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Eco-friendly larvicide source from *Acacia auriculiformis* and its antimicrobial activity against clinical pathogens

Dinesh Kumar Subbaraj[1*], Srinivasan Ponnuraj[1], Rajalakshmi Manickam[2], Gowtham Raj Rajendran[3], Sathiyamurthy Karuppannan[4]

***Corresponding author:**

Dinesh Kumar Subbaraj

[1]Department of Microbiology, Karpagam University, Coimbatore -641 021.
[2]Laboratory of Clinical Microbiology, Department of Microbial Biotechnology, Bharathiar University, Coimbatore – 641 046.
[3]PG and Research Department of Biotechnology, Sree Narayana Guru College, K.G Chavadi Coimbatore – 641 105.
[4]Department of Biomedical Science, School of Basic Medical Sciences, Bharathidasan University, Tiruchirappalli – 620 024.

Abstract

Mosquito remains as a global threat for millions of lives since they serve as vector for most of the deadly pathogens and parasites. Several methods such as indoor spraying, insecticidal usage, and nets are employed to evade mosquito threat. However, the chemicals present in such compositions not only have negative impact on the human health and environment but also paves the way for the development of resistance in the insect vectors. Under such circumstances, novel and safer alternative tools have been recently researched to improve mosquito control. Medicinal plants species have been screened as a potential source for secondary metabolites with larvicidal and antimicrobial properties. In view of such fact, the present study investigated the solvent extracts of *A. auriculiformis* for its antimicrobial, antioxidant and larvicidal properties against the malarial and Japanese encephalitis vector *Aedes albopictus* and *Culex quinquefasciatus* respectively. Results evidently showed that the ethanol, ethyl acetate, and water extracts inhibited the test pathogens significantly as compared to the other extracts at sub-MIC levels. DPPH and FRAP antioxidant studies elucidate, strong antioxidant potential of the ethanol and chloroform extracts of *A. auriculiformis*. TLC profile of the ethanol extract showed six prominent bands with respectively coloured band patterns in the UV spectrum which corroborated with the presence of more than one active principles in the extract. The larvicidal activity assay revealed that the ethanol extract significantly inhibited the mosquito vectors with an LC_{50} of 6.1 and 4.2 µg/ml respectively. Notably, the toxicity assay on the non target organism *G. affinis* showed low mortality at the tested concentrations (LC_{50} = 1670 µg/ml) which is ten times greater than the lethal concentration to the mosquito species. Overall, this study highlights the preliminary results which substantiates that *A. auriculiformis* is an important source of eco-friendly and biodegradable larvicides against two important mosquito vectors with low toxicity against non-target organism.

Keywords: *A. auriculiformis*, *Aedes albopictus*,*Culex quinquefasciatus*, *G. affinis*, Larvicidal activity, Antimicrobial activity.

Introduction

Herbal medicines had been the primary source of medicine for treating many ailments indigenously on a global scale. Several factors such as easy availability, non-toxic, eco-friendly and broad spectrum of activity has made the use of medicinal plants indispensable in the treatment of diseases [1]. India serves as the richest hub for the widest variety of the medicinal plants and a number of such plants have found their application in treating a wide range of human ailments. World Health Organization estimated that 25% of all modern medicines have their origin in medicinal plants [2].Traditional herbal medicines are used to maintain health, as well as to prevent, diagnose, improve physical and mental illnesses. About 61% of the 877 drugs introduced worldwide are observed to contain derivatives of herbal plants [3]. Extensive research on different plant species has revealed that the therapeutic principles of herbal plants are due to the presence of phytochemicals such as alkaloids, steroids, flavonoids, terpenoids and tannins Such secondary metabolites consists of diversified pharmacological properties which provide the plants with medicinal properties [4].

Development of novel drugs with plant derivatives are based upon the identifying the principle factors responsible for the medicinal and curative effect of the plants [5]. The advent of chemotherapeutic agents and their indiscriminate use in the treatment against bacterial infections has resulted in the emergence of multi drug resistance pathogens [6].

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Therefore the need for a simple and effective alternative medicine has become important and several studies were conducted to substantiate the determination of antimicrobial properties of various plant extracts during the past few decades [7]. Moreover mosquitoes serve as one of the main source for the transmission of pathogens and parasites. Diseases such as dengue, yellow fever, malaria, chikungunya, Rift Valley fever, Japanese encephalitis, avian malaria, Western equine encephalomyelitis, bancroftian filariae, Zika virus, canine heart worm disease and steariosis are some of the majormosquito borne deadly diseases [8]. Recent estimates reveal that despite many control measures every year the mortality rate due to mosquito borne diseases has increased beyond one million [9]. On average about 50 million dengue infection occurs among which the death due to dengue haemorrhagic fever leads to 22,000 deaths mainly among children [10]. Viral diseases such as chikungunya caused by arbovirus have emerged from less frequent endemic of East Africa to a wide spreading infection of Indian Ocean basin and Southeast Asia. In addition, Japanese encephalitis virus has spread to Indian subcontinent and Australia [11]. Such pivotal scenario necessitates a crucial vector control strategy. Organophosphates, microbial agents and insect growth regulators are employed to control the young mosquito instars. However, such chemicals negatively impact human health and environment along with the development of resistance in vectors [12,13]. Recent research has found novel and eco-friendly methods to improve mosquito control. Screening plants for the presence of secondary metabolites to be used as an alternative entomological and parasitological control agents has gained importance in the recent decade [14]. Plant derived molecules have been experimented for their mosquito repellent, ovideterrent and mosquitocidal properties as they are widely noted for their eco-friendly property, easy biodegradability and minimal side effects against other organisms [15 -17]. Notably, the oils derived from plants show significant ovicidal, larvicidal, pupicidal, oviposition deterrence, repellent action, antifeedant and adulticidal activity [18,19].

