Research Article

Larvicidal Effects of GC-MS Fractions from Leaf Extracts of *Cassia uniflora* Mill Non Spreng

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ABSTRACT

Invasive weeds are becoming dominant over the native plant communities. They are known to contain phytochemicals having different biocidal activities. Such phytochemicals are one of the focuses of research in the field of natural chemistry. Larvicides of plant origin may serve as an alternative bio-control method for mosquito control, as synthetic insecticides are becoming a serious concern due to their harmful effects. In present study, the larvicidal activity of ethyl acetate fractions of dry leaf powder of *Cassia uniflora* Mill non Spreng, was checked. Four fractions viz. 20%EA, 40%EA, 60%EA, 80%EA were obtained by column chromatography. The third instar larvae of Aedes aegypti were exposed to these fractions with concentrations ranging from 200ppm to 1000ppm. Mortality was observed initially after 8hrs of exposure and then after 24hrs, 48hrs, 72hrs and 96hrs of exposure. Among the four fractions, 20% and 60% fractions showed significant larvicidal activity at 1000 ppm concentration. Major compounds from 20% and 60% fractions were identified by GC-MS analysis. The 20% fraction was hexadecanoic acid ethyl ester that possesses antifungal, pesticidal, nematicidal and antioxidant activity. The 60% fraction had benzyl butyl phthalate, which is a known ecdysone agonist. Thus it revealed that such plant originated products could be used as effective vector control and hence the probable candidates in sustainable development.

Keywords: *Cassia uniflora*, Fractionation, GC-MS analyses, Larvicidal activity, Phytochemicals.

INTRODUCTION

Allelopathy is described as direct or indirect interaction between plants and other organisms, including microorganisms like bacteria, fungi and protozoa. It also refers to any adverse or beneficial effects of one plant on the growth of other plant due to release of biochemical compounds into the environment known as allelochemicals. Various studies showing allelopathic effects of different weeds have been carried out till date[1,2]. The observations on weed-weed interactions also have been reported[3]. Invasive weeds are non-native plants that have tendency to spread widely and sometimes causing harm to human health. Weeds are used as potential bio-cides because of their unproblematic availability and to control their undesirable growth[4]. They are characterized by group of allelochemicals such as terpenoids, flavonoids, phenolic compounds and essential oils[5-8]. Hence to manage the spread of weeds and with the intension of testing the activities and use of allelochemicals, weeds are widely used in allelopathic research. Many species belonging to the genus *Cassia* have been used for larvicidal studies and reported to produce the phytochemicals that act as general toxicants both against the larval and adult stages of mosquitoes. Further, few of these were also found to interfere with the growth, development and reproduction[9]. Synthetic larvicides are continuously applied for controlling mosquitoes but many of these are toxic to human, animal, and plant life.[10] Whereas the use of plant extracts for mosquito control have benefits such as easy degradability, less harmfulness to non-target organisms and rich source of other phytochemicals as well.

Cassia uniflora Mill Non Spreng belongs to family Caesalpiniaceae. It is a tropical South American leguminous under shrub, distributed widely in different parts of India. The plant is widely spread in Karnataka and Maharashtra states (India) and also found to be a road side weed in Mannanur forest in Mahabubnagar District Andhra Pradesh[11]. Besides controlling growth of *Parthenium*, *C. uniflora* young leaves are used as vegetable. The phytotoxic nature of aqueous leaf leachates of *Cassia*, affecting the growth of crop plants is well documented[5]. The GC-MS studies of *Cassia uniflora* Mill. Non Spreng, indicated the presence of steam volatile components as 2 pentadecanone, hexadecanoic acid, phytol, isobutyl phthalate, dioctyl phthalate as major compounds.[6] The aqueous leaf leachates of *C. uniflora* were phytotoxic against crop plants like mustard and radish[6]. *Aedesa egypti*, which is called yellow fever mosquito, is a known vector of diseases like Dengue, Chikungunya, Yellow fever and Zika fever. The mosquito can be easily recognized by white markings on its legs and a marking in the form of a lyre on the upper surface of its thorax. Mosquito control is an important public health practice followed throughout the world to eradicate their presence. Earlier, plant or plant-based products were the only insect-managing agents available to farmers around the world[12]. But now, the synthetic chemicals are used for the same. The control of mosquito larvae depends on

continuous application of organophosphates and insect growth regulators[13] that proved to be harmful to the environment[10] and on non-target organisms. Hence the non-toxic and eco-friendly practices of mosquito control can be chosen.

