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Standardisation and larvicidal activity of *Cleome viscose* Linn.

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Abstract
Progressive and ascending use of traditional systems of medicines has embarked a great question on quality of herbals. In present study, *Cleome viscose* Linn., (*Sticky Cleome*) was successively extracted; standardised and larvicidal activity was assessed against *A. stephensi, Cx. quinquefasciatus* and *Ae. aegypti*. A dose depended % mortality of the test solutions was established compared to standard and control. The chloroform extract had highest larvicidal activity with least LC_{50} values compared to other extracts. A greater sensitivity of the *Culex quinquefasciatus* was observed in contrast to other mosquito species. The biochemical effects on the larval lumen and respiratory gills were owed to alkaloids, tritepenoids and steroids. Standardisation of plant material and extracts is the first and foremost step in quality control of herbal drugs. The larvicidal activity insights potential use of chloroform extract of *Cleome viscose* Linn., a common weed as a vector bio-control agent and develop bio-pesticidal formulations.

Keywords: *Cleome viscose* Linn., larvicidal activity, mosquitoes, *A. stephensi, Cx. quinquefasciatus*

Introduction
Plant based drugs are the most primitive form of deriving remedies for human ailments. With sedentary lifestyle including the food habits, the longevity of life has substantially decreased. Though allopathic systems are chosen for symptomatic relief, on the long run herbal drugs are preferred. People around the world prefer herbal drugs as a source of medicine [1, 2, 3]. World Health Organisation estimates that over 80% of world population depend on plant sources for maintenance of health. Plants sources available locally are incorporated in to the indigenous systems of medicines and developed in to different systems of medicines. According to Indian system of medicine- Ayurveda, medicines are utilised for restoration of health in diseased or to inculcate a state of wellness in a living being. This principle is owed to the phyto- constituents [4, 5].

With progressive and ascending use of traditional systems of medicines, the quality of herbals embarks a great question [6, 7]. Herbal drug quality varies on various factors such as climate, geography, time and period of collection, processing and storage conditions. Herbals are greatly adulterated in this regard [8, 9]. Hence it becomes a bound responsibility for every personnel involved in herbal drug research to develop methods of standardisation for assessment of quality. Standardisation is the assessment of quality control parameters of the various medicinal plants used in traditional medicine is becoming more important today in view of the commercialization of formulations based on these plants [10, 11, 12]. World Health Organisation specifies guidelines for assessment of quality control of herbal drugs with various parameters [13].

Cleome viscose Linn. commonly called as "*Sticky Cleome*" is a well known weed found in tropical and subtropical regions of India [14, 15, 16, 17]. The plant is reported to have immuno-modulatory, antidiarrheal, psycho-pharmacological and wound healing properties [14, 15]. The standardisation profile of *Cleome viscose* Linn. from Western Ghats- a rich biodiversity and geographical hub has not been explored and reported earlier. Also, the literature sources lack in terms of parameters of chromatographic and phyto-chemical investigations in relation to its insecticidal properties. Reports on larvae of *Culex quinquefasciatus, Tribolium castaneum, Callosobruchus chinensis* are crude extracts like pet ether, acetone, methanol and water [18, 19]. Successive solvent extraction separates out phyto constituents based on its polarity to extract mosquito larvicidal constituents [20, 21].

Mosquitoes are a mayhem acting as vectors for deadly diseases [22]. Sudden outbreaks in high risk endemic areas cause high communal mortality [23]. Thus, vector control is the first and foremost step to interrupt further disease proliferation [24].

The larval stage of mosquitoes is easily accessible and is the longest stage in the mosquito life cycle. Synthetic chemical larvicides used turn out to be resistant and also grounds to ecological imbalance. Hence plants are considered as alternate sources since they contain plethora of insecticidal phyto-constituents [24, 25].

The plant was extensively reviewed and the gap analysis directed the research towards standardisation of plant drug, successive solvent extraction and comparative larvicidal activity of extracts on three primary mosquito vector species – *Anopheles, stephensi*, *Culex quinquefasciatus*, and *Aedes aegypti*. Hence such as attempt was put forth and the results have been presented in the article.

