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Original Research Article

Synergistic Anti-*Staphylococcus aureus* (Methicillin Resistant) Activity of Ethnomedicinal Plants from Shevaroy Hills (Eastern Ghats), South India

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Abstract

In the present study, ethanolic extracts of the leaves of the ethnomedicinal plants collected from Shevaroy hills (Eastern Ghats) in Tamil Nadu, *Anamirta cocculus, Ichnocarpus frutescens, Strychnos potatorum* and *Vitex altissima* were tested individually and in different combinations against Methicillin Resistant *Staphylococcus aureus* (MRSA). The results showed that the extracts tested individually against MRSA reveals that the crude ethanolic extract of *Anamirta cocculus* had maximum zone of inhibition and activity index. A maximum of 2.5 cm zone of inhibition was found in the extract combination of *Ichnocarpus frutescens* + *Vitex altissima* followed by other combinations. In the combination of three extracts of ethnomedicinal plants, the highest zone of inhibition (2.2 cm) was observed in *Anamirta cocculus* + *Ichnocarpus frutescens* + *Strychnos potatorum.* Antibacterial activity and activity index recorded in the ethanolic extracts in combination of all the four of selected plant leaves against MRSA showed 2.6 cm zone of inhibition and 0.93 activity index which was nearly close to the standard antibiotic vancomycin. These findings suggest that the plant extracts in combination show synergistic effect on MRSA.

Keywords

Antimicrobial activity
Ethnomedicinal plants
Eastern Ghats
MRSA
Synergistic effect

Introduction

Plants are the chief sources of natural compounds widely used in medicines (Gupta, 1999). The plants which are being used by traditional societies are known to possess various medicinal properties. Many such plants have been evaluated scientifically for their biological and pharmacological activities resulted in the discovery of several life-saving medicines (Balunas and Kinghorn, 2005). Folk knowledge or pre-existing data is highly useful in drug discovery. It is well known fact that the medicinal plants are the resources of promising drugs for many diseases.

Marathe et al. (2013) reported that their results offer a scientific basis for the traditional use of *Tabernimontana alternifolia* in the treatment of skin infections, showing that the plant extract has an enormous potential as a prospective alternative therapy against MRSA skin infections, and the study lays the basis for future studies, to validate the possible use of *Tabernimontana alternifolia* as a candidate in the treatment of MRSA infections.

The studies revealing the antimicrobial activities of several medicinal plants tested individually are available in plenty. Recent studies show that the plant extracts in combination of two or more are exhibiting effective antimicrobial activity against a wide range of microorganisms including drug resistant bacteria (Prakash et al., 2006a, 2006b; Karmegam et al., 2008; Karmegam et al., 2012). The effect of combination of plant extracts against microorganisms is considerably higher than the single plant or plant part extract. Keeping in this view, the present study has been carried out to find out the combinatorial anti-MRSA activity of four selective ethnomedicinal plants of Shevaroy hills (Eastern Ghats), Salem district, South India.

Materials and methods

Selection of plants

Based on the preliminary survey made on the ethnomedicinal plants of Shevaroy hills (Eastern Ghats), Tamil Nadu and on the availability of the plants, the following plants were selected for the present study. The list of plants selected for the study is given below in Fig. 1.

Collection and identification of plants

The leaves of the medicinal plants selected for the present study were collected Shevaroy hills (Eastern Ghats), Tamil Nadu and the identification was confirmed using standard local floras (Gamble and Fischer, 1957; Matthews, 1983). The leaves collected were immediately transported to the laboratory for further processing.

Preparation of crude extract

The leaves of the plants collected were individually washed with tap water, blotted with filter paper and spread over news paper for air drying under shade. After complete dryness, the leaves of individual plants were powdered using a mixer grinder. A known quantity of leaf powder (100 g) of each plant was taken in a 250 ml conical flask and added with 100-200 ml of ethanol (95%).

