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Synergistic antibacterial activity of the mangrove plant leaf extracts and non mangrove medicinal plant seed extracts against the multi drug resistant bacterial human pathogens

Harish Thumulla[1,*], Krishnamoorthy[1], S.V.Meenakshi[2], Udhaya[3] and Natarajan[3]

[1]Department of Microbiology, Katuri Medical College, Andrapradesh.

[2]Department of Biotechnology, Karpaga Vinayaga College of Engineering and Technology, Affiliated to Anna University, Tamilnadu.

[3]Department of Microbiology, Rajah Muthiah Medical College, Annamalai University, Chidambaram, Tamilnadu.

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ABSTRACT

This study was aimed to evaluate the synergistic antibacterial activity of the mangrove species Avicina marina and Rhyzophora apiculata leaf extracts tested with the non mangrove South Indian medicinal plants, Terminalia chebula, Punica granatum, and Vitis vinifera seed extracts against the multi drug resistant (MDR) bacterial isolates of clinical origin. The antibacerial synergistic activity of the chosen plant extracts were studied by agar well diffustion method. Compared to the single plant extracts, the antibacterial activity expressed by the combination of the Mangrove leaf extracts with the non mangrove medicinal plants seed extracts found to be greater due to the synergism . We recomond the T.chebula seed extract , which can be given / taken along with the mangrove plant leaf material to treat the ailments / drug resistant bacterial infections. We also suggest that the traditional medicine system can adapt the practice of giving the combination of the discrete plant materials / extracts to treat the bacterial infections especially to treat the drug resistant infections to achieve the success in their system of medical practice.

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Introduction

The extensive use of antibiotics results with the prevalence of drug resistance and creates problem while treating the bacterial infectious diseases. For the past few years, the interest of the researchers at global level turned towards the study of microbial drug resistance and related remedies (Bonjar 2004). The increasing use and misuse of the antibiotics result in antibiotic resistance among the bacterial strains and it was assessed and unanimously acknowledged by all researchers and scientists, and medical professionals. The rapid increase of microbial drug resistance pushing us to search for new remedies and to develop new innovative and novel antimicrobials.

The use of numerous medicinal plants to treat or cure the human diseases is one of the traditional practice among many countries for centuries together (Duraipandiyan et al 2006). According to the World Health Organization, about 80% of world population rely chiefly on plant based traditional medicine for their primary healthcare need (WHO). It become must for us to screen the anti microbial activity of the plant parts not only to create the scientific records of such qualities but also to translate these plant products as remidies for the drug resistant infections.

All over the world, the extracts of the different parts of the mangrove and mangrove associates have been used to treat various types of infections (Abeysinghe et al 2006). The stem of Avicinia marina used to treat ulcers. *Rhizophora apiculata* and *R.mucronata* have been used as alternative medicine to treat various diseases and they are considered to have astringent, antidiarrhoea, antiemetic and haemostatic properties (Kokpol et al 1990). Larvicidal and antiviral properties of these species are also reported [Kathiresan et al 1998]. Numerous research publications are available regards with the antimicrobial activities of the mangrove plant extracts (Nabeel et al 2010) and other south Indian medicinal plant extracts (Bandaranayake 1998).

Terminalia chebula is one of the most familier and important medicinal plants used in the traditional medicine for various types of Diseases. This is also called as black myrobalan a king of medicine. It is always listed first in the list of Ayurvedic Materia medica due to its extraordinary healing power (Anwesa et al 2013). The different parts of Terminalia chebula found to have different types of medicinal properties which includes the antimicrobial activity (sato et al 1997, Malckzadehet al 2001, Rani et al 2004 kim et al 2006, carounanidy et-al 2007, Parekh and chand a 2008, kannan et al 2009, Deepak et al 2010).

T .chebula is having many aptitudes and having a wide spectrum of pharmacological and medicinal activities. This south Indian medicinal plant possess different types of bioactive compounds with various chemical structure. Very little work has been done on the plausible medicinal applications of this plant against the diseases particularly on multidrug resistant bacterial pathogens. Hence extensive investigation is needed to exploit their therapeutic ability to combat diseases including drug resistant infections (Anwesa et al 2013).