Materials and Methods

Plant material- authentication, extraction & standardisation

Aerial parts of *Cleome viscose* Linn. were collected from Western Ghats of Karnataka (Sirsi district). Plant material was authenticated by a Plant taxonomist, Natural Remedies Pvt. Ltd. A voucher specimen was prepared and deposited at the herbarium of KLE College of Pharmacy, Bengaluru, India and Natural Remedies Pvt. Ltd., Bengaluru, India for future reference. The plant material was evaluated for various pharmacognostical characteristics and physico-chemical parameters.

The air dried plant material (1.40 kg) was pulverized in laboratory scale electrical blender to moderately coarse powder (#40). Each time 150g of plant powder was successively extracted with pet-ether (60-80º), chloroform, ethanol in soxhlet assembly and distilled water. The extracts were concentrated by rotary evaporator (Buchi R3) at 40°C. The yield of extracts were calculated and stored under refrigeration until further use. A standard protocol for standardization of plant material and successive solvent extracts including the organoleptic evaluation, physico-chemical evaluation including moisture content(LOD), ash values, extractive values, swelling index, foaming index and phyto-chemical screening, TLC analysis were followed according to WHO guidelines for assessment and quality control of herbal drugs.

Evaluation of mosquito Larvicidal activity

Collection of mosquitoes

The late 3rd/ early 4th instar larvae of *A. stephensi* (RS-Bangalore strain), *Cx. quinquefasciatus* (Bangalore strain) and *Ae. aegypti* (RS- Bangalore strain) were collected from cyclic colony of the insectory of National Institute of Malaria Research (ICMR), Bengaluru, India.

Preparation of test and standard solutions

100 ml of stock solutions (10 mg/ml) were prepared (10 ml DMSO for solubility) and were stored in capped volumetric flasks and covered with aluminium foil. Concentrations 10-1000 ppm were prepared and used for larvicidal activity. 1ml of DMSO in 99 ml tap water as negative control and 10-50ppm of azadirachtin (0.1% Neemark™) in tap water was used as positive control.

Larvicidal activity

Larvicidal bioassay was carried out on mosquito larvae according to the World Health Organization (WHO 2005) standard protocols [26]. 25 larvae of each of vector species were exposed to test, standard and control solutions. Each experiment was carried out thrice in triplicates. The % mortality was recorded every hr up to 6 hr and later at 6hr duration until 72 hr (3 days).

Results and Discussions

Standardisation of plant material and extracts

Standardisation should be the first and foremost step in research related to any herbals products. It segregates the most suitable drug, both qualitatively and quantitatively to be utilized as herbal remedy. With well established pharmacognostical, physico-chemical, biological evaluation methods every herbal drugs, extracts and formulations can be standardised. As an initiative step towards standardisation the above mentioned parameters were evaluated and are presented.

The plant material was authenticated by its morphological and taxonomical characteristics by plant taxonomist Dr. Santhan P, Natural Remedies Pvt. Ltd, Bengaluru as "*Cleome viscose* Linn". A voucher specimen, KLE/CV/221/2014 at the herbarium of KLE College of Pharmacy, Bengaluru and NR/CV/221/2014 at Natural Remedies Pvt. Ltd, Bengaluru. Pharmacognostical evaluation which comprises of organoleptic evaluation and foreign organic matter are presented in Table no.1. The picto-microgarphs of powder characteristics are presented in Figure no. 1. These parameters could be utilized as an authenticated reference for pharmacognostical evaluation of samples of *Cleome viscose* Linn., from the Western Ghats region of India. Ash values and foreign organic matter indicates the purity of sample and degree of adulteration in herbal drugs. Extractive values give an insight of quantity of phyto-constituents present in the crude drugs that can be extracted with specific solvent.