Ethanol was used for the extraction of phytochemicals because it has the ability to dissolve the phytochemical compounds like tannins, polyphenols, flavonols, terpenoids and alkaloids (Silva et al., 1997; Habtenmariam et al., 1993; Ivanovska et al., 1996). The ethanolic leaf extract of each plant was kept in beaker on a water bath at 45ºC until the solvent gets evaporated. A greasy final material (crude extract) obtained for each plant was transferred to screw cap tubes and stored under refrigerated condition till use.

Preparation of stock solution

By using digital electronic balance, 200 mg of each crude extract was carefully taken in a standard measuring flask and 5 ml of ethanol was added to dissolve the extract and one or two drops of emulsifier (Triton-X100) was added to completely dissolve the extract. Then it was made up to 200 ml by adding distilled water. This forms the stock solution of 1000 ppm (i.e., 1mg/ml).

Preparation of test solution

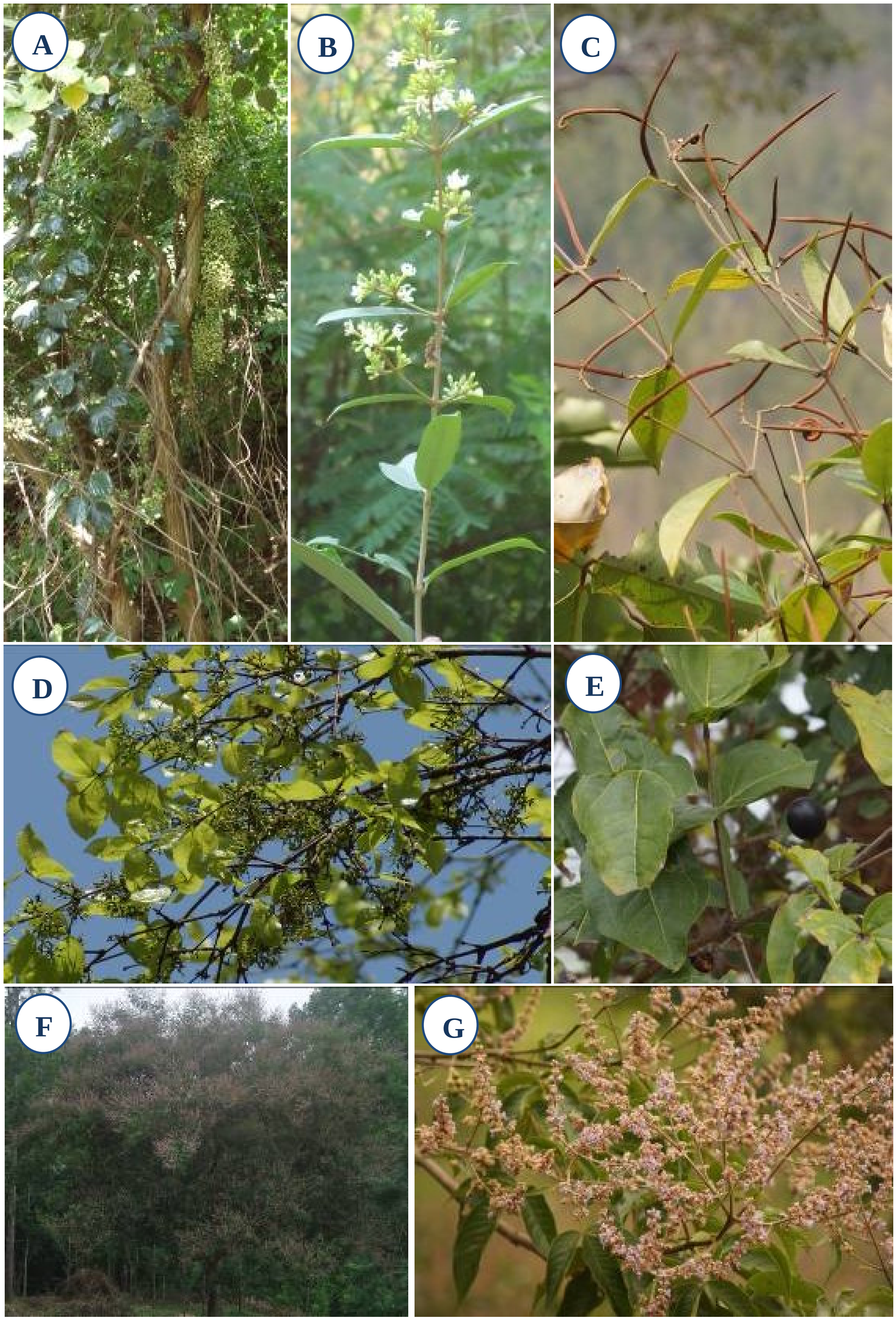
For the antimicrobial assay using individual plant extract, the stock solution of 1000 ppm was directly used. For studying combined antimicrobial activity i.e., synergistic effect of the plant extracts against test microorganisms, the test concentrations were prepared based on the number of extracts of the selected plants to be combined i.e., combination of extracts of any two plants, three plants and of four plants were mixed respectively in separate container. Care was taken to maintain 1000 ppm concentration of combined extract solution in total, by adding volumes of respective stock solution of crude extracts as calculated (Karmegam et al., 2008).

