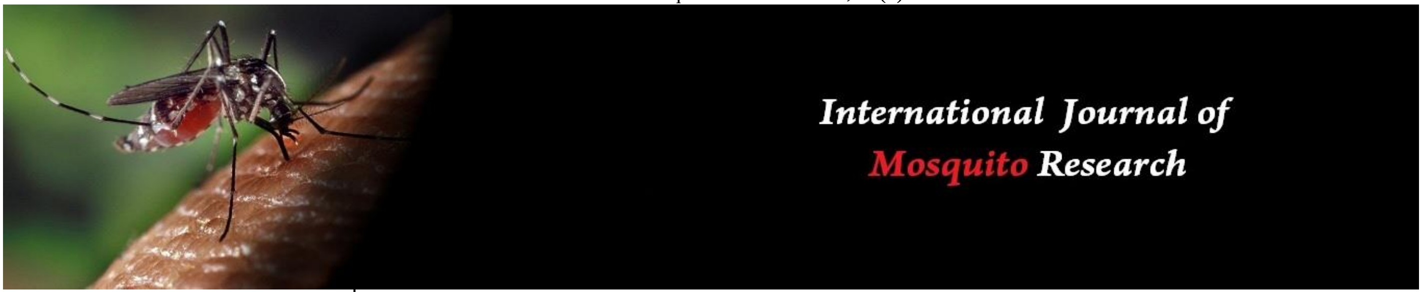

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Bio-efficacies of different solvent extracts of *Cosmos sulphureus* on 3rd instar larvae of *Aedes aegypti*

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Abstract
Mosquitoes are vector carriers of Dengue haemorrhagic fever, Malaria, Chikungunya, Yellow fever, Zika virus, Filariasis and Japanese encephalitis which are mainly found in tropical and sub-tropical countries. Mosquito borne disease have great impact on human health in terms of mortality and morbidity along with economic loss as per the survey conducted worldwide. The study of larvicidal activity of existing weeds mainly found on wastelands is very scanty. The potentiality of invasive weeds in controlling mosquito need to be explored on large scale. Hence, the present investigation was undertaken to evaluate larvicidal potential of different solvent crude (petroleum ether, chloroform, ethyl acetate, and methanol) of *Cosmos sulphureus* against 3rd instar larvae of *Aedes aegypti* L. *Cosmos* leaf extracts exhibited larval mortality with different solvents chloroform < ethyl acetate < methanol < petroleum ether with LC_{50} values as 2453.56 ppm, 4706.05 ppm, 5877.20 ppm and 8254.46 ppm respectively. In the results obtained chloroform extract has proved to be more effective than methanolic and ethyl acetate extracts. This study revealed that *Cosmos sulphureus* extracts in crude and pure formulations can further be used in controlling mosquitoes.

Keywords: *Aedes aegypti*, botanical pesticides, Bio-guided cold solvent extraction, *Cosmos sulphureus*, GC-MS analysis, Percentage mortality

Introduction
Mosquitoes belongs to Class Insecta are major vector of Dengue haemorrhagic fever, Malaria, Chikungunya, Yellow fever, Zika fever, Filariasis and Japanese encephalitis mainly in sub-tropical country like India [18, 25, 31, 35, 36]. Mosquito transmitted diseases are present in more than 125 countries and thus, all vector control strategies are mainly targeted on mosquitoes [38]. Mosquito borne disease have great impact on human health in terms of mortality and morbidity along with economic loss as per the survey conducted worldwide [3, 28, 30]. As per survey conducted by WHO in 2012 around 2/3rd or around 390 million of human population are at high risks from vector spreading diseases such as Dengue and Chikungunya and the cases reported were 15, 783 and 59,535 respectively, while 1-2 million death cases are due to Malaria [11, 24, 28, 30, 35,36].

Vector Borne Diseases (VBD) are mainly found in tropical and sub-tropical countries which is the most favourable environment for growth and proliferation of mosquito *Aedes aegypti* and other mosquito species such as *Anopheles* and *Culex*. VBD account for about 17% of 1 million population as per WHO survey 2014. Therefore, WHO has declared mosquitoes as “Public Enemy number one” [34].

