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Research Article

LARVICIDAL POTENTIAL OF *INDIGOFERA TINCTORIA* (FABACEAE) ON DENGUE VECTOR (*AEDES AEGYPTI*) AND ITS ANTIMICROBIAL ACTIVITY AGAINST CLINICAL ISOLATES

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ABSTRACT

Objective: The objective of this study was to evaluate the larvicidal potential of *Indigofera tinctoria* on dengue vector (*Aedes aegypti*) and its antimicrobial efficacy against clinical isolates.

Methods: The extract was tested at various concentrations 64, 128, 256, and 512 mg/ml for antimicrobial activity and 0.1 and 5 mg/L were prepared for larvicidal activity. The numbers of dead larvae were counted after 24 hrs of exposure.

Result: The lowest minimum inhibitory concentration (MIC) values of the extract were 128 mg/ml against *Klebsiella* spp. - 1 alone and rest of the clinical test pathogens execute MIC activity at 512 mg/ml. The extract also showed antifungal activity with MIC of 64 mg/ml against the *Candida albicans*. Larvicidal activity of *I. tinctoria* extract were tested against fourth instar larvae *A. aegypti* and larval mortality were found after 24 hrs with lethal concentration (LC_{50})=3.1870 and LC_{90}=5.3991 were observed.

Conclusions: These results indicated that the extract displayed larvicidal potential on *A. aegypti* and antimicrobial activity against clinical isolates.

Keywords: Infectious disease, *Indigofera tinctoria,* Antimicrobial activity, Larvicidal activity.

INTRODUCTION

Infectious diseases caused by bacteria and fungi affect millions of people worldwide. Today, infectious diseases account for one-third of all deaths in the world; the World Health Organization estimates that nearly 50,000 people die each day throughout the world from infectious diseases. The increasing incidence of multiple resistance in human pathogen due to the random use of commercial antimicrobial drugs commonly employed in the treatment of infectious diseases [1]. The development and spread of resistance to currently available antibiotics is a global concern. This has been forced scientist to search for new antimicrobial substances from various sources like the medicinal plants. Search for new antibacterial agents should be continued by screening many plant families. The screening of plant extracts and plant products for antimicrobial activity shown that higher plants represent a potential source of new antibiotic prototypes [2]. Herbal treatment would promise a greater viable solution for the effective treatment of infectious diseases caused by bacteria [3]. The most important antimicrobial compounds of plants are alkaloids, flavonoids, tannins and phenolic compounds [4]. Plant possesses higher antioxidant, antibacterial, pesticidal, and larvicidal property influence was documented [5]. This phenomenon has triggered and advised the development of alternative techniques using plant extracts as larvicides. Because plant contain various phytochemicals that are specific in killing mosquito larvae without harming other organisms, environment, cost effective, and do not pose any side effects [6]. Plants serve as a major therapeutic tool for Indian system of medicine and the information on this is found in earliest works [7]. Mosquitoes serve as vector for various tropical and subtropical diseases which cause destructive effects in human [8]. They not only transmit parasites and pathogens, but they also source of allergic reaction that includes local skin and systemic sensitivity [9]. The most common diseases associated with mosquitoes are dengue fever, chikungunya, and yellow fever. Dengue is one of the most important viral diseases transmitted by *Aedes aegypti* [10]. Plant based pesticides are less toxic, delay the development of resistance because of its new structure, and easily biodegradable. Plant derived compounds, such as saponines, steroids, isoflavonoids, alkaloids, and tannins has potential of mosquito larvicides [11].

The plant *Indigofera tinctoria* (fabaceae) is popularly known as indigo [12]. It contained alkaloids, flavonoids, tannins and phenolic carotenoids and coumarins [13]. The whole plant contains glycoside, indicom, indigotine, indirubin, and glalctomannan. Plants contain several phytochemicals which possess strong antioxidant activities [14]. *I. tinctoria* were traditionally used to treat various kinds of diseases such as anticancer, anti-hyperglycemic, anti-diabetic, anti-bacterial, antioxidant, cytotoxic effect, and hepatoprotective activity [15]. The plant was found to contain indirubin and indigtone where the juice extracted from the leaves is useful in the treatment of hydrophobia [16]. The present study is to evaluate *I. tinctoria* extract is potential against larvicidal activity on dengue fever vector *A. aegypti* and antimicrobial activity against clinical isolates.