Antimicrobial assay

Bacteria used

The test bacteria, Methicillin Resistant *Staphylococcus aureus* (MRSA) was obtained from the Microbial Type Culture Collection (MTTC) of Institute of Microbial Technology (IMTECH), Chandigarh, and was used for the

Fig.1 Ethnomedicinal plants selected for the present study: *Anamirta cocculus* (A); *Ichnocarpus frutescens*: (B) with flowers, (C) with fruits; *Strychnos potatorum*: (D) with flowers, (E) with fruit; *Vitex altissima*: (F) habit-entire, (G) flowering branch



present study. The bacterial species was maintained by sub-culture method in nutrient agar slants.

Kirby-Bauer disc diffusion technique

This technique was used to test the sensitivity of selected test organism to the ethanolic leaf extracts individually and in combination (Bauer et al., 1966).

Preparation of antimicrobial disc using crude extracts

Discs of 5 mm in diameter from a sheet of filter paper were punched out, placed in Petri dishes allowing a distance of 2-4 mm between each disc and sterilized in a hot air oven at 160ºC for 1 hour. After allowing the disc to cool, 20 µl (0.02 ml) of each test solution was added on to each disc and then the discs were dried at 37ºC in an incubator for one hour (Cheesbrough, 1984). For control set, the discs were added with distilled water (200 ml) containing 5ml ethanol + 2 drops of emulsifier at 20µl/disc.

Preparation of plates

The Petri plates of 100mm diameter with Muller-Hinton agar were swabbed with broth culture of MRSA by using sterile swab. Over this, prepared antimicrobial discs were placed under aseptic conditions. Control sets with standard antibiotic vancomycin (30 µg/disc) were simultaneously maintained along with methicillin (30 µg/disc) to show the resistance and sensitivity pattern of test organism. Also the discs without plant extract (discs prepared using 200 ml distilled water + 5 ml ethanol + one or two drops of emulsifier) were also maintained as another set of control for the test organism.

The plates were then incubated at 37ºC for 24 hrs and the zone of inhibition (ZI) was measured in cm and recorded. From the results activity index was calculated by comparing the zone of inhibition (ZI) of leaf extracts with standard antibiotic as follows:

$$\textbf{Activity Index} = \frac{\textbf{Inhibition area of test sample}}{\textbf{Inhibition area of standard antibiotic}}$$

Results

Based on the ethnomedicinal claims recorded in the present study, the following four plants were found available at the time of collection. The following ethnomedicinal claims has been procured on the selected plant species from tribal and non-tribal villagers in the study area.