Plants are considered as a natural source of various compounds and are known to contain larvicidal agents, which may act in combination or independently[14]. Earlier studies on crude aqueous leaf extracts of *Cassia uniflora* have demonstrated larvicidal effect[9]. Keeping this in view, present study was intended to check the larvicidal activity of the fractions of leaf extract of *Cassia uniflora* in *Aedes aegypti.* Hence for characterization and identification of an effective and affordable natural larvicide, fractionation of crude extract was carried out, to ensure action of each constituent of *Cassia uniflora* extract and fractions were tested against 3rdinstar larvae of *Aedes aegypti.*

MATERIALS AND METHODS

Material

Chemicals Used: AR grade Ethanol, Ethyl acetate, n-hexane, Iodine crystals were used for all experiments. For column chromatography 200mesh size Silica gel was used.

Method

Selection of plant

Cassia uniflora Mill non Spreng was taxonomically confirmed after collection from the field. Then mature green leaves of *Cassia uniflora* were collected from different areas of Pune city. The collected leaves were air shade dried after cleaning and spreading on cotton cloth. The composite sample of leaves was used for further study.

Preparation of Crude Extract (powder)

The dried leaves were ground, powdered and sieved through fine mesh. 500g of the powder was soaked in 1000ml of ethanol for 5 days then filtered using funnel through muslin cloth to collect the filtrate. It was concentrated using rotary evaporator the thick slurry obtained was then mixed with silica gel powder and coated with silica gel powder to make it dry. It was stored in a bottle for further use.

Fractionation of Crude Extract (powder) using Column Chromatography

In next step, fractions of crude extract at different concentrations of solvents were collected using Column Chromatography.

Preparation of solvents of different concentrations /Proportion

Solvents of different concentrations were prepared using n-hexane (nonpolar) and ethyl acetate (polar). N-hexane is nonpolar solvent so 100% n-hexane was used first, then the polarity of solvents was increased by adding or mixing different quantities of ethyl acetate to n-Hexane and lastly 100% ethyl acetate was used. Concentrations of 20%, 40%, 60%, 80% and 100% ethyl acetate were used as eluent.

Preparation of the Column

Column with dimensions 2.5”X 1.5’diameter was taken and arranged on a stand in vertical position. A cotton plug of suitable size was pushed down to the bottom of the column. Slurry of silica gel was prepared with n-hexane and poured gently into the column.

Loading of the Sample to the Column

25 g of crude powder coated with silica gel was loaded to the top of the column through the funnel. A portion of 100 ml 100% n-Hexane was added to the column and fractions were collected in the 100 ml conical flask. The fractions were continuously monitored by TLC. The procedure is repeated till column is free from first fraction (Ten times). Similar protocol is followed for 20%, 40%, 60%, and 80% ethyl acetate and 100% ethyl acetate solvent till the active compound was eluted out while 1000 ml 100% n-hexane was added continuously from the funnel to the top of the column.

Recovering the Constituents

Fraction (20% ethyl acetate) collected in different conical flask was transferred in round bottom flask and solvent was recovered using rotatory evaporator. Sticky mass was collected in the flask. Above procedure was repeated for 40%, 60%, 80% ethyl acetate fractions. For 100% n-hexane and 100% ethyl acetate there was no elution of components. Table 1 describes the quantities of fractions collected in ml and the weight of compound recovered from each of them.