Extraction of plant material is a critical step in preparation of extracts to separate medicinally active components. The choice of extraction method depends on nature, stability and cost of drug; concentration of phyto-constituents, therapeutic value, solvent and its recovery process. In consideration of such factors, the successive solvent method of exhaustive extraction was preferred (non-thermo-liable phyto-constituents). The plant material was grounded to sieve #40 in laboratory scale electrical blender to avoid much heat generation. Soxhlet method of successive solvent extraction was carried with non-aqueous solvents like pet-ether (60-80º), chloroform, ethanol (60% v/v). The solvent choices were based on polarity indices viz., petroleum ether (0.0), chloroform (4.1), ethanol (5.2) and water (9.0). Though benzene, hexane and other solvents are ranked higher in polarity charts (than our choices) the carcinogenicity, relative toxicities and side effects are note worthy [27, 28]. In concentration of extracts, drying under reduced pressure was preferred (rotavapour), to ensure safety and stability of the active constituents. The % yields of concentrated extracts were 4.226, 3.867, 3.228, 4.862% for solvents- petroleum ether, chloroform, ethanol and water respectively.

A standard protocol for standardization of plant material and successive solvent extracts in cluding the organoleptic evaluation, physico-chemical evaluation including moisture content (LOD), ash values, extractive values, swelling index, foaming index and phyto-chemical screening, TLC analysis were followed according to WHO guidelines for assessment and quality control of herbal drugs.

Table 1: Standardisation of aerial parts of *Cleome viscose* Linn.

Parameters (% w/w) ± SD		Aerial parts
Organoleptic Evaluation	Nature	Amorphous
	Color	Green
	Odour	Characteristic
	Consistency	Powder
Physico-chemical Evaluation	Moisture content *	2.568 ± 0.26
	Ash values	
	Total ash	4.336 ± 0.52
	Water soluble ash	0.765 ± 0.15
	Acid insoluble ash	1.036 ± 0.16
	Extractive values (EV) % w/w	
	PE soluble extractive values	3.899 ± 0.56
	CL soluble extractive values	2.197 ± 0.28
	ET soluble extractive values	4.215 ± 0.63
	AQ soluble extractive values	8.239 ± 0.35
Foreign organic matter		1.626 ± 0.25

% w/w- percentage weight/weight with reference to air dried material, SD- Standard deviation; PE-Pet ether 40-60˚C, CL-Chloroform, ET-Ethanol, AQ- Water extract, (*)- Loss on drying.

Table 2: Standardisation of Successive solvent extracts of *Cleome viscose* Linn.

Parameters (% w/w) ± SD		PE	CL	ET	AQ
% yield		4.226 ± 0.18	3.867 ± 0.22	3.228 ± 0.82	4.86 ± 0.24
Organoleptic Evaluation	Nature	Oily	Viscous liquid	Viscous liquid	Brown
	Color	Dark green	Pale green	Green- brown	Brown
	Odour	Characteristic	Characteristic	Pleasant	Characteristic
	Consistency	Sticky	Sticky	Sticky	Dry powder
Physico-chemical Evaluation	Moisture content*	0.0036 ± 0.12	0.0062 ± 0.11	0.0021 ± 0.15	0.0033 ± 0.23
	Phyto-chemical screening	Steroids, triterpenoids	Alkaloids, steroids	Flavonoids, tannins, phenolics	Carbohydrates, proteins

% w/w- percentage weight/weight, with reference to air dried material, SD- Standard deviation; PE-Pet ether 40-60˚C, CL-Chloroform, ET-Ethanol, AQ- Water extract, (*)- Loss on drying.