Anamirta cocculus (Linn.) Wight & Arn. (Eng.: Fish berries, Crow killer; Tam.: Kaakkamari, Kakkakollivithai). *Parts used:* Leaves, Fruits; *Uses:* Antifungal and antihelminthic.

Ichnocarpus frutescens (Linn.) R. Br. (Tam.: Udarkkodi, Paaravalli). *Parts used:* Leaves, Roots; *Uses:* Diuretic, skin diseases and general weakness.

Strychnos potatorum L.f. (Eng.: Clearing nut tree; Tam.: Thetham kottai). *Parts used:* Seeds and leaves; *Uses:* Seeds are used as stomachic, used in diarrhea; leaves are used for skin diseases.

Vitex altissima L. f. (Tam.: Mayilai nocchi). *Parts used:* Leaves and bark; *Uses:* antiseptic, antibacterial; also used for skin diseases.

The standard reference antibiotic vancomycin at 30 µg per disc showed 2.8 cm zone of inhibition against MRSA. The ethanolic leaf extracts of *Anamirta cocculus, Ichnocarpus frutescens, Strychnos potatorum* and *Vitex altissima* tested alone at 1000 ppm showed 2.2, 2.1, 2.0 and 1.7 cm zone of inhibition respectively (Table 1). The activity index of the ethanolic leaf extracts of *Anamirta cocculus, Ichnocarpus frutescens, Strychnos potatorum and Vitex altissima* tested individually showed 0.79, 0.75, 0.71 and 0.61 respectively against MRSA (Fig. 2). The highest zone of inhibition of 2.5 cm was recorded in the combination of *Ichnocarpus frutescens* + *Vitex altissima* (B+D) against MRSA followed by *Strychnos potatorum* + *Vitex altissima* (2.2 cm) = *Ichnocarpus frutescens* + *Strychnos potatorum* (2.2 cm) (Table 2). The least zone of inhibition was recorded in *Anamirta cocculus*+ *Strychnos potatorum* leaf extract combinations (zone of inhibition = 1.4 cm). The activity index also reflected the same results for the combination of two extracts was ranged between 0.50 and 0.89 (Fig. 3).

Table.1 Anti-MRSA activity of ethanolic leaf extracts of ethnomedicinal plants (used individually) collected from Shevaroy hills (24 hrs; Reference standard: Vancomycin)

Ethanolic extract of the plant leaves used	Zone of inhibition (cm)*
Anamirta cocculus (L.) Wight & Arn.	2.2
Ichnocarpus frutescens (Linn.) R. Br.	2.1
Strychnos potatorum L. f.	2.0
Vitex altissima L. f.	1.7
Vancomycin (30 µg per disc)	2.8

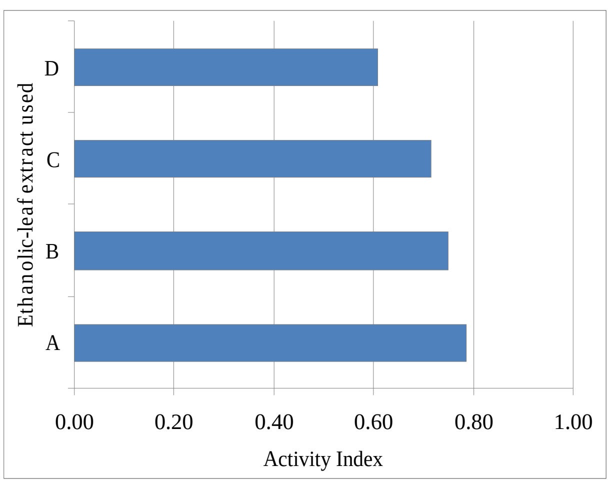
* Values are mean of three replicates.