Acacia auriculiformis (A.Cunn. ex Benth.) (Mimosaceae), commonly known as ear leaf acacia, is a fast-growing, crooked, gnarly tree native to Australia, Indonesia, and Papua New Guinea. The tree is compactly spread with multi stems and grow up to 30 m tall. Leaves of Acacia are alternate, simple, and blade-like with slight curvature, 11-20 cm in length with a dark green surface. Flowers in loose, yellow-orange spikes at leaf axils or in clusters of spikes at stem tips; flowers mimosa-like, with numerous free stamens. Fruit are flat, oblong pod, twisted at maturity, splitting to reveal flat black seeds attached by orange, string like arils. The plant contains rich quantities of methyl glucuronic acid, glucuronic acid, galactose, arabinose and rhamnose [20]. Some of the medicinal properties include central nervous system-depressant, spermicidal and filaricidal activities [21]. The present study is focused on the antibacterial and larvicidal activity of the solvent extracts of *A. auriculiformis* followed by extensive analyses of the active compounds with the help of various phytochemical tests.

Materials and Methods

Reagents

The reagents used for the study were of analytical grade as follows: Millons reagent (Himedia), Fehling A and B reagent (Himedia), Ferric Chloride (Himedia), Dimethyl Sulphoxide (Loba Chemie), DMSO (Fisher), Sodium Hydroxide (Himedia), Vanillin (Himedia), Ethanol, Sodium hydroxide (Himedia), Mayer's and Wagner's reagent (Loba Chemie), n-hexane (Himedia), Petroleum ether (Himedia), Chloroform (Himedia), Ethyl acetate (Himedia).

Collection of plant material and solvent extraction

Leaves of *A. auriculiformis* (A.Cunn. ex Benth.)were collected from the rural area of Tiruchirappalli, Tamil Nadu, India. The plant specimen was authenticated at the Botanical Survey of India, Southern Circle, Coimbatore and the voucher number was obtained. The collected leaves were washed thoroughly under running tap water, shade dried, powdered using kitchen blender and stored under air tight condition.

About 10 g the powdered leave sample was soaked separately in 100 ml (1:10) of different solvents such as water, chloroform, petroleum ether, ethyl acetate and ethanol. The extraction was performed using cold percolation method as follows. The mixture was kept in rotary shaking incubator for 48 hours at 28 C with 160 rpm. The samples were then separately filtered and the crude extracts were obtained. The filtrates were evaporated to dryness at 50-65 C in a rotary vacuum evaporator (Model: R150, Labomed, India) to obtain a dark coloured molten mass. The extracts were collected in sterile tubes and were stored at 4 C until further use. The yield percentage was calculated using the formula.

$$\text{Yield (g /100 g of dry plant material)} = (W_1 \quad 100) / W_2$$

Were, W_1 was the weight of the extract after the evaporation of solvent, and W_2 was the weight of the dry plant material.

Qualitative phytochemical analysis

The extract was tested for the presence of bioactive compounds by using following standard methods [22-24].

Test for proteins -Millon's test

Crude extract when mixed with 2 ml of Millon's reagent, white precipitate appeared which turned red upon gentle heating that confirmed the presence of protein.

Test for carbohydrates - Fehling's test

Equal volume of Fehling A and Fehling B reagents were mixed together and 2 ml of it was added to crude extract and gently boiled. A brick red

precipitate appeared at the bottom of the test tube indicated the presence of reducing sugars.

Test for phenols and tannins

Crude extract was mixed with 2 ml of 2 % solution of $FeCl_3$. A blue-green or black coloration indicated the presence of phenols and tannins.

Test for flavonoids - Alkaline reagent test

Crude extract was mixed with 2 ml of 2 % solution of NaOH. An intense yellow colour was formed which turned colourless on addition of few drops of diluted acid which indicated the presence of flavonoids.

Test for saponins

Crude extract was mixed with 5 ml of distilled water in a test tube and it was shaken vigorously. The formation of stable foam was taken as an indication for the presence of saponins.

Test for glycosides - Liebermann's test

Crude extract was mixed with each of 2 ml of chloroform and 2 ml of acetic acid. The mixture was cooled in ice. Carefully concentrated H_2SO_4 was added. A colour change from violet to blue to green indicated the presence of steroidal nucleus, i.e., glycone portion of glycoside.

Test for steroid

Crude extract was mixed with 2 ml of chloroform and concentrated H_2SO_4 was added sidewise. A red colour produced in the lower chloroform layer indicated the presence of steroids. Another test was performed by mixing crude extract with 2 ml of chloroform. Then 2 ml of each of concentrated H_2SO_4 and acetic acid were poured into the mixture. The development of a greenish coloration indicated the presence of steroids.