Tele:
E-mail address: udaviarokya@gmail.com

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Vitis vinifera is well known for their high levels of antioxidants and polyphenols and have also found to have antimicrobial activity (Brown et al., 2009), anti-cancer properties (Mertens-Talcott et al., 2006), anti-inflammatory activity (Greenspan et al., 2006) and antimicrobial activity against *Escherichia coli* O157:H7 (Kim et al., 2009) salmonella (ref). Study reports shown the anti H. pylori, activities of Vitis vinifera seed extracts (Brown et al 2009). Numereus studies have been performed on the health promoting and antioxidant effects of Vitis vinifera.

P.granatum, commonly known as pomogranate, have its historical and traditional values. It is used in several systems of medicine for a variety of ailments. Its therapeutic and medicinal values had been published in the past decades. The synergistic act of P.granatum has also been recognised (Jurenka 2008). Many publications indicating the wonderful potential effects of pomegranate including bactericidal, antifungal, antiviral, immune modulation, vermifuge, stimulant, refrigerant, astringent, stomachic, styptic, laxative, diuretic and anthelmintic (Meléndez et al 2006, Duraipandiyan et al ,2006, Lansky et al 2007, Braga et al 2005, Reddy et al 2007).

Compare to the scientific literatures published on the antimicrobial activity of the individual mangrove plants and the non mangrove south Indian medicinal plants, especially the seed part, the research publications related to the synergistic antibacterial activity of the mangrove plant extracts with other non mangrove medicinal plant extracts seems to be rare. In this situation we aimed to do study on the synergistic antibacterial activity of the mangrove plant species A.marina, R.apiculata leaf extracts combined with the non mangrove south Indian medicinal plants T.chebula , V.vinifera, and P.granatum seed extracts against the multi drug resistant (MDR) human bacterial pathogens of clinical source inorder to bring out the hidden values of these plants combinations.

Materials and Methods

Mangrove plants and parts used in this study

The mangrove plants species, Avicinia marina and R.apiculata leaf parts and the non mangrove south Indian medicinal plant species Terminalia chebula , Punica granatum, and Vitis vinifera seeds were chosen to prepare the extracts. The extracts were studied for the synergistic antibacterial activity against the multi drug resistant bacterial strains of the clinical source (fgi.1).

Mangrove plant preparation

The crude extracts of the mangrove leaves were prepared by the method of Meenakshi et al 2015 (Ph.D scholar, Marine biotechnology, C.A.S in Marine biology, Annamalai University), with modifications. The mangrove leaves were collected from the Pichavaram sea shore in a clean dry plastic bags and transferred to the laboratory. The leaves were plucked out from the branches and immediately washed three times with the tap water then three times with boiled-warm water in order to remove the adhered dust particles on the leaves. To obtain the better wash, the leaves were rotated in the water containing vessel, by clockwise and anti-clockwise movement. Then the leaves were weighed (1 kg each species) and crushed into small pieces and placed in separate glass bottles and subjected to dry for three days as follows. First day under the sunlight, second day under shade dry and the third day at 57°C for six hrs.The dried leaves were made as powder with the help of electronic mixer and kept in a closed container till it subjected to the extract preparation.

Non mangrove south indian plants seed extracts

The non mangrove south Indian medicinal plants, Terminalia chebula, Punica granatum, and Vitis vinifera were chosen and their seed parts was used to prepare the extracts. The seed powder was shared by the Ph,d scholar, Department of microbiology, Faculty of science, Annamalai University.

Extract preparation

Each 30 grams of the three different non mangrove medicinal plants seed powder and two different mangrove plant species leaf powder, were placed in a separate glass container, contained 100 ml of ethanol, The mixed content was placed in boiling water for 1 hr, and overnight at 4°C, then it was filtered by sterile what man no, 1 filter paper. The filtrate was finally reduced to 3 ml by evaporation, and this was considered as **Master extract solution** and stored in a sterile container and stored in the refrigerator.

