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Research Paper

Anti-larval effects of leaf and callus extract of *Dysoxylum binectariferum* against urban malaria vector, *Anopheles stephensi*

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ABSTRACT

Dysoxylum binectariferum Hook is a large tree endemic to Western Ghats of India and shows incredibly high yields of rohitukine. Methanolic extracts of leaves of this plant were tested against 3rd& 4th instar larvae of *Anopheles stephensi*, a malaria vector in urban areas of India. Attempts were also made in the present investigation to tissue culture the leaf and compare the efficacy of callus extract with that of the leaf extract against the larvae of *An. stephensi*. The dose mortality data were subjected to log probit regression analysis to determine median lethal concentrations, LC_{50} and LC_{90} after exposure for 24h. Accordingly, 97.5% mortality was observed at 18000 ppm concentration of leaf extract, with LC_{50} 13465 (95% CI: 12845-14182ppm) and LC_{90}18009 (95% CI: 16653-20368) ppm respectively. The callus extract showed 98.75% mortality at 2000 ppm with LC_{50} & LC_{90} values 907 (95% CI: 726-1133) ppm and 1961 (95% CI: 1478-3522) ppm respectively. Results revealed that both leaf and callus extract have activity against *Anopheles stephensi* larvae but compared to the leaf, callus appears more efficient. They could be potential source of herbal-based insecticide for control of disease vectors.

Keywords: *D. binectariferum; An. stephensi*; Larvicidal activity; Leaf extract; Callus extract.

INTRODUCTION

Mosquitoes are the single most important group of insects, which transmit serious human diseases such as malaria, filariasis, dengue, chikungunya, Japanese encephalitis, yellow fever, etc. One of the major global public health problems, malaria poses a great threat to human health in terms of morbidity and mortality. In India, malaria is an important cause of direct or indirect infant, child and adult mortality with about 2 million new cases reported every year. Approximately 2.8 billion (about 40% of the world's population) lives under the risk of malaria. India alone contributes 77% of the total malaria burden in Southeast Asia (Kumar, et al., 2007). To curb vector borne diseases, vector control is as important a component as are the diagnosis of illness and treatment of the cases. So far, chemical insecticides

have been the mainstay of the vector control programs, but resistance in target vectors and environmental pollution are two major concerns related to their frequent use. The selection pressure caused by repeated use of conventional insecticides is primarily responsible for enhancing resistance in mosquito vector populations besides causing environmental pollution (Brown, 1986; Pates and Curtis, 2005). Many plants are alternative source of insect-control agents because they contain a range of bioactive chemicals many of which act selectively or synergistically and also have little or no harmful effect on non-target organisms and the environment in general (Hedlin, et al., 1997). Hence natural products have long been a thriving source of drug discovery due to their chemical diversity and ability to act on various biological targets (Bhutani and Gohil, 2010). They also act similar to chemical insecticides and can kill target mosquito vectors expediently.

Dysoxylum, which belongs to family *Meliaceae,* is widely distributed from South East Asia to Australia. About a dozen species of *Dysoxylum* such as *D. binectariferum, D. malabaricum, D. hamiltonii,* etc. are found in India and many of the *Dysoxylum* species are known to possess good medicinal and antimicrobial properties (Anon. 1952; Kritikar and Basu, 1981; Hisham, et al., 2001; Parcha and Gahlot, 2003). *D. binectariferum* is a rare tree, endemic to Western Ghats of India and takes years to grow. The crude extracts of the tree have been found to be highly effective against ovarian and breast cancer lines (Mohanakumara, et al., 2010). Rohitukine, a chromane alkaloid, is a precursor of flavopiridol, a promising anti-cancer compound obtained from the *D. binectariferum* (0.9% dry weight). *Meliaceae* plants are also recognized for their insecticidal properties as exemplified by the extracts of callus, fruit and leaves of the Chinaberry tree, *Melia azedarach*, against adults of sweet potato whitefly, *Bemisia tabaci* (Gennadius) showing repellent activity of 58.9–67.7% (Hammad, et al., 2001). Several investigators have demonstrated biological effects of limonoids isolated from different species of *Dysoxylum* on insects (Russel, et al., 1994; Nathan, et al., 2006).