Table.2 Antibacterial activity recorded in the ethanolic extracts of selected plant leaves in combination of three against MRSA (24 hrs; Reference standard: Vancomycin)

Combination of plant extracts tested *	Zone of inhibition (cm)
A + B	2.0
A + C	1.4
A + D	1.7
B + C	2.2
B + D	2.5
C + D	2.2
A + B + C	2.2
A + B + D	1.9
B + C + D	2.0
A+B + C + D	2.6**

* Values are mean of three replicates; **Activity Index = 0.93; A = *Anamirta cocculus*; B = *Ichnocarpus frutescens*; C = *Strychnos potatorum*; D = *Vitex altissima*;

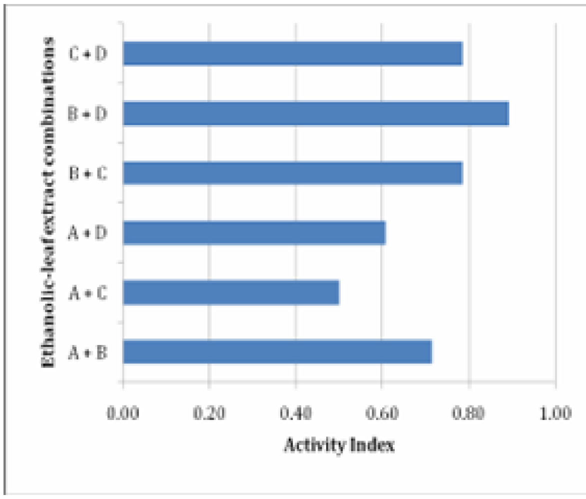
Fig.2 Antibacterial activity index recorded in the ethanolic leaf extracts of selected medicinal plants against MRSA



(24 hrs; Reference standard: Vancomycin; A = *Anamirta cocculus*; B = *Ichnocarpus frutescens*; C = *Strychnos potatorum*; D = *Vitex altissima*).

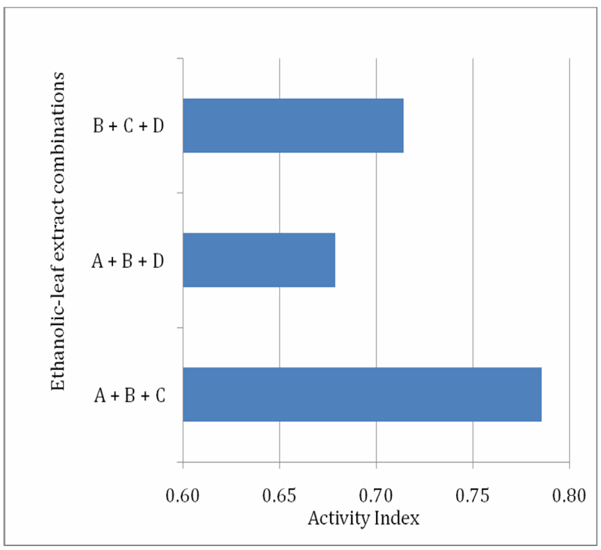
In the combination of three extracts of ethnomedicinal plants, the highest zone of inhibition (2.2 cm) was observed in *Anamirta cocculus* + *Ichnocarpus frutescens* + *Strychnos potatorum* (A+B+C) followed by *Ichnocarpus frutescens* + *Strychnos potatorum* + *Vitex altissima* (2.0 cm)> *Anamirta cocculus* + *Ichnocarpus frutescens* +*Vitex altissima* (1.9 cm) (Table 2). The highest activity index of 0.79 was found in *Anamirta cocculus* + *Ichnocarpus frutescens* + *Strychnos potatorum* (A+B+C) extract combination followed by *Ichnocarpus frutescens* + *Strychnos potatorum* + *Vitex altissima* (0.79)> *Anamirta cocculus* + *Ichnocarpus frutescens* + *Vitex altissima* (0.71) (Fig. 4). Antibacterial activity and activity index recorded in the ethanolic extracts in combination of all the four of selected plant leaves against MRSA showed 2.6 cm zone of inhibition and 0.93 activity index which was nearly close to the standard antibiotic vancomycin.