Test for terpenoids

Crude extract was dissolved in 2 ml of chloroform and evaporated to dryness. To this, 2 ml of concentrated H_2SO_4 was added and heated for about 2 min. A greyish colour indicated the presence of terpenoids.

Test for alkaloids

Crude extract was mixed with 2 ml of 1 % HCl and heated gently. Mayer's and Wagner's reagents were then added to the mixture. Turbidity of the resulting precipitate was taken as evidence for the presence of alkaloids.

Antimicrobial assays

Microbial cultures and culture conditions

About eight pure cultures of bacteria and three fungal isolates namely *Bacillus subtilis* (MTCC 121),*Escherichia coli* (MTCC 119), *Proteus mirabilis* (MTCC 9493), *Pseudomonas aeruginosa* (MTCC 1034), *Salmonella enteric* (MTCC 1164), *Shigella flexneri* (MTCC 9543), *Staphylococcus aureus* (MTCC 1430), *Vibrio cholera* (MTCC 3904) and three fungal strains such as *Aspergillus niger* (MTCC 4325), *Candida albicans* (MTCC 3017), *Cryptococcus sp.* (MTCC 9423)were obtained from IMTECH, Chandigarh and used for the antimicrobial assays. All the strains were cultured in their routine media and were maintained at 37 ºC and 25 ºC for bacteria and fungi respectively. Antimicrobial activity was performed on Mueller Hinton Agar (MHA, Himedia) for bacteria and Sabouraud Dextrose Agar (SDA, Himedia).

Determination of Minimal Inhibitory Concentration

The minimum inhibitory concentration (MIC) was determined, by using the micro dilution method in 96 well micro titre plates [25] with slight modifications. Briefly, 200 µl of freshly prepared Nutrient broth (Himedia) and Sabouraud Dextrose broth (Himedia) were added to the respective wells for determining the MIC. Then, 30 µl of microbial cultures at a concentration of 1 x 10^5 CFU/ml were added to each well. Plates were incubated at 25 ºC for bacteria and fungi respectively for 18 h. 50 µl of the crude plant extracts at a concentration (stock concentration: 1 mg/ml) ranging from 0.0625 to 8 mg/ml were dispensed on to the respective wells. Turbidity was measured using ELISA plate reader (ELX 800, Biotek, India) at 620nm. The lowest concentration of the plant extracts which showed absence of visible growth was considered as MIC.

Agar disc diffusion assay

Overnight bacterial cultures (0.5 O.D) were swabbed in MHA plates using sterile swabs. For anti-fungal activity, three days old fugal cultures were swabbed on SDA plates. Sterile discs (6 mm dia) were impregnated with 50 µl concentration of the plant extracts (Sub-MIC level) and placed on the pre-inoculated MHA plates. Ciprofloxacin (0.03 mg/ml) and nystatin (0.01 mg/ml) were used as positive controls for bacterial and fungal test organisms respectively. 80 % was used as negative control. The plates were incubated for 24 h at 37 C for bacteria and for 72 h for fungi at 30ºC. After incubation the zone of inhibition formed around the discs were measured [26].

Antioxidant activity assays

2, 2'-diphenly-1-picrylhydrazyl (DPPH) scavenging method

The DPPH radical scavenging assay was determined according to Leong and Shui [27] with slight modification. Briefly, 2 ml of 0.25 mM DPPH (in methanol) was added to the different concentrations of the extract (1 ml). The reaction mixture was incubated for 30 min after which its absorbance was measured at 517 nm, methanol was used as both a blank and negative control. Results were expressed as ascorbic acid equivalent antioxidant capacity (AEAC) and defined as mg ascorbic acid equivalents (AA)/100 g of fresh weight basis.

Ferric reducing antioxidant power (FRAP) method

The FRAP reagent was prepared by mixing 300 mM sodium acetate buffer (pH 3.6), 10.0 mM TPTZ solution and 20.0 mM $FeCl_3.6H_2O$ solution in a ratio of 10:1:1 in volume. Samples at different concentrations were then added to 3 ml of FRAP reagent and the reaction mixture was incubated at 37 C for 30 min. The increase in absorbance at 593 nm was measured. Fresh working solutions of $FeSO_4$ (10 to 100 µg/ml) were used for calibration. The antioxidant capacity based on the ability to reduce ferric ions of sample was calculated from the linear calibration curve and expressed as mmol $FeSO_4$ equivalents per gram of sample [28].

Larvicidal activity

The larvicidal activity of the effective plants crude extracts were evaluated as per the method recommended by World Health Organization,[29]. Batches of 25 third in star larvae were transferred to a small disposable 6 well plates, each well containing 5 ml of water. The appropriate volume of dilution was added to 5 ml water in the well to obtain the desired target dosage at a concentration ranging from 2 to 10 µg/ml. Triplicates were set up for each concentration, and an equal number of controls were set up simultaneously using 1 ml DMSO in tap water. Larvicidal activity was recorded at 24 h post exposure, during which the larvae was not provided with food. The LC_{50} and LC_{90} value was calculated by probit analysis [30].