METHODS

Drugs and chemicals

Agar and nutrient broth were purchased from Sigma-Aldrich. Co., St. Louis, USA. All the other chemicals were of analytical grade obtained from Sisco Research Laboratory, Mumbai, India.

Collection and identification

The plant *I. tinctoria* collected March to June 2013 from the KSG Enterprises, Tindivanam, Tamil Nadu and authenticated by Dr. D. Aravind, Department of Medical Botany, National Institute of Siddha, Chennai. Voucher specimens have been deposited at the Herbarium of National Institute of Siddha, Chennai Reg. No: NIS/MB/83/2013. The collected plant were separated from unwanted materials and dried in shade. The leaves were grounded to coarse powder with the help of a suitable grinder. The powder was then stored in an airtight container, kept in a cool, dark, and dry place until the analysis commence.

Extraction procedure

I. tinctoria fresh plant leaves of 30 g were extracted with 250 ml of sterile distilled water using the Soxhlet apparatus. The extracts were then filtered with Whatman No. 1 filter paper and then freeze dried

stored at 4°C for further investigation. The extraction efficiency was quantified by determining the weight of the extract and the percentage yield was calculated.

Antimicrobial screening

Organisms used

To evaluate the antimicrobial effect of aqueous extract of *I. tinctora*, a panel of microorganisms such as *Staphylococcus aureus*, *Escherichia coli*, *Salmonella typhi, Pseudomonas* spp., *Klebsiella* spp., and *Candida albicans* were tested in this study. Standard strains of *S. aureus* ATCC-25923 and *E. coli* ATCC-25922 were also included in the study. All the isolates were maintained as stock cultures in the respective growth medium at 4°C in the Department of Microbiology, Dr. ALM PG Institute of Basic Medical Sciences, University of Madras. Clinical isolates were collected from Government General Hospital, Chennai, India and speciated as per standard microbiological methods.

Preparation of inoculum

Each of the test organisms were freshly subcultured on their respective growth medium and incubated at 37°C for 24 hrs. Microbial inoculums were prepared by suspending a single colony from the freshly grown culture plates into sterile brain heart infusion broth (BHIB) and incubating at 37°C for 30 minutes. The turbidity of the suspension was adjusted with sterile BHIB by matching to McFarland 0.5 standard (1×10^6-5×10^6 CFU/mL) and density was measured using spectrophotometer (Nano Drop).

Minimum inhibitory concentration (MIC)

The antimicrobial activity of aqueous based crude extracts of *I. tinctora* was analyzed using broth dilution method (BDM). MIC was defined as the lowest concentration of the each extract showing no visible growth after incubation. MIC was determined by testing the various dilutions of test drug against the standard inoculum of test organisms. Two-fold serial dilutions of the test drug were prepared using sterile nutrient broth and the initial drug concentration was taken as 64 mg/ml. Various concentrations of aqueous extract like 64 mg/ml, 128 mg/ml, 256 mg/ml, and 512 mg/ml were prepared aseptically in sterile eppendorf tubes [17]. The extract was filtered through syringe filter unit (0.2 µm). Each tube with different concentration of crude extracts was inoculated with 5 µl of standardized suspension (prepared as above) of test isolates and incubated at 37°C for 24 hrs. Appropriate sterility control and growth control (culture and respective solvent) was included.

Spot assay

To clearly define the MIC of the extracts, 5 µl of the suspension from each test and control tubes was spotted onto the sterile Mueller-Hinton agar plates and incubated at 37°C for 24 hrs. Thus, the MIC was determined as the lowest concentration of each extract inhibiting the visual growth of test isolates on the agar surface. Each of the experiment was carried out in triplicates for better concordance.