Invitro synergistic antibacterial test

Extracts preparation for invitro synergistic antibacterial activity test

Every time just before do the invitro synergistic antibacterial test, the desired plant extract combinations (table.1) had been prepared by mixing equal quantity (1 ml each) of the individual plant extracts which was already stored in the refrigrator (Master extract solution) and used in the invitro test.

Bacteria tested and inoculum preparation

The human pathogenic bacteria isolated from different clinical specimens such as urine, pus, sputum, feces, wound swab, vaginal swab and indwelling medical devices were used in this study to check the antibacterial synergistic activity of the mangrove leaf and non mangrove medicinal plants seed extracts. All these bacterial strains were multidrug resistant (MDR) strains, had shown resistance to 5 or more than five individual drugs. Both gram positive and gram negative bacterial strains were included in this study (table.1). Totally (07) seven different bacterial species and (35) thirty five bacterial strains (5 strains from each species) were tested for the synergistic anti bacterial activity. Bacterial pathogens were maintained as stock cultures in a nutrient agar slopes and blood agar slopes in case of Str.pyogenes and pnemococci, and stored in the refrigerator till it get used.

Bacterial inoculum standardization

Prior to every invitro synergistic antibacterial assay, the bacterial stock cultures were subjected to subculture in the Brain Heart Infusion Broth (BHIB). For this a loop full of bacterial stock culture was taken and inoculated into the tube containing 1ml of BHIB and incubated at 37°C for three hours and the suspension was adjusted to Mac Far land opacity 0.5 which equvals to 10-8 cells /ml and used in the in vitro synergistic antibacterial test.

Agar well diffusion method

Agar well diffusion method was used to test the plant extracts for the synergistic antibacterial activity. For the primary synergistic actibacterial activity screening, to test both mangrove plants and non mangrove medicinal plants extracts', the innovative technique introduced by Meenakshi et al 2015, was followed. Briefly 124 mm sterile petri plate was used instead 90 mm petri plate. 30 ml sterile Muller Hinton agar was poured. After solidify, 25 micro liter of the standardised bacterial inoculum was delivered and it was uniformly spread on the agar plate. With the help of sterile micro tip, 4 mm wells were made. The cut wells were labled at back side of the petri plate. The known quantity (30 micro liters) of the

individual as well as the desired combination of the extracts were delivered to the wells separately. The inoculated plates were incubated at 37°C for 24 hrs under aerobic incubation and the synergistic antibacterial activity was assessed.

Synergistic antibacterial activity

Effect of the mangrove plant leaf extract with non mangrove medicinal plant extracts was assessed by measuring the diameter of the zone of inhibition (ZOI) around both wells which contains single the mangrove plant leaf extract and single non mangrove plant extract alone, and comparing with the diameter of the zone of inhibition formed around the well which contains both mangrove plant leaf extracts and the non mangrove medicinal plant seed extracts (table.1 &fig.2). If the measure of the diameter of the zone of inhibition around the well which contains the mixture of the two discrete extracts was exceeding the diameter of the zone of inhibition formed by the individual plant extracts, it was considered as positive for the antibacterial synergistic activity.

Antagonistic antibacterial activity

Either the diameter of the zone of inhibition formed by the plant extract combinations measures less than the diameter of the zone of inhibition formed by the individual plant extracts, or absence of the ZOI, was considered as antagonistic anti bacterial activity.

Results

Antibacterial activity of the individual mangrove and the non mangrove plant extracts

The results of individual and combination of the mangrove plant species leaf ethanolic extracts and non mangrove plants seed extracts against the multi drug resistant (MDR) bacterial pathogens is presented in tables 1 and 2, fig.2 & 3. The individual extracts of A. marina was not acted against all gram positive MDR bacterial strains we tested. While it acted against all gram negative MDR bacterial strains. The maximum ZOI formed by A.marina was recorded as 3mm. The R.apiculata extract failed to act against all Str.pyogenes and Pneumococcus MDR strains. 1mm ZOI was recorded with S.aureus and Lactobacilli.The maximum ZOI formed against the gram negative bacterial isolates was measured as 5mm.