Toxicity assay on aquatic organisms

The toxicity effect of the *A. auriculiformis* crude plant extracts were tested against the other non-target aquatic organisms such as mosquito predators i.e.,*Gambusia affinis* according to the method of Sivagnaname and Kalyanasundaram [31]. The mosquito fish was obtained from fish farms of Tamil Nadu Fisheries Development Corporation (TNFDC; Govt. of India), Aliyar, Tamil Nadu. The organism was maintained in plastic tubs 100 cm (W) X 50 cm (H) at 28 ± 2 C and Relative humidity of 75 %. The plant extract was tested at a dosage 50 times higher than the LC_{50} dosage exhibited against the mosquito larvae. Five replicates were maintained along with that of an untreated control. The organism was also observed for a total of 10 days post treatment to determine the effect on swimming and survivability at the tested concentration.

Thin Layer Chromatography (TLC)

A TLC system equipped with a sample applicator was used for application of samples according to Urakova, *et al.,*[32] Five microliters of each leaf extracts were separately applied on 4 16 cm chromatographic pre-coated silica gel plates (Himedia, TLC grade) as the stationary phase. The chromatograms were developed in a twin trough glass chamber containing ethyl acetate and *n*-hexane (6:4, v/v) as the mobile phase. The plates were removed after the solvent front has moved about 15 cm from the sample origin, and subsequently allowed to dry. The spots were visualized under visible (570 nm), short UV (250 nm) and long UV (360nm) light. Then, the plates were sprayed with vanillin-sulfuric acid reagent for colour reaction and allowed to dry. A scanner was used for photo-documentation at the used wavelengths after application of vanillin-sulfuric acid reagent. The movement of each separating spot of the extract was expressed by its retention factor (*Rf*). Values were calculated for each spot for the leaf extract using the formula:

Rf= distance travelled by the solute spot distance travelled by the solvent

Statistical analysis

Mortality data were analyzed using probit analysis. LC_{50} and LC_{90} values were calculated according to Finney [30] and the biotoxicity studies on non target organisms were calculated according to the method of Deo *et al.* [33]. The suitability index (SI) was calculated using the formula

SI = LC_{50} of non target organisms LC_{50} of target organism

All Data were subjected to statistical analysis using SPSS software (Version 21.0, USA). The values were expressed as mean ± S. E.

Results

Extraction yield and qualitative phytochemical analysis

The yield percentage and qualitative phytochemical composition of *A. auriculiformis* was determined and tabulated (Table 1). Results elucidated that the ethanol extract showed higher yield percentage followed by petroleum ether, water, chloroform and ethyl acetate.

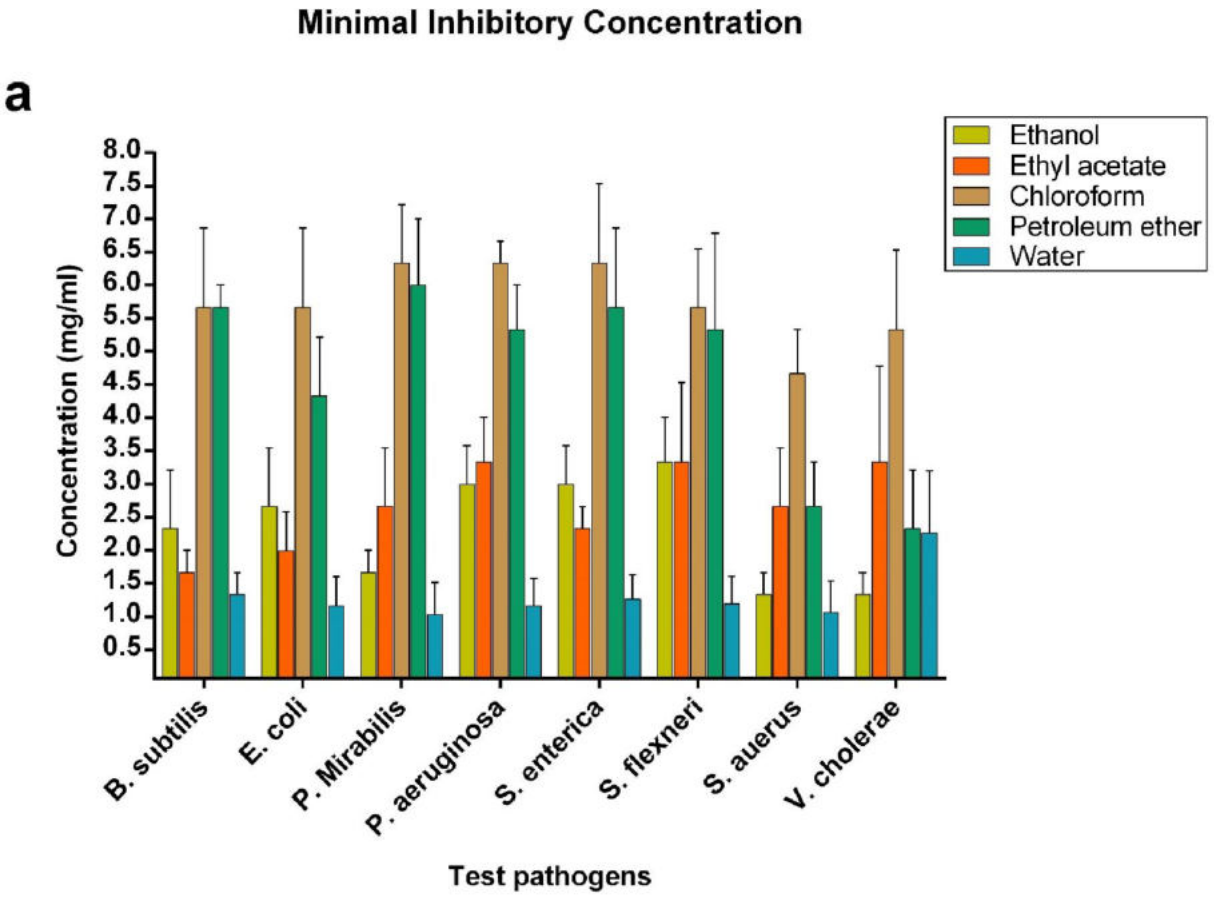
Table 1. Qualitative phytochemical composition and yield percentage of *A. auriculiformis* solvent extracts.