Larvicidal activity

Mosquito culture

Mosquito culture *A. aegypti* larvae were collected from Salem, Tamil Nadu, and India. The larvae were kept in plastic trays containing tap water and were maintained, in the laboratory; and all the experiments were carried out at 27±2°C and 75-85% relative humidity under 14:10 light and dark cycles. Larvae were fed with yeast and dog biscuits. They were maintained and reared in the laboratory.

Larvicidal bioassay

Larvicidal bioassay was carried out according to WHO (1996). The test was performed by placing 25 mosquito fourth instar larvae into 200 ml of sterilized double distilled water with *I. tinctoria* aqueous plant extract into a multi-vial tray. *I. tinctoria* aqueous plant extract solutions were diluted using double-distilled water to the desired concentrations (0.1, 0.3, 0.5, 1, and 5 mg/L). Each test included a set control group (distilled water) with three replicates for each individual concentration. Mortality was assessed after 24 hrs to determine the acute toxicities on fourth instar larvae of *A. aegypti* mosquitoes.

Dose-response bioassay

Based on the preliminary screening results, crude leaf extracts of *I. tinctoria* were subjected to dose-response bioassay for larvicidal activity against *A. aegypti*. Different concentrations ranging from 0.1 mg/L to 5 mg/L (plant extracts) were prepared for larvicidal activity. The numbers of dead larvae were counted after 24 hrs of exposure and the lethal concentration 50 (LC_{50}) and LC_{90} were calculated. However, at the end of 24 hrs, the selected test samples turned out to be equal in their toxic potential.

Data analysis

Larval mortality data were subjected to probit analysis for calculating LC_{50}, LC_{90}, and other statistics at 95% fiducial limits of upper confidence limit and lower confidence limit, and Chi-square values were calculated using the software developed by SPSS 11. Results with $p<0.01$ were considered to be statistically significant.

RESULT

Antimicrobial activity

Aqueous extracts of *I. tinctoria* plant were tested for the antimicrobial activity against pathogenic isolates isolated from various clinical sources. The extract was tested at different concentration like 64, 128, 256, and 512 mg/ml by BDM. MIC of crude extracts of *I. tinctora* leaves was found to be satisfactory (Table 1). MIC obtained against test isolates indirectly depicts the amount of antimicrobials present in the aqueous extract. All Gram-negative organisms isolated from various sources were found to be inhibited at the concentration tested when compared with the control plate. Of 6 *E. coli* strains tested, 1 strain was inhibited at 256 mg/ml; 5 strains at 512 mg/ml. ATCC *E. coli* strain was found to be inhibited at 256 mg/ml. Among the 2 *Klebsiella* spp. tested, 1 strain was inhibited at 128 mg/ml; 1 strain at 512 mg/ml. All the *Pseudomonas* spp. (2/2) were found to be inhibited by *I. tinctora* aqueous extract at 256 mg/ml. On the other hand, 1/3 *S. typhi* strains tested was inhibited at 256 mg/ml and 2/3 *S. typhi* strains was inhibited at 512 mg/ml. Among the Gram-positives tested, 1/6 *Staphylococcus* spp. were found to be inhibited at 256 mg/ml concentration; 5/6 were found to be inhibited at 512 mg/ml concentration. ATCC *S. aureus* was inhibited at 512 mg/ml concentration. *C. albicans* doesn't show any growth at the concentrations <64 mg/ml tested.

Larvicidal assay

I. tinctoria leaf aqueous plant extract for larvicidal efficacy (Table 2). Aqueous extract produced high larval mortality in *A. aegypti* (LC_{50}=3.1870; LC_{90}=5.3991) after 24 hrs exposure. The results obtained were statistically significant with $p<0.01$ (Table 2).