Fig.3 Antibacterial activity index recorded in the ethanolic extracts of selected plant leaves in combination of two against MRSA



(24 hrs; Reference standard: Vancomycin; A = *Anamirta cocculus*; B = *Ichnocarpus frutescens*; C = *Strychnos potatorum*; D = *Vitex altissima*).

Fig.4 Antibacterial activity index recorded in the ethanolic extracts of selected plant leaves in combination of three against MRSA



(24 hrs; Reference standard: Vancomycin; A = *Anamirta cocculus*; B = *Ichnocarpus frutescens*; C = *Strychnos potatorum*; D = *Vitex altissima*).

Discussion

The use of and search for drugs and dietary supplements derived from plants have accelerated in recent years. Ethnopharmacologists, botanists, microbiologists and natural-products chemists are combing the Earth for phytochemicals and "leads" which could be developed for treatment of infectious diseases. While 25 to 50% of current pharmaceuticals are derived from plants, none are used as antimicrobials. Traditional healers have long used plants to prevent or cure infectious conditions; Western medicine is trying to duplicate their successes.

Plants are in a wide variety of secondary metabolites such as tannins, terpenoids, alkaloids and flavonoids which have been found *in vitro* to have antimicrobial properties. Based on the above facts, the present study has been carried out to find an alternative solution for controlling micro-organisms by using combinations of crude plant extracts.

In the present study, ethanolic extracts of the leaves of the ethnomedicinal plants, *Anamirta cocculus, Ichnocarpus frutescens, Strychnos potatorum and Vitex altissima* were tested individually and in different combinations against Methicillin Resistant *Staphylococcus aureus* (MRSA). The results on the effect of the extracts tested individually against MRSA reveals that the crude ethanolic extract of *Anamirta cocculus* had maximum zone of inhibition and activity index as shown in Table 1 and Fig. 2. The parallel results have also been reported by Karuppusamy et al. (2001) while screening ethanolic extracts of 25 medicinal plants of the family Asclepiadaceae and reported that out of 25 plant extracts screened, 13 extracts showed strong antimicrobial activity whereas rest of the plant extracts showed no activity or very minimum antimicrobial activity against different bacterial strains.

The studies on screening the extracts of specific plant parts alone obtained by many workers fall in line with the present investigation: different solvent extracts of *Zapoteca portoricensis* (Agbafor et al., 2011), antibacterial ester from root bark extracts of *Vitellaria paradoxa*, methanolic leaf extracts of *Anogeissus leiocarpus* (Ichor and Ekoja, 2011) and antibacterial activity of *Artemisia dracunculus* essential oil against multi-drug resistant *Acinetobacter baumannii* (Jazani et al., 2011). A maximum of 2.5 cm zone of inhibition was found in the extract combination of *Ichnocarpus frutescens* + *Vitex altissima* followed by other combinations.

In the combination of three extracts of plants, the highest zone of inhibition (2.2 cm) was observed in *Anamirta cocculus* + *Ichnocarpus frutescens* + *Strychnos potatorum* (A+B+C) followed by *Ichnocarpus frutescens* + *Strychnos potatorum* + *Vitex altissima* (2.0 cm)> *Anamirta cocculus* + *Ichnocarpus frutescens* +*Vitex altissima* (1.9 cm) and the same has been reflected in the activity index (Table 2 and Fig. 4). Synergistic activity of different combinations of ethanolic extracts of the plants in the study showed different degrees of antimicrobial activity against MRSA and it has been compared with a standard antibiotic vancomycin also reveals the same in the form of activity index.