Observations are also on record about accumulation of secondary metabolites in the stationary phase of callus growth (Satio and Mizukami, 2002). Significant research work has been reported wherein the bioactive compounds obtained from callus extract act against various types of vectors (Ee, et al., 2004; Kamal and Mangla, 1993; Chapagain, etal., 2008; Sarin, 2004; Kamiabi, et al., 2013). So far there are no reports on bioactivity *of D. binectariferum* against larvae of *An. stephensi*. Taking the lead, attempt was made in the present investigation to tissue culture the leaf explant of *D. binectariferum* and compares the efficacy of aqueous callus extract with that of leaf extract against larvae of *An. stephensi*.

MATERIALS AND METHODS

***Plant source and sterilization*:** Fresh, mature, healthy leaves as well as seeds were collected from the trees of *D. binectariferum* growing in Ambegao village near Kolhapur, Maharashtra, India in March 2009. The lead author physically carried plant parts for taxonomic identification to Prof. Dr. S.R. Yadav, Head of Botany Department, Shivaji University, Kolhapur, Maharashtra, India.

Sterilization and tissue culture: Seeds collected from the site were washed thoroughly under the tap water and dried at room temperature for further use. The seeds were again washed with Tween-20 followed by 70% ethanol for 3 minutes as per Ahuja, 1994. Finally they were rinsed for three to four times with distilled water.

Surface disinfection of the seeds was carried out by 0.1% aqueous $HgCl_2$ solution for 3 minutes followed by rinsing with several changes of sterile double distilled water. Sterilized seeds were planted in plastic pots containing garden soil and were kept in the laboratory for germination (Figure-1A). Young plants obtained from the germinated seedlings were a continuous source of leaf as explants for tissue culture.

Callus induction: Young leaves obtained from four week old germinated seedlings were surface sterilized using 0.1% $HgCl_2$ for half a minute and then rinsed with sterile distilled water. Leaf discs (Figure-1B) from the midrib region of approximately 10 mm diameter were obtained using cork borer under aseptic conditions (Patil, 2003). Discs were placed with abaxial side upwards on the solid ½ strength MS medium consisting of basal salts and vitamins with 3% (w/v) sucrose, 0.8% agar supplemented with five different compositions and concentrations of growth regulators. They were maintained at 22 ± 2°C and 75% humidity for 9 hr photoperiod of cool white florescent light at intensity of 1350 lux (Murashige and Skoog, 1962; Bhojwani and Rajdan, 1983; Ahuja, 1994).

Callus was sub cultured periodically every 4th week. The response of explants for callus induction was expressed as percentage of callus induction. For each hormonal treatment 10 explants were used and all the experiments were repeated thrice. Calli from most responsive media were harvested after eight weeks for extraction and bioassays.

Methanolic extract of leaf: Methanol extracts of leaves were obtained according to the method of Warthen et al., (1984). Initially leaves were crushed to a fine particle size and dried in shade at room temperature. 100g of dried crushed material was stirred in 1 L of methanol. The solution was left to rest overnight and then filtered through Whatman no. 40 filter paper. The solvent was evaporated at 45°C and a dark green residue from leaves was obtained. This crude extract was then used to prepare a stock solution. Crude extract was dissolved in methanol and volume was made to 500 ml. A drop of Tween-20 was added to the extract for complete solubility in the solution. The stock solution was then diluted to required percentage with water.

Methanolic extract of Callus: Extraction of callus was carried out by following basic method of Warthen et al., (1984) with some changes with regard to volume of methanol. Calli were harvested after eight weeks of culture and were kept for drying in the oven at 50ºC. The dried crushed material was stirred in 20 ml of methanol. The solution was kept to rest overnight and then filtered through Whatman no. 40 filter paper. The procedure was repeated for solid filtration and two filtrates were combined. The solvent was evaporated at 45ºC and a light green residue from callus was obtained. This residue was then dissolved in 5 ml of distilled water and used for bioassay experiments.

