaldehyde ($C_{12}H_{21}NO_3$), 4-Cyclopropylbenzaldehyde ($C_{10}H_{10}O$), 3,5-Dimethylcyclohexanone ($C_8H_{14}O$), Digoxigenin ($C_{23}H_{34}O_5$), Methyl 4-piperidineacetate ($C_8H_{15}N0_2$), 2',6'-Dihydroxyacetophenone ($C_8H_8O_3$).

Table 1. Percentage mortality of mosquito larvae of *An. stephensi* and *Cx. quinquefasciatus* exposed to different concentrations of different solvent leaf extracts of *P. granatum*.

Extracts	*An. stephensi*		*Cx. quinquefasciatus*	
	Concentration (ppm)	mortality±SD[a]	Concentration (ppm)	mortality±SD[a]
Hexane	Control	0.00±0.0[f]	Control	0.00±0.0
	50	4.2±1.7[a]	50	5.6±1.8[a]
	100	10.6±2.6[a]	100	16.2±2.2[a]
	150	25.8±2.7[ab]	150	37.8±1.7[ab]
	200	48.6±2.6[bc]	200	56.8±2.2[c]
	250	76.8±2.7[cd]	250	85.8±1.7[d]
Ethyl acetate	Control	0.00±0.0	Control	0.00±0.0
	50	15.8±2.4[a]	50	18.8±3.8[a]
	100	32.6±3.1[ab]	100	36.2±2.1[ab]
	150	54.4±2.3[bc]	150	57.8±3.6[c]
	200	76.4±2.3[cd]	200	78.6±3.5[cd]
	250	97.2±1.7[e]	250	99.2±0.8[e]
Chloroform	Control	0.00±0.0	Control	0.00±0.0

	50	6.8±2.6[e]	50	12.2±2.6[a]
	100	17.8±2.4[a]	100	26.4±3.2[ab]
	150	39.6±2.5[ab]	150	48.8±2.2[bc]
	200	60.8±2.5[c]	200	69.4±2.5[cd]
	250	86.6±2.1[d]	250	90.8±2.1[de]
Ethanol	Control	0.00±0.0	Control	0.00±0.0
	50	19.2±3.2[a]	50	24.8±2.7[a]
	100	37.4±2.5[ab]	100	42.6±2.6[b]
	150	58.2±2.6[c]	150	64.2±3.8[c]
	200	78.8±3.9[cd]	200	86.6±1.9[d]
	250	98.4±1.6[e]	250	100±0.00[e]

[a] Values are mean ± SD of four replicates. Within each row, different letters indicate significant differences (ANOVA, Duncan's new multiple range method test)

Table 2. LC_{50}, LC_{90}, slope, regression and chi squire analysis of larvicidal activity of *P. granatum* extracts against *An. stephensi* and *Cx. quinquefaciatus*

Species	Extracts	LC_{50} (mg/L)	95% Confidence limits		LC_{90} (mg/L)	Slope	Regression	χ^2
			LCL	UCL				
An. stephensi	Hexane	198.07	187.40	210.27	299.88	3.356793	y=0.919x+1.829	1.04[a]
	Ethyl acetate	134.71	124.65	144.46	232.83	3.775939	y=3.864x+1.140	4.30[a]
	Chloroform	171.27	161.33	181.78	271.49	3.52617	y=1.625x+1.604	1.05[a]
	Ethanol	125.78	115.25	135.71	225.98	3.832026	y=4.732x+1.022	5.35[a]
Cx. quinquefaciatus	Hexane	176.49	166.57	187.12	275.71	3.578278	y=1.354x+1.709	1.82[a]
	Ethyl acetate	126.68	116.43	136.42	224.34	4.100648	y=4.592x+1.041	7.00[a]
	Chloroform	151.06	140.70	161.49	256.75	3.366214	y=2.955x+1.266	1.13[a]
	Ethanol	110.36	106.34	129.18	212.28	3.987976	y=6.053x+0.889	6.04[a]

Values represent mean of five replications. Mortality of the after 24 h of exposure period LC_{50}= Lethal Concentration brings out 50% mortality and LC_{90}= Lethal Concentration brings out 90% mortality. LCL= Lower Confidence Limit, UCL= Upper Confidence Limit, χ^2 = Chi-squire,

[a] Significant at $p<0.05$

Table 3. Repellent activity of the *P. granatum* extracts against *An. stephensi* and *Cx. quinquefasciatus* at 3.5 mg/cm^2

Species	Extract	% of repellency					
		Time post application of repellent (min)					
		40	80	120	160	200	240
An. stephensi	Hexane	100±0.00	100±0.00	96.6±1.94	87.6±2.30	77.2±2.16	66.2±1.48
	Ethyl acetate	100±0.00	100±0.00	100±0.00	100±0.00	100±0.00	96.8±2.48
	Chloroform	100±0.00	100±0.00	100±0.00	96.8±1.30	86.2±3.27	75.2±1.64
	Ethanol	100±0.00	100±0.00	100±0.00	100±0.00	100±0.00	100±0.00
Cx. quinquefasciatus	Hexane	100±0.00	92.8±2.16	83.2±2.68	72.2±2.16	61.6±2.30	49.2±2.28
	Ethyl acetate	100±0.00	100±0.00	100±0.00	100±0.00	100±0.00	92.8±2.38
	Chloroform	100±0.00	100±0.00	94.4±1.81	84.2±2.38	72.6±2.60	61.8±2.48
	Ethanol	100±0.00	100±0.00	100±0.00	100±0.00	100±0.00	100±0.00

Values represent mean ± SD of the five replications

Table 4. List of identified phytocompounds in the *P. granatum* ethanol leaf extract