In the combination of two of the plant extracts, six different combinations were tested against the test bacterium, in which, the combination with *Vitex altissima* leaf extract with any of the other plant extracts showed higher activity. So, it is clear that effective synergistic activity of plant extracts relies on the particular combination of different plants even though any one of the combined plant that showed least activity while used alone. The effectiveness of ethanolic extracts used in combination may be the cumulative effect of active principles in these plants. The plants used in the study had also been studied for various biological activities individually and showed promising results (Table 3).

Table.3 Various biological activities of the ethnomedicinal plants used in the present study

Part used	Activity studied	Reference
***Anamirta cocculus* (L.) Wight & Arn.**		
Fruit extract	Anti-inflammatory activity	Satya and Paridhavi (2013a).
Fruits	Wound healing activity	Satya and Paridhavi (2013b).
***Ichnocarpus frutescens* (Linn.) R. Br.**		
Leaves	Antioxidant activity of polyphenolic extracts	Kumarappan et al. (2012a).
Leaf powder	Modulatory effect of polyphenolic extracts	Kumarappan et al. (2012b).
Whole plant	Chemomodulatory effect	Stalin et al. (2013).
Leaves	Antiobesity effect	Saravanan and Ignacimuthu (2013).
Leaves	Insulin secretagogue effect	Subash-Babu et al. (2008).
***Strychnos potatorum* L.f.**		
Seeds	Hepatoprotective and antioxidant	Sanmugapriya and Venkataraman (2006).
Seeds	Antiplasmodial activity	Philippe et al. (2005).
Seeds	Antiulcerogenic potential	Sanmugapriya and Venkataraman (2007).
Seeds	Diuretic activity	Biswas et al. (2001).
Seeds	Antihyperglycemic and antioxidant action	Mishra et al. (2013).
Seeds	Anti-diarrhoeal activity	Biswas et al. (1992).
***Vitex altissima* L. f.**		
Leaf extract	Antibacterial activity	Kannathasan et al. (2011a).
Leaves	Mosquito larvicidal activity	Kannathasan et al. (2011b).

These findings are in coherence with the study reported earlier on synergistic activity of six different plants against pathogenic bacteria by Karmegam et al. (2008). Synergistic activity of aqueous and ethanolic extracts of selected plant leaves, in combination of two, three, four, five and six against test organisms ranged from 0-2.8 cm zone of inhibition. The highest ZI of 2.8 cm was observed against *Staphylococcus aureus* in ethanolic leaf extract combinations of *Balanites aegyptiaca* + *Lobelia nicotianaefolia* (Karmegam et al., 2008). Similarly, Prakash et al. (2006b) reported that the ethanolic leaf extracts of *Catharanthus roseus*, *Lawsonia inermis* and *Chrysanthemum odoratum* showed least activity against methicillin resistant *Staphylococcus aureus* (MRSA) when used individually. Whereas, the combination of these three plant-extracts exerted a higher activity of 26 mm zone of inhibition followed by *Catharanthus roseus* +

Lawsonia inermis (2.3 cm) and *Lawsonia inermis* + *Chrysanthemum odoratum* (2.0 cm) extract combinations against MRSA. Recently, Karmegam et al. (2012) showed that the zone of inhibition of 2.7 cm was observed in ethanolic leaf extract combination of *Cassia auriculata* + *Cissus quadrangularis* against *Staphylococcus aureus*. All these studies suggest that the combination of leaf extracts of different plants exert enhanced activity against selective bacteria that may be useful for the development of a new and effective antibacterial drug.

Conclusion

Synergistic activity of different combinations of ethanolic extracts of the plant species showed different degrees of antimicrobial activity. Among different combinations tested, the combination of all the four plant extracts showed enhanced antimicrobial activity with a maximum inhibition zone of 2.6 cm against MRSA. There is a scope to use ethanolic extracts of the leaves of *Anamirta cocculus*, *Ichnocarpus frutescens*, *Strychnos potatorum* and *Vitex altissima* in combinations against MRSA.

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