Mosquito culture: Bioassays were carried out against the larvae of the mosquito species, *An. stephensi*. These mosquitoes were reared in the insectary of the National Institute of Malaria Research, Field Unit, Campal, Panaji-Goa as per WHO, 1975. Larvae were fed on a diet of commercially available baby food of trusted brand mixed with powdered fish food in a ratio of 2:1. Late 3rd and early 4thinstar larvae were used to screen the larvicidal activity of the methanolic extract of the leaves & callus.

***Larvicidal bioassay of methanol leaf extract of D. binectariferum*:** Bioassay of methanolic leaf extract was carried out against 3rd and 4th instar larvae of *An. stephensi* using various concentrations of extract. To perform larvicidal activity, 25 healthy larvae of 3rd/4thinstar were introduced into each 300ml capacity plastic bowls

containing 200 ml of leaf extract of desired concentration adjusted in water. Four such replicates per concentration were used for all the experiments and control. Observations were recorded after 24 hours of exposure and dead larvae were counted and recorded for further analysis. All the experiments were carried out at room temperature of 27 ± 2°C and relative humidity of 75–85%. Bioassays were performed as per WHO (2005) procedure with some modification as per the method of Rahuman, et al., (2000).

***Larvicidal bioassay of callus extract of D. binectariferum*:** Bioassays were performed on 3rd and 4thinstar larvae of *An. stephensi* using various concentrations of callus extracts. From 5 ml of callus extract various dilutions were prepared in distilled water in the range of 500 - 2000 ppm with relevant controls run concurrently. A minimum of 25 larvae per dilution were used per 50 ml of the solution for all the experiments with 4 similar replicates. Observations on mortality of larvae were recorded after 24h of exposure. The above bioassay experiment was repeated thrice.

Statistical analysis: % mortalities were corrected using Abbott's formula (Abbott, 1925) and the average larval mortality data were subjected to probit analysis for calculating LC_{50} and LC_{90}, 95% confidence limits and Z test values by using the SPSS (SPSS-PASW-1.8.0) software. $P<0.05$ was considered to be statistically significant.

RESULTS

Callus induction: Callus derived from leaf explants cultured on ½ strength MS medium fortified with hormonal combinations 2,4-dichlorophenoxyacetic acid (2,4-D), Naphthaleneacetic acid (NAA) and Kn; 2,4-D (A), 2,4-D, NAA and Kin(B); 2,4-D, indole-3-acetic acid (IAA) and Kn (C). Indole-3-butyric acid (IBA), indole-3-acetic acid (IAA) and Kn in the range of (2 - 0.5mg/l) has yielded good induction and growth. Media A with 2,4-D, IBA, Kin gave callus induction up to 95.66% in 10 days from the day of inoculation. Media B-2,4-D, NAA and K in gave 73.02% and media C-2,4-D, IAA; Kin gave 69.55% of callus induction. The leaf discs used as an explant gave pale green and friable callus (Figure1-B) in all the three compositions. The other two compositions gave less percentage and took long time for callus inductions.

Larvicidal bioassay of methanol leaf extracts of D. binectariferum: Leaf extract derived was tested against 3rd and 4th instar larvae of *An. stephensi*. Accordingly, 97.5 % mortality at concentration of 18000 ppm of leaf extract was observed against the larvae of *An. stephensi* (Table.1) with LC_{50} 13465 ppm and LC_{90} 18009 ppm respectively (Table.2).

***Larvicidal bioassay of callus extract of D. binectariferum*:** The extract obtained from calli and tested against 3rd and 4th instar larvae resulted in 98.75% mortality at a dose of 2000 ppm (Table.1) with LC_{50} & LC_{90} values of 907 ppm and 1961 ppm respectively (Table. 2).

DISCUSSION

Prolonged use of insecticides based on synthetic chemicals for vector control has induced resistance in vectors (Brown, 1986; Pates and Curtis, 2005; Nathan et al., 2005). Globally this has resulted in increasing demand for environmentally safe, target-specific and degradable products without undesirable side effects. Biological control plays an important role currently in vector control programs although alone, it does not provide a complete solution in all situations. Plant based insecticides are

gaining importance as they are generally considered harmless and eco-friendly. Hence there is a major effort to promote plant based products.