The maximum measure of the ZOI formed by the non mangrove south Indian medicinal plant seed extracts was recorded as 32mm, 18mm, and 5mm by T.chebula, V.vinifera and P.granatum respectively (table.1).

Synergisstic antibacterial activity among mangrove plant extracts plant

The combination of the A.marina and R.apiculata leaf ethanolic extracts expressed their synergistic antibacterial activity on both the gram positive and gram negative MDR bacteria. However it had shown minimum and the maximum of its synergistic antibacterial activity and the ZOI measure ranged between 1 mm to 10 mm diameter.

Synergisstic antibacteril activity among mangrove plant extracs with non mangrove medicinal plant seed extracts

The combination of the A.marina leaf extracts and T.chebula seed extracts expressed their synergistic antibacterial activity equally against the gram positive and gram negative bacteria and the maximum zone of inhibition formed was measured as 33 for the gram positive and 35mm for gram negative MDR bacterial strains.The combination of the R.apiculata leaf extracts and the T.chebula seed extracts also acted on the gram positive and gram negative MDR bacteria. Their synergistic antibacterial activity was recorded as maximum 35mm .

The mixed extracts of A.marina and V.vinifera formed ZOI and the measure was ranged as 5mm to 15 mm and 3 mm to 9 mm for gram positive and gram negative bacteria respectively. For R.apiculta plus V.vinifera combination, the ZOI was ranged between 8mm, to 15 mm and 8mm to 18mm. Very minimal synergistic antibacterial the as well as antagonistic antibacterial activity was expressed by the A.marina plus P.granatum and synergistic antibacerial activity was recorded as maximum 5mm ZOI in dia meter. R.apiculata plus P.granatum extracts combination shown the active synergism to the gram negative bacteria than the gram possitive bacteria. The maximum ZOI formed by this compination was recorded as 6 mm and 15 mm to the gram possitive and gram negative bacteria respectively.

Fig 1 . multi drug resistant Staphylococcus aureus.

Fig 2 . Innovative methodology - used large petri plate (124zmm) to sreen the synergistic antibacterial activity of the manrove and non mangrove plant extracts. With one plate tested 16 extracts (Meenakshi etal 2015).

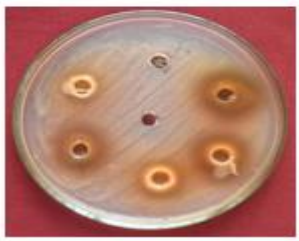

Fig 3 . Individual extracts of A.marina & R.apiculata (top and bottom well) formed 1mm and 5mm zone of inhibition.Combination with T.chebula extract (right bottom - R.apiculata + T.chebula and top well – A.marina + T.chebula) formed 35 mm and 33 mm zone of inhibition due to their synergism.

Discussion

The increasing use and misuse of the antibiotics result in antibiotic resistance among the bacterial strains and it was assessed and unanimously acknowledged by all researchers and scientists, and medical professionals. The rapid increase of microbial drug resistance pushing us to search for new remedies and to develop new innovative antimicrobials.

Abdelraouf et al 2011, studied the synergistic effect of the medicinal plant extracts against human pathogens. It is well understood that the methodology described by them involves time consuming while testing more number of extracts.

Table 1. Synergistic and the antagonistic antibacreial activity of the mangrove plants leaf extracts along with the non mangrove medicinal plants seed extracts

S. No	Plant extracts	Zone of inhibition (mm in diameter)								
		S.aur	Str.pyo	Pneumo	Lacto	Kleb	E.coli	V.cho	S	A
1	A.marina	-	-	-	-	1	3	2	*	*
2	R.apiculata	1	-	-	1	5	5	3	*	*
3	A.marina + R.apiculata	4s	1	1	2s	10s	1A	9s	+	+
4	T.chebula	30	28	25	25	32	31	26	*	*
5	A.marina + T. Chebula	35S	30S	30S	29S	33S	35S	30S	+	-
6	R.apiculata +T.chebula	33S	32S	33S	30S	35S	35S	33S	+	-
7	V.vinifera	12	2	2	1	18	12	9	*	*
8	A.marina + V.vinifera	15S	8S	5S	12S	9A	8A	3A	+	+
9	R.apiculata V.vinifera	15S	12S	8S	6S	18S	12S	8S	+	
10	P.granatum	-	-	-	-	3	5	1	*	*
11	A.marina + P.granatum	-	-	1	1	2	3	5	+	+
12	R.apiculata +Punica granatum	6	-	1	1	15	8	3	+	-