DISCUSSION

Antibacterial activity

Infectious diseases are the major cause of mortality worldwide. Bacterial pathogens are increasingly becoming resistant to antibiotics used in empirical therapy, which leads to multi-drug resistance followed by treatment failure. Such increase has been accompanied by the indiscriminate use of broad-spectrum antibiotics, immunosuppressive agents, intravenous catheters in hospitalized patients, and immunosuppression accompanied with organ transplantation and human immunodeficiency virus infections. There is an urgent need to discover new antimicrobial compounds with diverse chemical structures and mechanisms of action due to increasing new and re-emerging infectious diseases.

These studies were accompanied to investigate the antimicrobial properties of aqueous extract of *I. tinctoria* against pathogenic isolates.

Table 1: Lowest concentration which did not show any growth of the tested microorganism after macroscopic evaluation was determined as the MIC of aqueous extract of *I. tinctoria* at the respective concentration

S. no	Organism	64 mg/ml	128 mg/ml	256 mg/ml	512 mg/ml
1	*E. coli*-1	+	+	−	−
2	*E. coli*-2	+	+	+	−
3	*E. coli*-3	+	+	+	−
4	*E. coli*-4	+	+	+	−
5	*E. coli*-5	+	+	+	−
6	*E. coli*-6	+	+	+	−
7	ATCC *E. coli*	+	+	−	−
8	*Klebsiella* spp.-1	+	−	−	−
9	*Klebsiella* spp.-2	+	+	+	−
10	*Staphylococcus* spp.-1	+	+	+	−
11	*Staphylococcus* spp.-2	+	+	−	−
12	*Staphylococcus* spp.-3	+	+	+	−
13	*Staphylococcus* spp. IE-1	+	+	+	−
14	*Staphylococcus* spp. IE-2	+	+	+	−
15	*Staphylococcus* spp. IE-3	+	+	+	−
16	ATCC *Staphylococcus*	+	+	+	−
17	*Pseudomonas* spp.-1	+	+	−	−
18	*Pseudomonas* spp.-2	+	+	−	−
19	*S. typhi*-1	+	+	+	−
20	*S. typhi*-2	+	+	+	−
21	*S. typhi*-3	+	+	−	−
22	*C. albicans*	−	−	−	−

+: Growth, −: No growth, *I. tinctoria: Indigofera tinctoria, S. typhi: Salmonella typhi, E. coli: Escherichia coli, C. albicans: Candida albicans*, MIC: Minimum inhibitory concentration

Table 2: Larvicidal potential of plant extract against dengue vector *A. aegypti*

Solvent	n[a]	LC_{50}	LC_{90}	LC_{50}		LC_{90}		χ^2	df
				LCL	UCL	LCL	UCL		
Aqueous extract I. tinctoria	375	3.1870	5.3991	2.936	3.3647	5.003	5.9852	0.979	3

A. aegypti: Aedes aegypti, I. tinctoria: Indigofera tinctoria LCL: Lower confidence limit, UCL: Upper confidence limit, LC: Lethal concentration

Klebsiella spp. were found to be the most susceptible organism among the test isolates with the lowest MIC value such as 128 mg/ml against aqueous extracts of *I. tinctoria* (Table 1). In the recent times, *Klebsiella* spp. become increasingly resistant to antibiotics and it was labeled as multidrug-resistant *Klebsiella* spp. In this study, the extract showed an effective response against Gram-negative bacteria than Gram-positive bacteria. Our results corroborates with the reports of Rehnuma *et al*. [18] in which the growth inhibition activity of aqueous extract also started at lower concentration against Gram-negative bacteria (*Klebsiella* spp.).