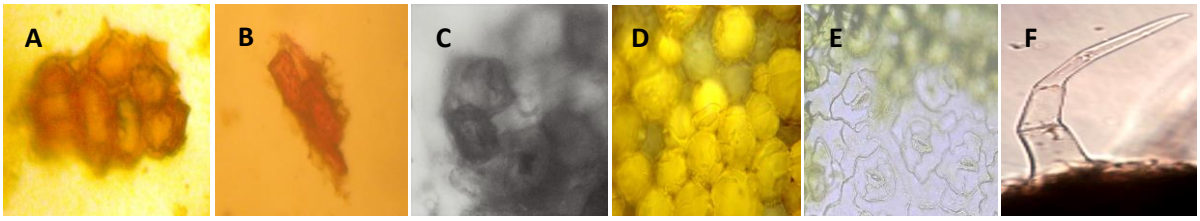
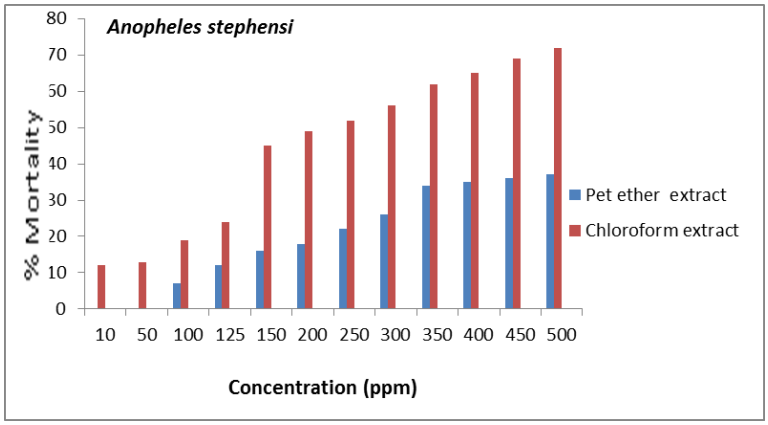


Fig 1: Powder microscopy of aerial parts of *Cleome viscose* Linn. A- Lignified sclerids; B- Pitted parenchyma; C- Prisms of calcium oxalate; D- Spongy parenchyma; E- Anamocytic stomata; F- Covering trichomes

Larvicidal activity

The successive solvent extracts were subjected to larvicidal bioassay according to WHOPES scheme. A dose depended % mortality was established compared to standard and control. A comparison of the % mortality of all the extracts is represented in Figure no.2. Among the extracts assayed, the chloroform extract had the highest larvicidal activity at less concentration compared to pet-ether, ethanolic and aqueous extracts. The graph clearly indicates greater sensitivity of the *Culex quinquefasciatus* than other mosquito species. The order of sensitivity is *Culex quinquefasciatus* > *Anopheles stephensi* > *Aedes aegypti*. In ethanolic and aqueous extracts dose depended mortality was observed over 3000ppm. Hence are considered as inactive larvicides.