* Single extracts not applicable for synergism and antagonism results

S- synergistic act – A - Antagonistic act, S.aur-Staphylococcus aureus --Str.pyo – Steptococcus pyogenes, Pneumo – pneumococcus , Kleb – Klebsiella , E.coli – Escherichia coli, V. cho – Vibrio cholerae

Table 2 .The best extract combination shown the synergistic antibacerial ativity against human pathogenic MDR bacreial strains (zone of inhibition –mm in dia)

Sl. No.		Gm+bacteria	Gm-bacteria
1.	A.marina +R.apiculata	4	10
2.	A.marina +T.chebula	32	35
3.	R.apiculata+T.chebula	35	35
4.	A. marina+V.vinifera	15	09
5.	R.apiculata+Vitis vinifera	15	18
6.	A.marina+P.granatum	1	05
7.	R.apiculata+P.granatum	6	15

* - The best extract combination acted against the gram +ve and gram –ve MDR bactreia

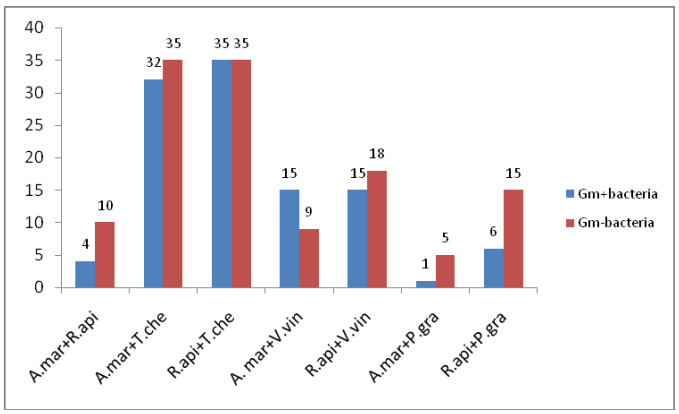

Fig .4

In our study, in addition to the traditional agar well diffusion method, to screen and to compare the synergistic activity of the plant extracts, we have used the innovative technique introduced by Meenakshi Annamalai, 2015, PhD scholar, Department of Marine biotechnology, C.A.S in marine biology, Annamalai University. We could experienced the advantage of the above technique by using the large pertidish (size 124 mm, Fig.2). We felt it is less time consuming, easy to perform and economic also while testing more number of the extracts.

The non mangrove south Indian medicinal plant T.chebula and V.vinifera and P.granatum were the plants of our choice to study the synergistic antibacterial activity along with the mangrove species A.marina and R.apiculata, since these plant parts found to have the bioactive compounds proven to have antimicrobiol effect and also found to be non toxic to human. The oral intake of mangrove plants A.marina and R.apiculata is found as non toxic for the cattle, and human and for the marine fishes. The informations gathered from the villagers living near the mangrove forest of the Pichavaram seashore reveals the fact of the use of these mangrove plant leaves for various purposes mainly for their health ailments to get relief.

Ethyl acetate and ethanolic mature leaf extracts of *A. marina* was found to be effective to control clinical isolate of *E. coli* growth. Petroleum ether and chloroform extracts of *A. marine* failed to exhibit inhibition against same tested organism (Abheysinghe *et al.*, 2006). In our study we have used ethanolic extracts to test the synergystic antibacterial activity of the selected plant species, since the ethanolic extracts of these individual plants shown its maximum antibacterial activity than acetone , methanol, and ethyl acetate extracts.