Table 1: Fractionation of Ethanol extract of *Cassia uniflora* leaves powder

No.	Fraction	Net weight of each eluted fraction after drying in mg
1	20% ethyl acetate	281
2	40% ethyl acetate	457
3	60% ethyl acetate	144
4	80% ethyl acetate	40

After pooling each fraction, it was concentrated by rotary evaporator. Weight of each fraction was measured and then it was blended with DMSO and the stock solutions of 100 mg/ml were prepared and stored. These were further used to prepare working solutions of various concentrations.

Selection of mosquito larvae

The mosquito species selected for the present study was *A.aegypti.* The III instar larvae were procured from Department of Entomology, National Institute of Virology, Pune and were used for experiment. The live larvae were discarded by killing them using 10% formaldehyde solution.

Treatments

Stock solution of each fraction was used for preparing different concentrations by adding 20µl, 40 µl, 60 µl, 80 µl, 100 µl in bowls containing 10 ml water to make 200, 400, 600, 800 and 1000ppm solutions respectively. Ten III instar larvae were released into each bowl. Mortality was observed initially after every 2 hrs till 8hrs of exposure and then after 24hrs, 48hrs, 72hrs and 96hrs of exposure. The bowls were kept at room temperature and two sets of 10 larvae each were arranged as control - one set in 10 ml water and another in 9.9ml of water and 100 µl of DMSO. Each treatment set was replicated four times.

Statistical Analysis

The larval mortality data was used to calculate LC_{50} values and 95% confidence limits by Reed and Muench method[15].

GC-MS Analysis

Make - model: Agilent Technologies7000 GC/MS Triple Quad:

The 7000 Series Triple Quad GC/MS is a standalone capillary GC detector for use with the Agilent 7890A Series gas chromatograph. Inbuilt Software installed for qualitative analysis of MS is Mass Hunter.

Working procedure for test sample:

10 ppm solution of *C.uniflora* dry leaf powder was prepared separately. The solution was filtered through 0.2 µ nylon filter and filtrate sample was injected. Details of Installation processing unit are as below:

Specifications:

Injector temperature	-	250°C
Mode	-	Spitless
Pressure	-	11.052 psi
Total flow	-	101.2 ml/min
Injection volume	-	2 µl
Oxillary temperature	-	285°C
Colum details /250-micron X 0.5-micron	-	Agilent DB 5MS 30m
Column flow	-	1.2 ml / min
Carrier gas	-	Helium
Collision gas	-	Nitrogen

Oven Programme-

Rate /Time	Temp	Hold time
0 min	70°C	1 min
2°C/min	150°C	8 min
3°C/ min	200°C	3 min
8°C/min	280°C	4 min

Total run time is 52.292 min.

RESULTS

Larvicidal Activity

All four Ethyl Acetate fractions showed lethal effect on *A. aegypti* larvae, however, they showed toxicity at different concentrations as well as different time of exposure.

At the lowest concentration (200 ppm), A. aegypti larvae exposed to 20% and 60 % EA Fractions showed more than 50% mortality (70% and 52.5% respectively), after 96 hrs of exposure. On the contrary the larvae exposed to 40% and 80% EA fractions showed less than 50% mortality (12.5 and 40% respectively) even after 96 hrs of exposure (Fig.1).

At the highest concentration (1000 ppm), *A. aegypti* larvae exposed to 20% EA Fraction showed 100% mortality after 48 hrs; those exposed to 60% EA fractions showed 100% mortality after 72 hrs. On the contrary the larvae exposed to 40% and 80% EA fractions showed less mortality (50 and 80% respectively) even after 96 hrs of exposure (Fig.2).

Comparative analysis indicated that 60% fraction was the most toxic to show 7.5% mortality at the lowest concentration (200ppm) after just 4 hrs of exposure and was observed to increase substantially up to 96 hours. Similarly, 20% fraction was observed to be highly toxic at 200ppm causing 27.5% mortality after just 8 hours of exposure. Remaining two fractions viz. 40% and 80% were found to be less toxic at 200 ppm to cause mortality from 48 hours of exposure (Fig.1).