Pests controlling organic synthetic chemicals such as DDT, pyrethroids especially for different species of mosquitoes [WHO 1996] have developed varying degree of physiological resistance in all species [3, 7, 11]. Organo-chlorines, Organophosphates, Carbamates such synthetic compounds have created problems within the ecosystem such as residual effects, destabilisation, biomagnification, environmental pollution, toxic impact on non-target organisms like fishes, crabs and copepods [7, 28, 30,36].

Considering the overall cause and effect of pests controlling agents over many decades, development of plant- based botanicals insecticides, mosquito controlling formulations which functions through lethal mechanisms on vectors is of utmost importance and need for sustainable future [24, 31, 35, 41].

Plant rich in secondary metabolites which are considered to be non-functional in plant growth, metabolism and reproduction are used for plant defence, prevention against herbivory (non-palatable), also possess some insecticidal properties. Plants-based formulations have been used in traditional practises effective against pests for generations with promising results. Botanicals such as Chrysanthemum, Pyrethrum, Derris, Quassia, Nicotine, Hellebore, Azadiractin and Turpentine have been used before DDT was introduced [36, 38]. According to studies conducted between plant-insect interactions it is observed that plants possess repellent, deterrent, fecundity, suppressant, ovicidal/ egg hatchability and insecticidal property [3, 30, 34].

Plant families like Asteraceae, Cladophoraceae, Lamiaceae, Meliaceae, Rutaceae, Solanaceae [25, 36]. Myrtaceae, Piperaceae, Poaceae, Verbenaceae, Apiaceae, Zingiberaceae [21] possess insecticidal and repellent properties against few species of mosquitoes. The application of phytochemicals of plants belonging to families Lamiaceae, Fabaceae, Asteraceae, Piperaceae and Euphorbiaceae have been tested against *Aedes aegypti* larvae [16, 38].

Phytochemicals such as alkaloids, coumarins, flavonoids, quinines, saponins, steroids and terpenoids prove toxic to immature mosquito larvae. Liu *et al*. (2012) in their studies conducted against *Aedes albopictus* mentioned alkaloids as one of the principal group of compounds amongst other different classes of phytochemicals present in plants to be toxic against mosquito larvae.

Researchers have mentioned that these pesticides are referred as “green pesticides” which are biodegradable, economical, recyclable and eco-safe. Such green pesticides are non -toxic to non-target organisms as fishes and copepods. These natural based formulations will reduce risks of cross-resistance and will thus will be target specific [31, 37, 41].

The study of larvicidal activity of existing weeds mainly found on wastelands is very scanty therefore selected for study [28,30]. Another reason for selecting weeds is their availability in abundance, no permission is needed for their research work and therefore will be beneficial in Integrated Pest Controlling programs (IPM) in future.

Cosmos sulphureus Cav is an ornamental plant (Asteraceae) native of Mexico and is used as a traditional medicine against *Plasmodium vivax* a malarial parasite in Brazil [13]. It is a potential herbicide due to presence of sesquiterpenes in leaves [29] and possesses larvicidal activity [20, 26]. Other properties studied are anti-microbial, anti-oxidant, anthelminthic, anti-diabetic, hypolipidemic and anti-proliferative activity, allelopathic effect on agricultural crops [4, 13, 9, 17, 23]. It also has anti- inflammatory, anti-fungal, hepato-protective potential as stated by Botsaris (2007) probably due to presence of quercetin, caffeic acid and chlorogenic acid.

Genus *Aedes* (Order Diptera, Family Culicidae) includes more than 950 species capable of transmitting diseases in human populations. *A. aegypti* L. (1762) mainly has been originated from tropical belts of Ethiopian regions of Africa, endemic to other countries like America, Pacific Island areas and S-E Asia has invaded all tropical and temperate regions mainly through shipments. It has been reported that the species can tolerate temperature ranges from 14 ℃-30 ℃ with 70% optimal relative humidity. Temperature plays an important role in metamorphic stages of *A. aegypti*.