Peak	Compounds	MF	MW	RT(min)*	Concentration (%)
1	Cardanolide	$C_{23}H_{36}O_2$	344.539	12.17	9.75
2	N-Boc-4-piperidineacetaldehyde	$C_{12}H_{21}NO_3$	227.304	13.26	2.43

3	4-Cyclopropylbenzaldehyde	$C_{10}H_{10}O$	157.213	14.2	26.82
4	3,5-Dimethylcyclohexanone	$C_8H_{14}O$	126.199	14.45	21.95
5	Digoxigenin	$C_{23}H_{34}O_5$	390.52	15.33	2.12
6	Methyl 4-piperidineacetate	$C_8H_{15}N0_2$	146.189	16.19	34.14
7	2',6'-Dihydroxyacetophenone	$C_8H_8O_3$	152.149	17.9	4.87

*RT- Retention Time (min), MF- Molecular Formula, MW- Molecular Weight.

The parasite is transmitted by the bite of an infective female Anopheles mosquito. *P. falciparum* and *P. vivax* species cause the most contaminations around the world [40]. The results of present investigation showed that pure compound Methyl 4-piperidineacetate was more than 2-fold highest active than ethanolic leaf extract of *P. granatum* in larvicides, and repellent against *An. stephensi* and *Cx. quinquefasciatus*. The present investigation is comparable with some of other reports that the LC_{50} and LC_{90} values of 85.44 and 159.73 mg/L, from citronella component from *Melissa officinalis* were tested against *An. stephensi*. In the same way, highest larvicidal activity (LC_{50} values) were 136.75, 140.56, 144.90 and 149.89 mg/L for *Ageratina adenophora* ethyl acetate extract with I, II, III, IV instar larvae of *Cx. quinquefasciatus* [39]. Further, ethanol fractions of *Eichhornia crassipes* displayed the larvicidal and pupicidal activity against *Cx. quinquefasciatus* analyzed to solvent extracts and fractionates with LC_{50} values were 71.43, 94.68, 120.42, 152.15 and 173.35 ppm for first, second, third, fourth instars and pupae respectively. Presences of metabolites like flavonoides, alkaloids, anthroquinones and anthocyanins in the proved extracts might be the reason for the larvicidal and pupicidal action of the plant extracts and fractions of water hyacinth. The plant realm is considered as an asset for various kinds of potential drugs. In ancient days, many of the diseases were cured using plant products [39, 41-43]. Repellent action was not exhibited by these extracts at the tested concentrations. In potential, *Eichhornia crassipes* aquatic extract was successful in the control of the filarial vector, *Cx. quinquefasciatus* [44]. The bioactive compounds have been utilized to the development of environmentally safe vector managing agents. The extracts from aromatic plants are rising as possible mosquito vector control agents, since there are cheap, easy to administer and with hazard free properties [45, 46]. The compounds were eugenol, α-pinene and β-caryophyllene from *Plectranthus barbatus*. It is appeared to be most effective against *An. subpictus* (LC_{50}= 25.45, 32.09 and 41.66 µg/ml), followed by *Ae. albopictus* (LC_{50}= 28.14, 34.09 and 44.77 µg/ml) and *Cx. tritaeniorhynchus* (LC_{50}= 30.80, 36.75 and 48.17 µg/ml) [47].

Among the tested compounds, eucalyptol (1,8-cineole) and α-terpinyl acetate were considered to be inactive as the LC_{50}>50.0 mg L^{-1} [48]. Larvicidal leaf extract of *Gymnema sylvestre* showed the highest mortality in the concentration of 1000 ppm against *An. subpictus* (LC_{50}=166.28 ppm) and the maximum efficacy was observed in gymnemagenol compound isolated from *Gymnema sylvestre* petroleum ether extract with LC_{50} values against *An. subpictus* at 22.99 ppm and *Cx. quinquefasciatus* at 15.92 ppm, respectively [49]. The investigated compounds were β-pinene, sabinene, germacrene D, estragole and linalool in *Clausena anisata* against *An. stephensi, Ae. aegypti* and *Cx. quinquefasciatus* with LC_{50} values range from 11.01 to 42.28 µg/ml [50].

The phytochemical components and larvicidal activity to confirm the presence of various photochemical was studied for glycosidase, saponin, fixed oil & fats, protein, carbohydrates and tannin. The most effective larvicidal activity with concentrations 0.4% *Cassia tora* extracts gave 80% mortality in the larvae of *An. Stephensi* [51]. The larvicidal action of components of essential oils against mosquito species is due to the monoterpenes β-asatone, ρ-cymene, (+)-limonene, linaly acetate, myrcene, α-phellandrene, (+)-β-pinene, (-)-β-pinene, γ-terpinene and terpinolene, α-terpinene, phenylpropenes safrole and eugenol, and the sulfur containing compound daillyl disulfide on one or more species of mosquitoes[52]. Compounds were limonene, cis-carveol and carvone from *Mentha spicata* against *Cx. tritaeniorhynchus, Ae. albopictus, An. subpictus* and LC_{50} values range from 9.82 to 36.33 µg/ml [52-54] reported that major phytochemical compound, phytol isomer in chloroform extract of

Terminalia chebula leaf, which have potential mosquito larvicides and pupicides on *Cx. Quinquefasciatus* [55-57].

In conclusion, generally, this research provides useful information for the safer mosquito control properties and development of newer ones. Concerning the composition of the *P. granatum* ethanol extract, it was mainly composed by Methyl 4-piperidineacetate compound. Mosquitocidal activity clearly noticed the toxicity of *P. granatum* ethanol extract against *Cx. quinquefasciatus* larvae, even at low dosages. Further studies needed to validate and develop efficient mosquito larvae and adults with least impact on human health and environment.

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