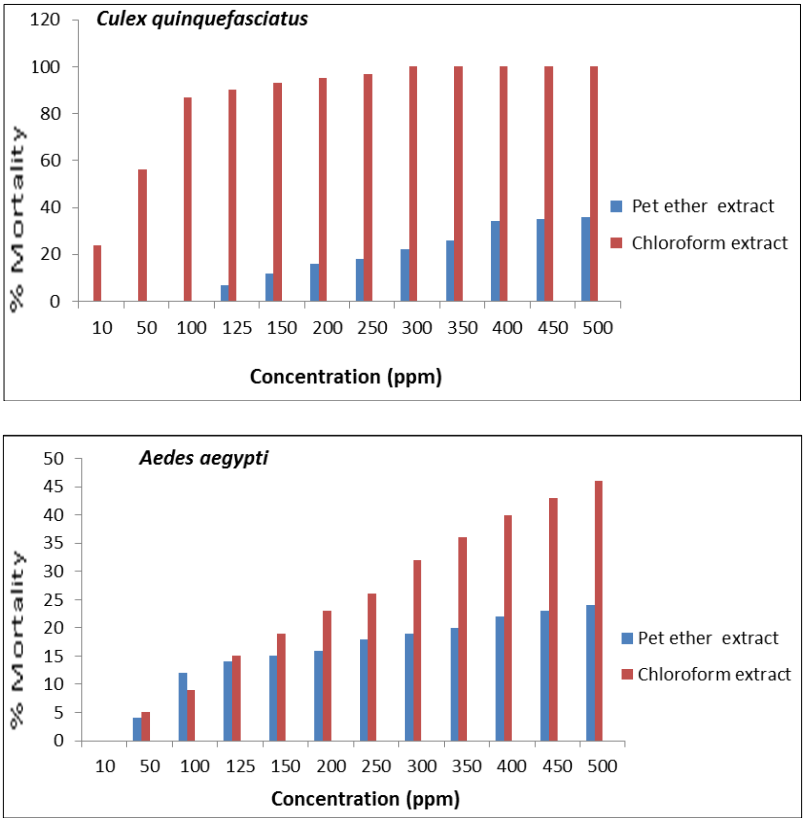


Fig 2: Comparison of % Mortality at 10-500ppm of successive solvent extracts on *Anopheles stephensi*, *Culex quinquefasciatus* and *Aedes aegypti* respectively.

The larval mortality affected by the chloroform extract was also observed microscopically. The chloroform extract induced erosive effects on inner lining of gastric lumen of anterior, posterior mid gut and on anal respiratory gills (Figure no. 3). Table no. 2 represents the probit analysis of the % mortality conversion of the linear regression line to the log-dose to give a dose response curve (DRC). Thus the LC_{50}, LC_{90} and limits of LC_{50} values can be calculated. The results were significant ($p \leq 0.05$) with higher chi-square values at 3 and 4 degrees of freedom. Though the LC_{50} values are higher compared to standard azadirachtin, the similar biochemical effects on the larval lumen and respiratory gills were observed.

Based on phyto-chemical screening, the most active chloroform extract was eluted in various solvent systems. TLC analysis in toluene: ethyl acetate (8:2) gave best separation of 14 phyto constituents after derivatized with Vanillin sulphuric acid reagent (Figure no. 4 & Table no. 3). These results indicate the phyto-constituents such as alkaloids and steroidal compounds on the chloroform extract may have induced the larvicidal effects on mosquito larvae similar to many plant drugs like *Zanthoxylum lemairei* [29], *Sphaeranthus indicus*, *Cleistanthus collinu* [30], *Murraya koenigii* [31], *Piper longum* [32].

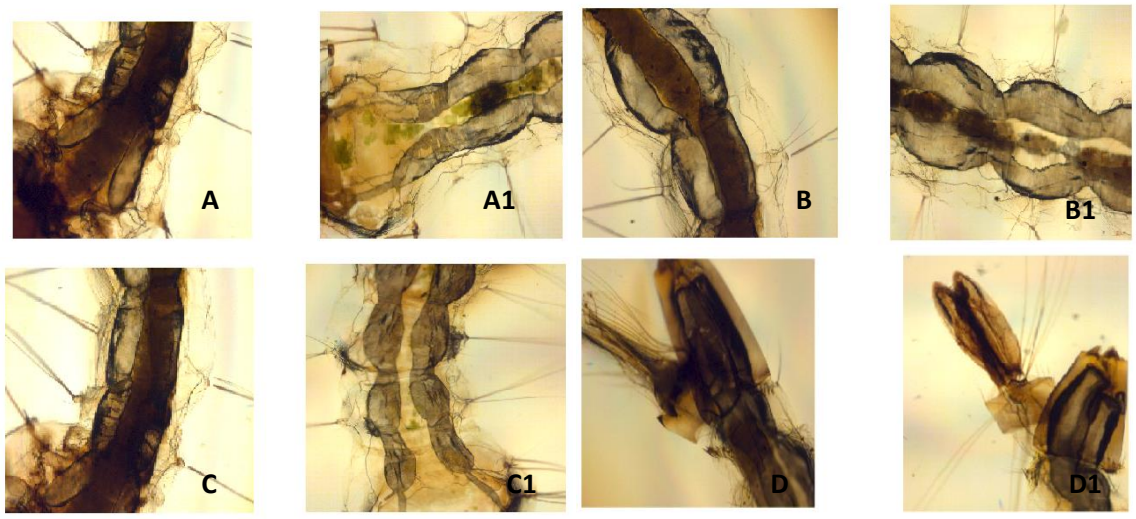


Fig 3: Larvicidal effects of chloroform extract of *Cleome viscose* Linn. on *Aedes aegypti;* A,B,C,D- control; A1, B1, C1- effects of chloroform extract at 100 ppm on anterior and posterior mid gut; D1- effects of chloroform extract on anal respiratory gills