Components	Ethanol	Ethyl acetate	Chloroform	Petroleum ether	Water
Protein	-	-	-	-	+
Carbohydrate	+	+	+	+	+
Phenols and tannins	+	+	+	+	+
Flavonoid	-	-	-	-	+
Sapnonin	+	+	+	+	-
Glycoside	+	+	-	+	+
Steroid	+	+	+	+	-
Terpenoid	+	-	-	-	+
Alkaloid	+	+	-	-	-
Yield Percentage	12.5	7	9	10	9.5

+; Presence -; absence of phytochemical

Minimal Inhibitory Concentration

MIC was determined against the test organism for the solvent extracts of *A. auriculiformis* using the MTP method (Figure 1a and Figure 1b). The results revealed that the mean MIC value of the solvent extracts such as ethanol, ethyl acetate, chloroform, petroleum ether and water extract were 2 mg/ml, 3 mg/ml, 6 mg/ml, 5 mg/ml, 1 mg/ml against the test bacterial samples while the mean MIC value was 2 mg/ml, 2 mg/ml, 6 mg/ml, 5 mg/ml, 1 mg/ml against the test fungal samples respectively.



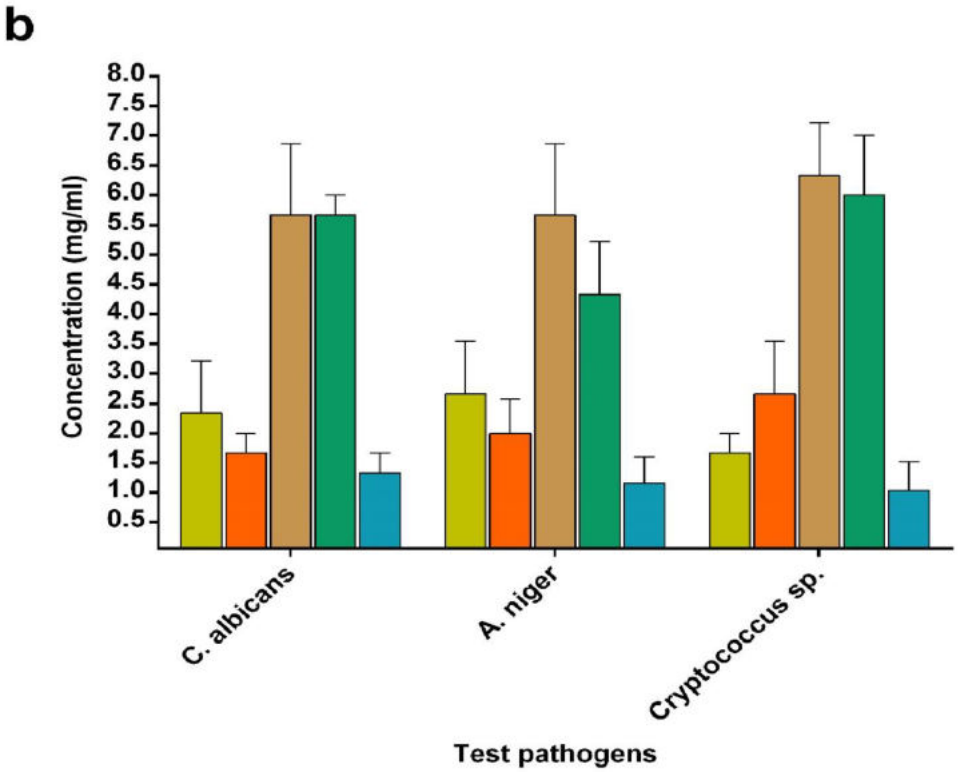


Figure 1. Minimal inhibitory concentration assay of *A. auriculiformis* extracts against the test organismsa) bacteriab) fungi. Values of represented as mean ± S.E

Agar disc diffusion assay

The antibacterial and antifungal activity of the *A. auriculiformis* leaf solvent extracts were tested at MIC using the agar disc diffusion assay against bacterial and fungal pathogens. As represented in the Table 2 among the tested solvent extracts water, ethanol and ethyl acetate extracts showed antimicrobial activity against all the tested pathogens at MIC concentration. However, chloroform and petroleum ether extract did not inhibit some of the tested pathogens as represented in the Table 2.

Table 2. Antibacterial and anti-fungal activity of *A. auriculiformis* solvent extract against clinical pathogens.