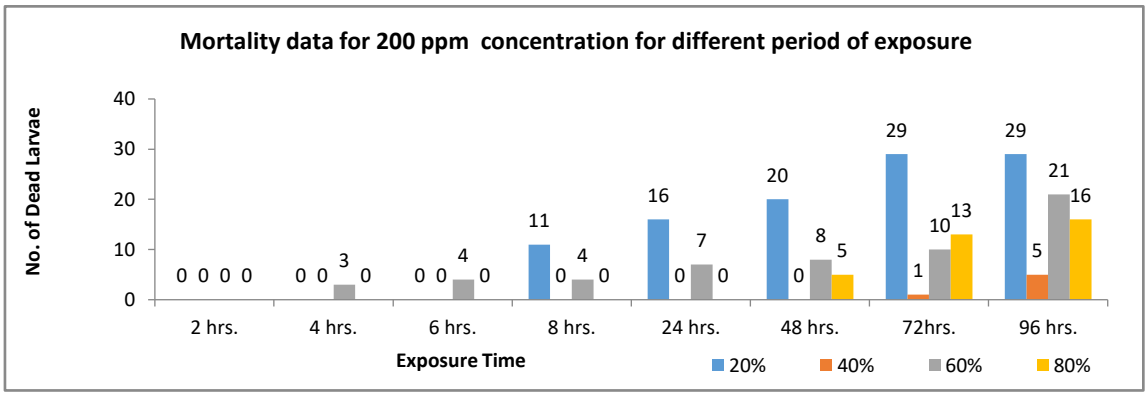


Figure 1: Graphical representation of mortality data for lowest concentration for different period of exposure

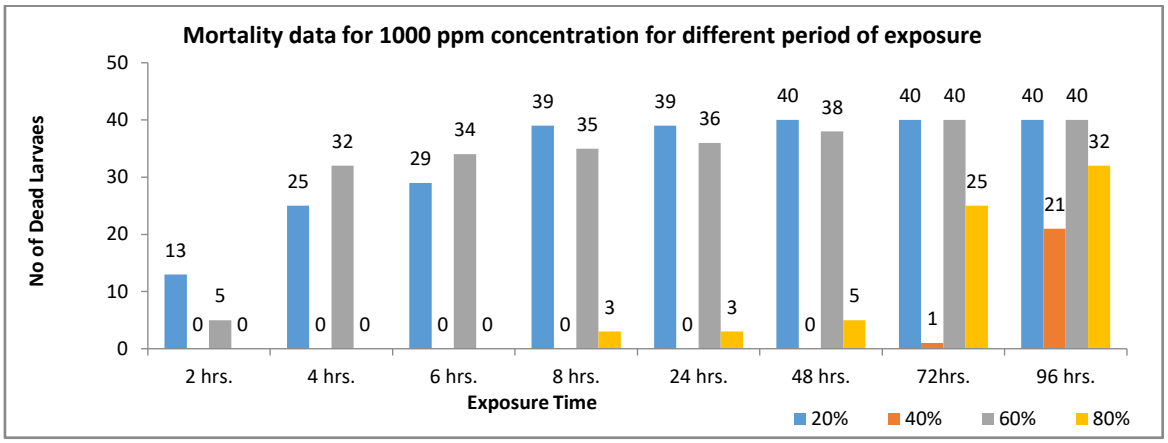


Figure 2: Graphical representation of mortality data for highest concentration for different period of exposure