The plants of Meliaceae family contain a variety of compounds with insecticidal properties. Many investigators have isolated limonoids from species of *Dysoxylum* genus of family Meliaceae (Singh, et al., 1976; Mulholland and Naidoo, 2000; Hisham et al., 2001; Luo et al., 2002). Apart from genus *Dysoxylum*, several other genera of Family Meliaceae are source of limonoids whose biological efficacy against insects has been demonstrated by several investigators (Butterworth and Morgan, 1971; Morgan and Thornton, 1973; Vanucci et al., 1992; Xie et al., 1994). However, their bioactivity against mosquitoes remained largely unexplored.

The present investigation was therefore undertaken to study the effect of *D. binectariferum* against larvae of *An. stephensi* in search of an effective natural larvicide having malaria vector control potential. Our studies with the methanolic leaf extract of *D. binectariferum* showed high mortality against the larvae of *An. stephensi,* an important urban malaria vector in India with LC_{50}13465 ppm and LC_{90}18009 ppm respectively. Similar studies on *D. malabaricum* carried out by Nathan et al. (2006), showed 97% mortality in first instars *Anopheles* larvae with 4% plant extract. Our results are although comparable with above study but showed high mortality in 3rd and 4th instar larvae instead of 1st instar larvae used in above study which is generally more susceptible than the higher instar larvae.

The appeal of using natural products for medicinal purposes is increasing and researchers are aiming to produce substances through tissue culture technology (Singh, 2011). In the present investigation the callus extract of *D. binectariferum* showed 98.75% mortality at 2000 ppm with LC_{50} and LC_{90} values of 907 ppm and 1961 ppm respectively. Though not against insect larvae, observations have been made by Balian et al., (2006) on the methanolic leaf and callus extracts of the plant, *Silybum marianum* against oedema in the rats. In that study, *in vitro* culture generated callus extract showed greater inhibition of rat oedema as compared to methanolic extract of leaves. In our study although both leaf and callus extract of *D. binectariferum* showed high anti-larval activity against larvae of *An. stephensi,* but callus produced higher mortality at very low concentration. It appears to be due to the synthesis of higher amounts of secondary metabolites/bioactive compounds in the tissues of callus as compared to the leaf. There have been various reports of callus extract showing more antimicrobial activities when compared to the extracts of different parts of the plant (Radfar et al., 2012; Singh, 2011).

CONCLUSION

The present study reveals that both leaf and callus extracts of plant *D. binectariferum* have anti-larval activity against malaria vector *An. stephensi*. Callus being more effective can be further exploited for micro-propagation as well as for enhancement in secondary metabolite accumulation. The leaf and callus extract of this plant therefore could be a potential source of herbal larvicide for vector control.

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Table-1: Percentage mortality of *An. stephensi* larvae treated with different concentrations of leaf and callus extracts of *Dysoxylum binectariferum*.

Leaf		Callus	
Concentration (ppm)	**Percentage mortality**	**Concentration (ppm)**	**Percentage mortality**
18000	97.50	2000	98.75
14000	42.50	1000	36.25
12000	27.50	500	25.00
10000	16.25	-	-

Table-2: Log Probit analysis of the bioassay results: LC_{50} and LC_{90} values of leaf and callus extracts against *An. stephensi* $3^{rd}/4^{th}$ instar larvae.

	LC_{50} (ppm)	**95% Confidence limit (lower-upper)**	**LC_{90} (ppm)**	**95% Confidence limit (lower -upper)**	**SE**	**Z**	**p**	**S/NS**
Leaf extract	13465	12845-14182	18009	16653-20368	1.013	10.021	.000	S
Callus	907	726-1133	1961	1478-3522	0.432	8.859	.000	S

- SE= Standard Error, Z= Z-test, P= Probability, S=/ Significant NS= Not Significant

A **B**

Figure-1: (A) Saplings and (B) Leaf explant discs of *D. binectariferum* on medium showing callus induction.