Test Pathogens	Zone of inhibition (mm)						
	Solvent extracts						
	Ethanol	Ethyl acetate	Chloroform	Petroleum ether	Water	PC‡	NC†
Antibacterial activity							
B. subtilis	18	14	-	10	14	24	-
E. coli	19	17	10	14	12	25	-
P. mirablis	18	16	12	11	10	22	-
P. aeruginosa	17	12	-	-	10	21	-
S. enterica	15	13	-	-	09	23	-
S. flexneri	19	14	-	-	11	22	-
S. aureus	20	14	15	12	12	25	-
V. cholerae	16	07	12	15	11	24	-
Antifungal activity							
Candida albicans	22	14	10	14	10	24	-
Cryptococcus sp.	24	13	12	15	09	26	-
Aspergillus niger	21	15	12	14	11	27	-

‡Positive Control – Ciprofloxacin (0.03 mg/ml); nystatin (0.01 mg/ml)†NegativeControl – (80 %) DMSO

DPPH scavenging activity

The scavenging effect of *A. auriculuiformis* solvent extracts on DPPH radical is expressed in terms of AEAC under the experimental condition was calculated and data presented in Table 3. All the solvent extracts exhibited scavenging effect on DPPH radicals. The scavenging effect of *A. auriculiformis* extracts on the DPPH radical decreased in the order of Ethanol>chloroform> ethyl acetate >water. Among the extracts, the methanol extract exhibited stronger radical scavenging activity.

Table 3. DPPH scavenging activity of *A. auriculuiformis* solvent extracts.

Concentration of the extract (µg/ml)	*Acacia auriculiformis* solvent extracts				
	Ethanol	Ethyl acetate	Chloroform	Petroleum ether	Water
25	3525 ±12^{c}	2695 ±12^{b}	2559 ±10^{b}	2075 ±11^{b}	1565 ±12^{a}
50	4075 ±12^{c}	3265 ±12^{b}	3341 ±10^{b}	2875 ±12^{a}	2085 ±12^{a}
75	4605 ±12^{c}	4075 ±12^{b}	4141 ±11^{b}	3605 ±11^{a}	3545 ±12^{a}
100	5545 ±12^{c}	5045 ±12^{b}	5272 ±11^{c}	4105 ±12^{a}	4045 ±12^{a}
125	6305 ±12^{c}	5885 ±12^{bc}	6063 ±12^{c}	4585 ± 11^{a}	5075 ±11^{b}
150	7045 ±11^{c}	6445 ±12^{b}	7045 ±12^{bc}	5105 ±11^{a}	6105 ±11^{b}

Values are the mean ± standard error of triplicate experiments. All the values in the table significantly ($p < 0.05$) differ among extracts; different letters in the same row are significantly different. ^{a}(0.05); ^{b}(0.01); ^{c}(0.001). DPPH activity of the solvent extracts are expressed as mg Ascorbic acid/100 g final weight.

FRAP activity

Based on the FRAP value obtained, methanolic extract of *A. auriculiformis* was observed to possess strong activity than other solvent extracts but, in dose-dependent manner as shown in Table 4. There were significant ($p < 0.05$) differences in the FRAP values, among the type solvents incorporated in the extraction. The antioxidant activity for different leaf extracts used increased in the order: Ethanol<Chloroform<Ethyl acetate <Petroleum ether < water.

Table 4. FRAP scavenging activity of *A. auriculuiformis* solvent extracts

Concentration of the extract (µg/ml)	*Acacia auriculiformis* solvent extracts				
	Ethanol	Ethyl acetate	Chloroform	Petroleum ether	Water
25	4215 ±14^{c}	3659 ±15^{b}	4107 ±14^{c}	2130 ±14^{ab}	2018 ±14^{a}
50	4875 ±15^{c}	4124 ±10^{b}	4724 ±15^{c}	2868 ±15^{ab}	2323 ±15^{a}
75	5854 ±10^{c}	4854 ±10^{b}	5774 ±10^{c}	3209 ±10^{ab}	3031 ±10^{a}
100	6342 ±18^{c}	5137 ±18^{b}	6272 ±18^{c}	4038 ±18^{ab}	3736 ±18^{a}
125	6995 ±10^{c}	5578 ±10^{b}	6749 ±10^{c}	4775 ±10^{ab}	4120 ±10^{a}
150	7414 ±10^{c}	6034 ±10^{b}	7253 ±10^{c}	5015 ±10^{ab}	4829 ±10^{a}

Values are the mean ± standard error of triplicate experiments. All the values in the table significantly ($p < 0.05$) differ among extracts; different letters in the same row are significantly different. ^{a}(0.05); ^{b}(0.01); ^{c}(0.001).FRAP values are expressed as mmol FeSO4/g DW of the solvent extracts respectively.

Larvicidal activity

The larvicidal activity of *A. auriculiformis* leaf extract was tested for ethanol extracts at various concentrations as it showed significant antimicrobial and antioxidant activities. As shown in the Table 5 the ethanol extract exhibited significant larvicidal activity against *A. albopictus* and *C. quinquefasciatus* in a dose dependent manner after 24 h incubation. LC_{50} values were observed to be 6.1 and 4.2 µg/ml whereas the LC_{90} values were observed to be 8.5 and 9.42 µg/ml against the two respective test larval species.

Table 5. Larvicidal activity of *A. auriculuiformis* ethanol extract.