Aedes aegypti L is a fresh water breeding mosquito mainly found in tropical and sub-tropical country like India. It mainly breeds by onset of monsoon and proliferates rapidly under given favourable conditions. *Aedes aegypti* is well known to spread vector borne diseases like Dengue fever, Chikungunya, Zika fever affecting human health. Larvae are taxonomically identified by 3 pairs of setae in ventral brush, antennae that are not greatly flattened and lack of enormous setae on the thorax. This particular species possesses white markings on legs and a lyre marking on the thorax.

In this context, the present investigation has been undertaken to establish the larvicidal activity of different solvents leaf extracts of *Cosmos sulphureus* Cav against 3rd instar larvae of *Aedes aegypti* L.

2. Materials and Methods

2.1 Plant Sample Collection

Comos is an annual, herb now encroaching dominant weed found near agricultural fields, wastelands and mainly along roadsides. *C. sulphureus* fresh leaves were collected along the roadsides from Tahsil Haveli, Dist. Pune region in its vegetative and few at its flowering stage during September-October. Leaves were washed thoroughly with tap water, later dried with filter paper and air shade dried for 15-20 days and leaves were grinded to fine powder and further stored in air tight containers.

2.2 Preparation of different solvent extracts

Dried powder 1kg was soaked with 2.5 liters solvents with increasing polarity (AR) in sequential order for 72 hours at room temperature. Bio-guided cold percolation method was used for extraction to obtain extracts. The solutions were filtered through muslin cloth and original stock solutions were subjected to Rotary evaporator under reduced pressure for complete evaporation of solvents. Thick sticky greenish extracts were obtained from each solvent and stored at 4℃ till further analyses.

2.3 Preparation of mosquito larval culture

The larval egg strips of *Aedes aegypti* were procured from research lab at Ross Life Sciences, Bhosari, Pune. The egg strips were dipped in tray containing normal tap water maintained in laboratory conditions with temperature of 25± 2 ºC and humidity 60-70% for hatching of larvae. Eggs were observed to hatch as 1st instar after 24 hours of incubation. Larvae were fed with appropriate amount of dog biscuit. Early 3rd instar larvae were used for further study.

2.4 Larvicidal bioassay method

Larval insecticide bioassay is the method employed to explore the mosquito larvae that are rear and maintained in laboratory. To evaluate the biological activity, Dimethyl sulphoxide (DMSO) was added to extract to make it soluble in aqueous medium. Various concentrations 2000, 4000, 6000, 8000, 10000, 12000, 140000 ppm were further prepared from original stock solution. 25 larvae were transferred to 100 ml of each concentration prepared from stock solution. Normal tap water was set as control for every experiment along with treated control with DMSO. Triplicates of every concentration were set and cumulative data was used to obtain LC_{50} values. Dead and moribund larvae after 24 and 48 hours were counted to calculate percentage mortality. No mortality was observed in control and treated control sample. Laboratory bioassays were conducted as per the guidelines for laboratory and field testing of mosquito larvicides [39, 40].

2.5 Statistical analysis
Statistical analyses for calculating percentage mortality, LC_{50}, confidence interval with 95% for upper and lower limit, p values were performed with Microsoft Excel.

2.6 GC-MS analysis
GC-MS is used to study liquid and solid samples for analyzing chemical mixtures in drug screening. Gas chromatography allows different molecules of a sample to separate as it travels through the column. In present study GCMS was used to analyze the methanol solvent leaf extract of *Cosmos sulphureus*. Gas chromatography-Mass spectroscopy (GC-MS) analysis was performed to evaluate and quantify volatile compounds with other details as retention time, area %, height of eluted compound for further comparison of indices with those of stored data of National Institute Standard and Technology (NIST 4) and Wiley 9 (Vandendool and Kratz 1963) online library.