The mangrove plant species A.marina and R.apiculata leaf enhanolic extracts when individually acted against the MDR bacterial strains, R.apiculata had shown its better antibacterial activity (mm) than A.marina (mm). Both of these mangrove species leaf extracts acted on the gram possitive and gram negative MDR bacterial strains. The maximum antibacterial activity of the individual extracts of A.marina and R.apiculata was recorded in terms of ZOI and the average score of the ZOI was measured as 3mm and 5mm respectively (table.1). From this we can suggest that the translation of these plant extracts for the use to treat the MDR bacterial infections, is felt essential and the plant extracts combination which shown to have antagonism may be identified and avoided. Further it is felt essential to perform the extensive study about the separation, purification and identification of the bioactive compounds of the same plant species in order to fine the synergism and antagonism of each bioactive compounds of these plants

However, A.marina plus Vitis vinifera, A.marina plus P.granatum extracts combination expressed both synergistic as well as antagonistic antibacterial activity to these gram positive and gram negative MDR bacterial strains. Whereas the combination of R.apiculata and Vitis vinifera had shown only synergism in its act. The mixture of R.apiculata and P.granatum seed extract shown its synergism as well as the additive activity (Table).

It has been found and reported that the fruit of T.chebula, prevents the liver toxicity caused by sub-chronic administration of rifampicin, isonized and pyrazinamide in combination (Tasduq et al, 2006). So the toxicity preventing act of T.chebula is one of the natural shield for the human to prevent the negative effects of the drus. Those thes who are under medication with multiple drugs can have the T.chebula along with that in view if preventing the bad effects of the multiple drus. Pertain to our present study, along with the marine plants leaf extracts, T. Chebula seed extracts shown the maximum synergistic antibacterial activity and it is expecting that the negative effects or the toxicity of the mangrove species A.marina R.apiculata if it possess, that may be nullified by the compination of the T. chebula seed extracts .Future study in this field is felt essential to bringout the hidden values of these plant extracts compination.

In our study it is observed that the synergistic antibacterial activity of the mangrove plants A.marina and R.apiculata leaf with the seed extracts T. chebula, Vitis vinifera, and Punica granatum extract was varied with each bacterial species and it is quite interesting to note that the synergistic antibacterial activity was found to be varied with the MDR bacterial strains of the same species. The synergistic antibacterial activity presented in table 1 and 2 is the average score of the measure of ZOI formed by bacerial strains of the same species. From this we come to know that the synergistic antibacerial activity expressed by A.marina and R.apiculata along with T.chebula, Vitis vinifera and R. apiculata seems to be bacterial strains specific but not species specific.

To best of our knowledge most of the research article published are included single strain of the single species in their studies. In our study we have included each five strains from the same species. And we could able to observe that the difference in their suceptibility pattern to the tested plant extracts. Hence we recomomnd to include multiple strains of the same species while screening / testing for their sensitivity pattern towards any testing agents such as plant extracts , antibiotics , synthetic chemical agents etc.

Abheysinghe *et al.,* 2006 in his study, they were able to record the Combination of various extracts exibited their additive/ antagonistic activity against tested organisms. Most of the extracts in combination showed antagonistic results where as individual of same extract were found to be much effective in their study. In contrast, in our study, we could observe the expresion of the synergistic and additive antibacterial activity of the marine species A.marina, R.apiculata leaf extracts with the non mangrove south Indian medicinal plants T.chebula, V.vinifera and P.granatum seed extracts combination to most of the tested bacterial strains. And the antagonistic effect was exibited to very few bacterial strains.

Conclusion

On the whole from our study results, we conclude that the interaction of the individual mangrove plant extracts or the combination of the different mangrove species A.marina and R.apiculata leaf extracts can act against the MDR bacterial strains at low level. But when they combine with other non mangrove medicinal plants T.chebula,V.vinifera and P.granatum seed extracts , the total antibacterial activity is greater than the antibacterial activity of the individual mangrove plant leaf extracts. And we recomond the T.chebula seed extract , which can be given / taken along with the mangrove plant leaf material to treat the ailments / drug resistant bacterial infections. Further the extensive study is felt essential in order to rule out the bioactive compounds responsible for the synergism and antagonistic antibacterial activity of these plant extracts. And we also suggest that the traditional medicine system can adapt the practice of giving the combination of the discrete plant materials to treat the bacterial infections especially to the drug resistant infections to achieve the success in their system of medical practice.

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