Test species	Concentration (µg/ml)	Mortality (%)*	LC_{50} (µg/ml) (LCL-UCL)	LC_{90} (µg/ml) (LCL-UCL)	Slope	Regression equation
Aedesalbopictus	2	10.7 ± 0.12	6.1 (5.2 to 6.3)	8.5 (8 to 10.5)	3.12	y =10.34 +9.15x
	4	17.3 ± 0.12				
	6	28.0 ± 0.21				
	8	54.7 ± 0.12				
	10	88.0 ± 0.20				
Culexquinquefasciatus	2	13.3 ± 0.12	4.2 (3.5 to 4.9)	9.42 (8.9 to 10.1)	2.45	y =4.17 +9.01x
	4	26.7 ± 0.23				
	6	53.3 ± 0.12				
	8	64.0 ± 0.20				
	10	82.7± 0.23				

Control group showed no mortality,*Values are the mean ± standard error of triplicate independent experiments. LC_{50} = lethal concentration at which 50% of the exposed organisms were killed. LD_{90} = lethal concentration at which 90% of the exposed organisms were killed. LCL = Lower Confidence Level, UCL = Upper Confidence Level

Biosafety of herbal larvicides on non-target mosquito predators

The biosafety of the herbal larvicides were tested against a non-toxic mosquito predator namely *G. affinis* and the results were tabulated (Table 6). LC_{50} and LC_{90} value were1670 µg/ml and 3450 µg/ml respectively. In addition, no significant changes were observed in the survival and swimming activities of the test organism during the exposure at LC_{50} and LC_{90} doses.

Table 6. Toxicity of *A. auriculiformis* ethanol extract against non-target organism.

Non-target organism	Concentration (µg/ml)	Mortality (%)*	LC_{50} (µg/ml) (LCL-UCL)	LC_{90} (µg/ml) (LCL-UCL)	Slope	Regression equation
Gambusiaaffinis	800	18.7 ± 0.11	1670 (1610 to 2090)	3450 (3130 to 3970)	3.99	y =12.14 +0.12x
	1600	42.7± 0.23				
	2400	53.3 ± 0.11				
	3200	64.0 ± 0.20				
	4000	94.6 ± 0.11				

Control group showed no mortality,*Values are the mean ± standard error of triplicate independent experiments. LC_{50} = lethal concentration at which 50% of the exposed organisms were killed. LD_{90} = lethal concentration at which 90% of the exposed organisms were killed. LCL = Lower Confidence Level, UCL = Upper Confidence Level

Thin Layer Chromatography

The TLC profiles of the ethanol leaf extracts of *A. auriculiformis* are presented in Table 7. A total of five spots were observed on the TLC plates and their R*f* values were calculated. Based on the photo documentation of the TLC plates a green spot was observed at 570 nm while at 360 nm two blue, pink and a red spot was observed. No coloration was observed at 250 nm. Also, the vanillin-sulfuric acid spray resulted in the development of grey and black spots.

Table 7. TLC profile of *A. auriculiformis* ethanol extract.

R*f* value	570 nm	360 nm	250 nm	Vanillin-sulfuric acid spray
0.51	-	blue	-	Black
0.11	-	blue	-	Grey
0.32	-	pink	-	Grey
0.71	Light green	-	-	-
0.62	-	red	-	Black

R*f*= Relative factor value;Mobile phase solvent system: ethyl acetate: n-hexane (6:4, v/v).

Discussion

Although a vast advancements had been made in the modern medical systems, herbal medicines play a centric role in the providing a promising cure against a variety of disease. However, most of the medicinal plants still remain underexploited and are yet to be scientifically validated for their medicinal properties. The present study deals with two sets of experimental design to validate the antimicrobial and larvicidal properties of the solvent extracts of *A. auriculiformis* in the aim of developing a safe natural drug candidate. The bioactive constituents of the medicinal plants depends greatly on its phytoconstituents. Preliminary qualitative phytochemical analysis indicated the presence of different classes of secondary metabolites in the leaf extracts namely terpenes, alkaloids, tannins, phytosterols, saponins, flavonoids and glycosides. The presence of the plant active phyto components in the solvent extracts aids in the various biological activities [34]. Extract yields calculation revealed higher yield percentage in ethanol extract among other solvent extracts. Higher yield percentage could be attributed to the concentrated active components in the solvent extracts based on the polarity of the solvents [35]. Flavonoids are most diverse and widespread compounds in the plant extracts. Prasad*et al.,*[36] reported presence of high flavonoid level in the solvent extracts of *Acacia sp*. Phenol compounds are widely present in plants and are known to exhibit free radical scavenging activity. The presence of phenol in the solvent extracts elucidate antioxidant property of the plant [37]. Results in the Table 1 shows that all the extracts except water contains saponins and tannin. According to Hoong*et al.,* [38] saponins and polyphenol molecules are used in several industrial applications such as leather tanning and wood adhesions. Our results on the phytochemical studies correlated with the studies of Silva *et al.,*[39], and Ferengrino Perez *et al.,*[40] who reported the presence of high level of phenol, tannin and saponins in the solvent extracts of *Acacia* sp.