Working Procedure
Specifications:
Column Oven Temp: 60 °C
Injection Temp: 250 °C
Ion Source Temp: 230 °C
Interface Temp: 300 °C
Injection Mode: Spintless
Carrier Gas Saver: Off
Sampling time: 1 min
Oven Temp Program

3. Result and Discussion
The present investigations were carried out to assess the larvicidal efficiency of different solvent extracts of *Cosmos sulphureus* Cav (Asteraceae) against early 3rd instar larvae of *Aedes aegypti* L (Diptera: Culicidae). Bioactivity of each plant extract is specific to plant parts and polarity of extraction solvent used and it also significantly differs with different larval stages [16]. The mosquito species was selected for study as they can be easily colonized in laboratory as fresh water breeding mosquito, less susceptible to insecticides, robust than other mosquito species like *Culex* and *Anopheles* [25]. Mosquito species can be controlled at larval stages because at this stage the mosquito larvae are immobile and can be easily targeted [36].

3.1 Larvicidal bioassay
Desired concentrations 2000, 4000, 6000, 8000, 10000, 12000, 140000 ppm were prepared from extracts obtained from petroleum ether, chloroform, ethyl acetate and methanol. Dead and moribund larvae were recorded after 24 and 48 hours of exposure. *Cosmos* leaf extracts exhibited larval mortality in order of chloroform < ethyl acetate < methanol < petroleum ether with LC_{50} values as 2453.56 ppm, 4706.05 ppm, 5877.20 ppm and 8254.46 ppm respectively. The extract with low ppm value is more effective. In the results obtained chloroform extract proved to be more effective than methanolic and ethyl acetate extracts (Fig 1). No mortality was observed in control and treated control. The comparative results are summarised in Table 1.

Table 1: Percent larval mortality of 3rd instar larvae of *Aedes aegypti* L

Plant Sample	Percentage Mortality (LC_{50}) (ppm)			
	Pet ether	Chloroform	Ethyl acetate	Methanol
Cosmos sulphureus	8254.46	2453.56	4706.05	5877.20
Slope	2.8	4.3	3.3	4.7
Intercept	-10.9	-14.5	-12.2	-17.7
p value	0.06	0.001	0.004	0.52
95% CI Lower limit	-1.92	22.6	11.36	-32.0
95% CI Upper limit	32.0	49.8	31.7	53.2