Table 2: List of compounds obtained by GC –MS analysis of 20% EA fraction

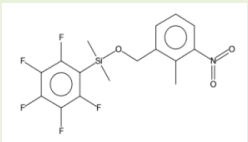
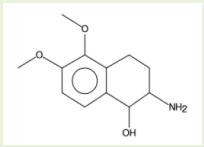
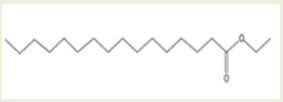
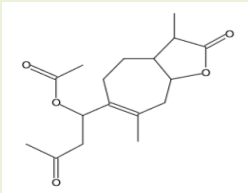
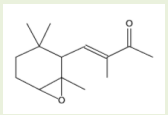
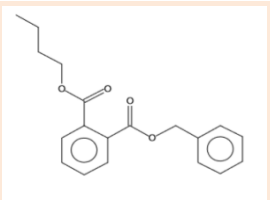
Sr.No	Retention Time	IUPAC Nomenclature	Molecular Formula	Molecular Weight	Structure
1	22.445-23.247 min	2-Methyl-3-nitrophenyl)methanol, dimethylpentafluorophenylsilyl ether	$C_{16}H_{14}F_5NO_3Si$	391	
2	24.005-24.659 min	α-Tetralol, 2-amino-5,6-dimethoxy	$C_{12}H_{17}NO_3$	223	
3	27.606 min	Hexadecanoic acid, ethyl ester	$C_{18}H_{36}O_2$	284	
4	29.765-30.586 min	Dihydroxanthin	$C_{17}H_{24}O_5$	308	
5	31.427 min	3-Buten-2-one, 3-methyl-4-(1,3,3-trimethyl-7oxabicyclo[4.1.0]heptan-1-yl	$C_{14}H_{22}O_2$	222	

Table 3: List of compounds obtained by GC –MS analysis of 60% EA fraction

Sr.No	Retention Time	IUPAC Nomenclature	Molecular Formula	Molecular Weight	Structure
1	22.635 - 23.037 min	Benzyl butyl phthalate	$C_{19}H_{20}O_4$	312	

The LC50 values of 20% and 60% EA fractions at 8 hrs of exposure were found to 350.73 ppm and 649.16 ppm respectively. As substantial mortality was not observed before 72 hours of exposure for both 40% and 80% fractions, the LC 50 values were calculated at 96 hours of exposure for these fractions. The 96 hr LC50 values for 40% and 80% EA fractions were 634.25 ppm and 446.39 ppm respectively.

GC-MS analysis of 20% EA and 60% EA fraction

For identification of bioactive compounds in 20% EA fraction of *C. uniflora* leaf powder GC-MS analysis was carried out using ethyl acetate as a solvent. It revealed the presence of five compounds (Table 2). The constituents found were 2-Methyl-3-nitrophenyl) methanol, dimethylpentafluorophenylsilyl ether (RT: 22.445-23.247), α-Tetralol, 2-amino-5,6-dimethoxy (RT: 24.005-24.659), Hexadecanoic acid, ethyl ester (RT: 27.606), Dihydroxanthin (RT: 29.765-30.586) and 3-Buten-2-one, 3-methyl-4-(1,3,3-trimethyl-7-oxabicyclo[4.1.0]heptan-1-yl (RT: 31.427).

The only compound identified by GC MS analysis of 60% fraction of *C. uniflora* leaf powder was Benzyl butyl phthalate (RT: 22.635-23.037) (Table 3).

DISSCUSSION

Use of synthetic insecticides and various chemicals in mosquito control is very common. The extensive use of these insecticides shows deleterious effects on the surrounding environment[16]. It is known that synthetic pyrethroid 'cypermethrin' shows toxic effects on common carp and contaminate aquatic ecosystem. The compounds which are used as larvicide if sprayed or added to water bodies can cause damage also to soil microbes, crops in vicinity. Phytochemicals derived from plants play role as toxicants against larval and adult stages of mosquitoes. They also act as growth inhibitors or repellents or attractants[17]. Essential oils extracted from *Zingiber officinalis* rhizome and leaves and stem of *Achyranthes aspera* showed significant larvicidal, repellent, oviposition deterrence activity against *Aedes aegypti* and *Culex quinquefasciatus*[18]. Phytochemicals which possess mosquitocidal properties are known to have an effect on nervous system and affect the mid gut epithelium in mosquito larvae[19]. Botanical derivatives or phytochemicals show their disrupting activity by interacting with cuticle membrane of larvae[20]. Depending upon the extraction procedure and the solvents used, effectiveness of the phytochemicals produced from same plant may vary significantly[21]. In present study ethyl acetate fractions of *C. uniflora* were used to check larvicidal activity. The similar ethyl acetate extract of leaves of *Ocimum sanctum* produced significant mortality against *A. aegypti* and *C. quinquefasciatus*[22]. Ethyl acetate extract of the leaves of *Aegle marmelos (L)* possessed high larvicidal properties against *Anopheles subpictus* and *Culex tritaeniorhynchus*[12]. Studies conducted with ethyl acetate fractions[23] reported that they were toxic to *A. aegypti, Anopheles stephensi, C. quinquefasciatus*.