The results of the MIC assay and anti-microbial activity assays indicated that the leaf extracts of *A. auriculiformis* can be used as an alternative source for developing anti-microbial metabolites against the tested pathogens. To our knowledge this is the first report on the anti-fungal activity of *A. auriculiformis* and anti-bacterial activity against multidrug resistant bacteria. MIC was 1-2 mg/ml in water extract and 2 – 3 mg/ml in the ethanol extract. Moreover, it was observed that the average MIC for ethyl acetate, petroleum ether and chloroform was 4-6 mg/ml. The difference in the MIC values of the different plant extract well corroborated with the varying degree of solubility of the phytoconstituents in the solvents used in the experiment. The MIC and antibacterial activity values of the leaf extracts revealed that the ethanol extract exhibited higher antibacterial and antifungal activity against the tested pathogens compared to the other extracts which could be related to the presence of large concentration of polar antimicrobial constituents [35].

In the present study *A. auriculiformis* leaf extracts were subjected to the antioxidant assays such as DPPH and FRAP. Results indicated that the ethanol and chloroform extract exhibited significantly ($p < 0.05$) higher antioxidant activity as compared to the other extracts. Several reports have pointed out the antioxidant and reducing potential of the genus *Acacia.* Singh *et al.* [41,42] and Tung *et al.,*43] reported that reducing potential of the *A. auriculiformis* bark was lower than that of *A. nilotica* pods. The results of the present study substantiated that the antioxidant potential of the *A. auriculiformis* solvent extracts were high as compared to the barks and pods. According to Naik*et al.* [44] the significant antioxidant potential of *A. auriculiformis* is due to the presence of high content of oxidizable components in its composition which could also be the basis of the higher antioxidant results obtained in the present study. In addition, Ferengrino Perez *et al.* [40] also reported the significant lower quenching activity of *A. auriculiformis* barks against the standards such as gallic acid and ascorbic acid. These reports elucidated that the leaf extracts show higher antioxidant potential than the bark and pod extracts of *A. auriculiformis*. The composition of the bioactive compounds present in the ethanol extract of *A. auriculiformis* was assessed using the TLC technique. TLC finger printing elucidates the chemical composition of a specific plant extract and is used in the standardization of pharmacological preparations from medicinal plants [45,46]. The results of TLC in the present study showed disctinct spot on the silica gel plate with R*f* values as mentioned in Table 7. Most of the spots are observed in the UV wavelength and after the spraying of vanillin sulphuric acid mixture. Phenolic compounds, being aromatic in structure, intensely absorb UV region of the spectrum thereby generating green, yellow, white to pale yellow, purple, pink, red, blue, grey, brown or black spots [47].The larvicidal activity of the ethanol leaf extract of *A. auriculiformis* revealed significant ($p < 0.05$) results against the test organisms. The results of the LC_{50} and LC_{90} values evidently showed that the ethanol extract significantly inhibited the larvae of *C. quinquefasciatus* as compared to *A. albopictus*. Seo *et al.* [48] highlighted that essential oil from medicinal plants significantly inhibited the growth of the *A. albopictus* larvae with an IC_{50} value of 190 µg/ml. The results of the present study revealed that the ethanol extract of *A. auriculiformis* inhibited the larval growth at a much lower concentration which could be attributed to the presence of rich bioactive molecules in the solvent extract. Tabanca *et al.* [49] elucidated that the coumarins extracted from medicinal plants had toxic effect to the *A. aegypti* larvae after 24 h post treatment at a concentration of 49.6 ppm. As *A. auriculiformis* leaves has been well reported for the presence of coumarins by Saini *et al.* [50] it is suffice to hypothesize that the coumarins in the ethanol extract could have actively contributed to the larvicidal activity against the test organisms. The results of the acute toxicity study on the non target organism has elucidated that the ethanol extract has not affected the growth and swimming activity. It has been recently reported that medicinal plant extracts are safe for the predatory fish, *G. affinis*. In agreement with this fact, the ethanol extract of *A. auriculiformis* was tested at a concentration ten times greater than LC_{50} value. Results revealed that the ethanol extract was considerably safe for the tested non target organism. Similar results were observed by Pavela and Govindarajan, [51] who tested the essential oil extracts of medicinal plants against for its toxicity against *G. affinis*. Haldar*et al.* [52] reported

the nontoxic nature of *Drypetes roxburghii* fruits against aquatic insects namely *Chironomus costatus* and *Diplonychus rusticus*. On the other hand, *A. monophylla* extract showed toxicity towards five nontoxic mosquito predators and modified their swimming pattern [31]. Notably many reports has implicated the low toxicity of plant extracts compared to the conventional insecticides [53,54]. Based on the result of this study we believe that the toxicity towards the studied test organisms could be due to the combined active principles of the ethanol leaf extract and its components.

Conclusion

Medicinal plants can be considered as an important source of alternative insecticides for the control of mosquito larvae. In conclusion, our results showed that the crude extracts of *A. auriculiformis* has exhibited significant antimicrobial and antioxidant properties. Among the extracts, ethanol extract has notably shown larvicidal activity against *Aedes albopictus* and *Culex quinquefasciatus* without disturbing the activities of non target organism *G. affinis*. Therefore, these results can be used in the selection of novel, highly specific and biodegradable larvicides. However, further studies are necessary to narrow down the active compound in the solvent extracts responsible for the antimicrobial and larvicidal properties.

Conflict of Interest

The authors declare no conflicts of interest.

Compliance

This study was conducted by following all the applicable international and national guidelines for the care and use of animals. Ethical standards of the concerned institutions were followed while performing experiments.

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