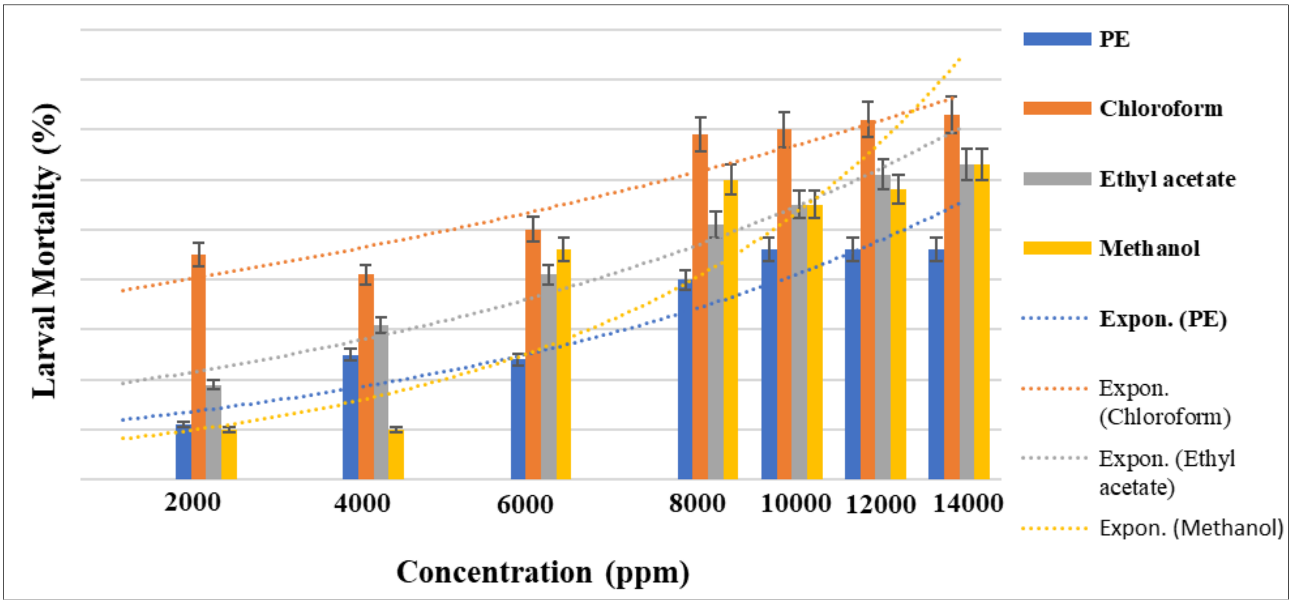

Fig 1: Bio efficacies of extracts of *Cosmos sulphureus* on 3rd instar larvae of *Aedes aegypti*

Mosquito larvae showed peculiar behavioural changes after exposure. They are anal biting with ring formation, restlessness, sluggish behaviour in movement and inability to come to surface, slow wriggling movement and unresponsiveness even with slight touch to siphon. Similar changes were observed with leaf extracts of *Calatropis procera* on *Aedes aegypti* larvae [20, 30]. Retraction of body parts, sluggish behaviour, loss of equilibrium and inability to come to surface with response to light are similar behavioural changes observed on exposure to high doses [42].

Larvicidal activity of ethanolic extracts of 83 plants from Asteraceae family were evaluated against *Aedes fluvialitis* [9]. *C. sulphureus and E. heterophylla* ethanolic extracts showed no larvicidal activity above concentration 1600 mg/l against four mosquito vector species [20]. *C. sulphureus* methanolic extract larvicidal toxicity against 3rd & 4th instar larvae of dengue vector *Aedes aegypti* with LC_{50} value (7063.175 ppm) was reported [26].

Larvicidal, pupicidal and insecticidal properties of *Cosmos bipinnatus, Foeniculum vulgarae* and *Tagetes minuta* on *Culex quinquefasciatus* mosquitoes. Ethanol extracts showed LC_{50} at 0.10, 1.17, 1.18 mg/ml for *F. vulgarae, T.minuta* and *C. bipinnatus* respectively followed with n- hexane at 1.03, 1.01 and 1.27 mg/ml for the same sequential order. *C. bipinnatus* pupicidal LC_{50} values for ethanol and n-hexane extracts were 1.14 mg/ml and 1.16 mg/ml respectively. No lethal or knock down effect was observed with aqueous extract of *Cosmos bipinnatus* on larvae or pupae [18].

Ethyl acetate fractions of invasive weeds *Synedrella nodiflora* were more effective than *Cassia uniflora* extracts probably due to presence of allelochemicals terpenoids, steroids, flavonoids and essential oils [5].

The variations in phytochemicals depends on plant part, age of the plant, phenological stage, geographical location, seasonality, mosquito species, and method of solvent extraction consequently affecting insecticidal activity [16, 27]. Steroids and alkaloids are extracted with mid-polar solvents [36]. Alkaloids present in plants possess insecticidal properties at very low concentrations by constricting blood vessels and affecting the autonomous nervous system [41]. Polar solvents extract polar molecules while no-polar solvents extract all fats and oils.

3.2 GC-MS analytical studies

GC-MS of methanolic leaf extract of *C. sulphureus* in our present study revealed the presence of 19 major compounds as β- sitosterol, γ-sitosterol, stigmasterol, hexadecenoic acid, Cholesta-5-22-diene-3-ol (3-beta)-, hexadecenoic acid, octadecanoins acid / stearic acid, phytol, ethyl oleate, eicosanoic acid, methyl ester, 9,12-Octadecanoic acid, propyl ester, E E Z-1, 3, 1, 2- Nonadecatriene 5,14-diol (Table 2).