Table 3: Larvicidal bioassay of successive solvent extracts

Mosquito species	Extracts	LC_{50} values (ppm)	LC_{90} values (ppm)	χ^2	Limits for LC_{50} (ppm)	
					LCL	UCL
Anopheles stephensi	PE	1259.33	1697.78	13.98	2180.93	2337.74
	CL	141.99	649.56	34.54	90.35	474.22
	ET	1617.76	2754.94	42.12	1494.73	1752.52
	AQ	4422.34	6788.45	51.43	3985.67	6932.77
	AZ	58.58	86.66	28.48	48.78	128.62
Culex quinquefasciatus	PE	736.29	4080.48	36.99	327.16	1022.72
	CL	35.68	368.75	21.22	20.73	52.85
	ET	1033.61	2694.99	65.27	943.55	1108.06
	AQ	3345.65	6298.45	23.65	2977.56	3647.69
	AZ	47.43	85.53	43.45	32.78	96.56
Aedes aegypti	PE	898.31	2083.62	22.07	468.99	1326.06
	CL	386.32	937.93	34.46	98.17	590.43
	ET	1777.73	2020.70	45.44	1487.50	1991.82
	AQ	3802.56	6890.51	28.38	3598.46	6946.64
	AZ	110.36	146.34	27.33	97.66	145.42

PE- petroleum ether, CL-chloroform, ET- ethanol, AZ- standard azadirachtin LC_{50}- Lethal concentration at which 50% larvae are dead, LC_{90}- Lethal concentration at which 90% larvae are dead, χ^2- Chi-square values, LCL- Lower confidence limit, UCL- Upper confidence limit; ($p \leq 0.05$)

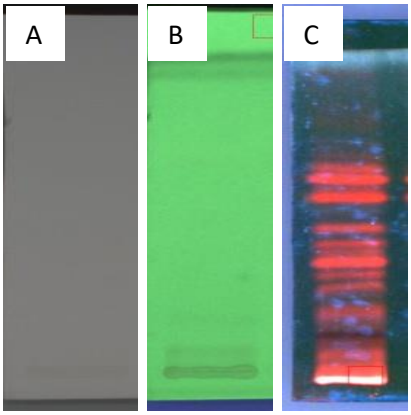


Fig 4: TLC of chloroform extract of *Cleome viscose* Linn. in A) day light; B) 254nm; C)366nm after derivatization with vanillin sulphuric acid reagent

Table 4: TLC profile of chloroform extract of *Cleome viscose* Linn.

Detection: Toluene : Ethyl acetate (8:2)					
Day light		UV 254nm		Vanillin Sulphuric acid reagent	
Spots	R_f value	Spots	R_f value	Spots	R_f value
2	0.75 (light yellow), 0.80 (light green)	2	0.75 (fluorescent orange), 0.80 (fluorescent orange)	14	0.03 (dark brown), 0.07 (dark brown), 0.12 (brown), 0.19 (brown), 0.33 (brown), 0.47 (purple), 0.52 (purple), 0.54 (purple), 0.68 (purple), 0.73 (purple), 0.77, 0.84 (yellow), 0.85 (light green), 0.91 (green)

Conclusions

The study not only explored the standardisation of *Cleome viscose* Linn from a rich bio-diverse geographical region of Western Ghats but also the larvicidal activity at concentrations of less than 10 ppm on *Culex quinquefasciatus* and *Anopheles stephensi* and 50 ppm on *Aedes aegypti*. This insights the potential use of *Cleome viscose* Linn., a common weed for mosquito larval control in breeding sites.

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