From earlier studies of *C. uniflora* crude leaf extract was found to be toxic to third instar larvae of *A. aegypti* [9]. In the present study further GC-MS analysis of ethyl acetate extract was carried out and compounds were identified as Paramomycin, Hexanal,4-Acetyloxyimino-6,6-dimethyl-3methylsulfanyl-4,5,6,7tetrahydrobenzo[c]thiophene-1-carboxylic acid methyl ester. According to some researchers[23], second instar larvae of the *A. aegypti* showed significant mortality at the highest concentration (200ppm) of methanolic seed extract of *Terminalia chebula.* The study concluded that there was alteration in midgut tissues of *A. aegypti* larvae, when exposed to crude methanolic seed extracts of *T. chebula*. This seed extract was subjected to GC-MS and revealed the presence of Paramomycin as one of the compounds. *C. uniflora* fractions were checked for larvicidal activity against *A. aegypti* and showed significant toxic effect in 20%EA and 60%EA fractions in the current research. From 20%EA fraction, five compounds were identified by GC-MS analysis. Among these compounds hexadecanoic acids ethyl ester was found as major component (more than 80%). So, probably, hexadecanoic acid ethyl ester is responsible for larvicidal activity seen in 20% EA fraction. In similar study[24] hexadecanoic acid, ethyl ester was reported to show antifungal, pesticidal, nematicidal and antioxidant activity[25, 26]. From chromatographic fractions of *Feronia limonia* leaves the pure active compound, identified was n-hexadecanoic acid which also showed the larvicidal activity on *C. quinquefasciatus*, *A. stephensi* and *A. aegypti* larvae[27].

The only compound identified in 60% fraction of *C. uniflora* was Butyl benzyl phthalate (BBP). It is mainly used as a plasticizer in the polyvinyl chloride (PVC) industry and the manufacturing of many other products and its presence in the aquatic environment is expected from decades[28]. It has potential to disrupt normal development and reproduction. Its effects have been observed in one or more animal species[29]. Long-term BBP exposure in rats showed increase in the liver and kidney weight of rats and reduction in body weight as well as carcinogenicity[30]. Some researchers[28] are of the opinion that BBP has capacity to mimic the action of steroid hormones, like ecdysone or estrogen, which can cause major endocrine disorders in different organisms. Larvicidal activity of BBP, and hence the 60 % EA fraction of *C. uniflora*, is perhaps due to its ecdysone agonist behaviour. Ecdysone agonists are hormonally active insect growth regulators that disrupt development of pest insects and have potential for development as insecticides[28]. They have confirmed its endocrine disrupting capacity in insects, with the ability to act as an ecdysone agonist. These compounds may prove to be valuable insect growth regulators for control of mosquitoes to decrease the frequency of pathogen transmission to humans. Hence the described properties of the compounds identified by GCMS can be the reasons for mortality of *A. aegypti* larvae, as observed in the current studies.

CONCLUSION

The ethyl acetate fractions of *C. uniflora* were studied for larvicidal activity against *Aedes aegypti* mosquitoes. The toxicity and bioactivity of *C. uniflora* fractions may be because of high content of n-hexadecanoic acid ethyl ester in 20% EA and Benzyl butyl phthalate in 60%EA. With the results of this experiment it is concluded that toxic effects shown by *C. uniflora* are probably due to endocrine disrupting mechanism of Benzyl butyl phthalate in insects. Thus 20% and 60% EA fractions of *C. uniflora* could be used

as probable larvicide / insecticide for mosquito control. It would be interesting to check the effects of these extracts on chitin formation during larval growth of *A. aegypti* and other mosquito species.

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