Table 2: GC-MS of methanolic leaf extract of *Cosmos sulphureus*

Sr. No.	IUPAC name	Mol. Formula	Ret. time	Nature of compound	Activity
1.	β-sitosterol	$C_{29}H_{50}O$	19.385	Phytosterol	Antioxidant, Antimicrobial, Angiogenic, Antioxidant, Immunomodulatory, Anti-diabetic, Anti-Inflammatory, anticancer, insecticidal, larvicidal, toxic [8, 10, 12, 22, 31, 32, 33]
2.	Gamma-Sitosterol	$C_{29}H_{50}O$	19.385	Phytosterol	Anti-diabetic [12]
3.	Stigmasterol	$C_{29}H_{48}O$	18.825	Phytosterol	Cytotoxic, Toxic [8]
4.	Cholesta-5-22-diene-3-ol (3-beta)-	$C_{27}H_{44}O$	18.825		
5.	Octadecanoic acid 2-hydroxy-1-hydroxy methyl ethyl ester	$C_{21}H_{42}O_4$	13.840		Not reported
6.	Hexadecanoic acid, 2-hydroxyl-1-(hydroxy methyl) ethyl ester or Glycerol 1-palmiate	$C_{19}H_{38}O_4$	11.890	Mono-acyl-glycerols	Toxic [8]
7.	Octadecanoic acid	$C_{18}H_{36}O_2$	8.795	Stearic acid	Toxic, Insectistatic [8]

15 major compounds isolated from leaves and 5 from roots of *C. sulphureus* are sesquitepenes lactones, costunolide, reynosin and santamarin along with stigmasterol, phenylpropanoids by GC-MS analysis [23]. As per literature terpenoids, alkaloids, phenylpropanoids, thipoenes and amides possess higher larvicidal activity [11]. High contents of phenols and flavonoids are present in *C. sulphureus* leaves which possess anti-oxidant property. Hybrid variety of marigolds contains chalcones, butein 4'- O-glycoside, 2 flavones, eriodictyol 7-O-glycoside and 7-O- glucuronoide while flowers have aurones and sulfuetin-6-O-glucoside [17]. As per the literature β-sitosterol, a ubiquitous phytosterol present in all plants, animals and fungi belonging to group of 4-desmethyl sterols possess many biological activities as anti-microbial, anti-inflammatory, cytotoxic and insecticidal/ toxic, larvicidal properties [8, 32]

Rahuman *et al.*, [22] reported moderate larvicidal activity of five plants *Jatropha gossypifolia, Abutilon indicum, Aegle marmelos, Euphorbia thymifolia* and *Solanum torvum* against *Culex quinquefasciatus* larvae. β-siotsterol was identified as novel mosquito larvicidal sterol from petroleum ether extracts of *Abutilon indicum*. Subramaniam *et al.*, [33] reported all four crude extracts (sequential extraction of hexane, diethyl ether, dichloromethane and ethyl acetate) of *Abutilon indicum* showed larvicidal activity with maximum mortality results from hexane extract against *Aedes aegypti*. β-sitosterol-3-O-β-D- glucoside isolated from *Acanthus montanus* resulted in 100% larval mortality of *Ae. aegypti* at 1.25 μg/ml concentration [1].

4. Conclusion

Many studies have been conducted on immature larval stages of mosquito vectors but, further isolation of bio-active compounds and assessing their larvicidal activity of these compounds needs to be done in order to understand the action of biopesticide on large scale production. Formulations derived from weeds will lead to eradication of these invasive weeds which are found gregariously growing near agricultural crop fields and hampering yield of crops. Bio-pesticides of plant origin will be economically available at an affordable price, environmentally safe and reduce the cost of synthetic chemicals. With the new pharmaceutical technique silver-based nanoparticles can be synthesized and studied for their bio-larvicidal activity. This study reveals that *Cosmos sulphureus* Cav pure compound formulations can be studied for mosquito controlling programmes.

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6. Conflict of interest: The authors declare no conflicts.

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