The active phytocompounds responsible for antimicrobial activity of aqueous extract of *I. tinctoria* still remained to be exactly elucidated. Other Gram-negative bacteria were found to be susceptible in <256 mg/ml (Table 1). *S. typhi* is a pathogenic Gram-negative bacteria and it causes enteric fever and invade intestinal mucosa and multiply in macrophages in intestinal lymph follicles predominately found in the intestinal lumen. Extract have potential to inhibit *S. typhi* at lower concentration. On the other hand, in the study carried out by Renukadevi and Suhani Sultana [15]. The antibacterial, antioxidant and cytotoxic activity of the leaf extract of *I. tinctoria* was found to be promising and it inhibits the growth of Gram-positive bacteria namely *S. aureus, Bacillus pumilus* and *Streptococcus pyogenes*. Rest of the tested microorganisms (*Klebsiella* spp.-2, *E. coli*-2, *E. coli*-3, *E. coli*-4, *Staphylococcus spp*-1, *E. coli*-5, *E. coli*-6, *Staphylococcus* spp. IE-1, *Staphylococcus* spp. IE-2, *Staphylococcus* spp IE-3, *S. typhi*-1, *S. typhi*-2, *S. aureus* ATCC-25923, and *Staphylococcus* spp.-3) were inhibited in the concentrations of 512 mg/ml (Table 1). *Candida* species, *Pseudomonas aeruginosa*, *E. coli* and *Staphylococci* were most frequently encountered nosocomial pathogens. *S. aureus* was responsible for a wide variety of diseases, including pneumonia, skin and soft tissue infections, and diabetic foot infections [19] and it rapidly acquired resistance to antibiotics. Magesh *et al.* [20] reported that methanol extract of *I. tinctoria* exhibited antibacterial activity against *S. aureus.* The antimicrobial activity of *I. tinctoria* is high against *Staphylococcus* spp., *E. coli,* and *S. typhi* at high concentration and it may be potential against nosocomial pathogens. In case of *Pseudomonas* spp., the antimicrobial activity differs with the concentration employed [21]. In general, among the tested microbial strains, *C. albicans* was found to be more sensitive to many of the test agents than bacteria. Nosocomial BSI in the United States (40-50%) due to *C. albicans* species may be increasing [22]. The present study shows that *I. tinctoria* aqueous extract possess antimicrobial activity and it can be used as an alternative to antibiotics to treat infectious diseases and protect the host from microbial infections. Many biological and pharmaceutical groups have already begun to perform the laboratory work on the development of analytical methodology from common herbal remedies [23]. Antibacterial activity of aqueous extract of *I. tinctoria* may possess biological active components (phenols and flavonoids) which could be the reason for enhanced antimicrobial activity. It has been documented that well-known plants' metabolites like phenols and flavonoids were responsible for antimicrobial and antifungal activity [24].

Larvicidal assay

I. tinctoria leaves of aqueous extract have highest larval mortality was found. Vector control is facing a serious threat due to the emergence of resistance in vector mosquitoes to conventional synthetic insecticides or development of newer insecticides [25]. Synthetic insecticides failed due to the development of resistance among mosquito species [26]. Bioactive crude extracts or isolated phytoconstituents from the plant could be used as an alternative to the currently used synthetic insecticides. The biological activity of plant extracts might

be due to various compounds, including phenolics, terpenoids, and alkaloids present in plants [27]. Highest larval mortality was found in the aqueous extract of *I. tinctoria* after 24 hrs exposure (Table 2) (LC_{50}:LC_{90}). Therefore, by increasing the concentration of the extract shrinking of the larva were observed. The findings of present study are quite comparable with previous reports of Kamal and Mangla [28] rotenoids (deguelin, dehydrodeguelin, rotenol, rotenone, tephrosin, and sumatrol) isolated from *I. tinctoria* were found to be toxic to larvae of *Anopheles stephensi.* This the first study that shows larvicidal activity of using *I. tinctoria* aqueous extract against dengue vector mosquito species *A. aegypti.*

CONCLUSION

The antimicrobial activities of the leaf extract display the ability to inhibit the growth of both bacteria and fungus clinical isolates from human patient. The larvicidal effect of leaf extract on *A. aegypti* shows the virtuous percentage of larva viability. Therefore, by increasing the concentration of the extract shrinking of the larva were observed. The present study aqueous extract of *I. tinctoria* showed antimicrobial and larvicidal activity and this may form a primary platform for further pharmacological drug designing studies.

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