



Synergistic Antibacterial Activity of Terminalia chebula Vitis vinifera and Punica granatum Seed Extracts Against Certain Human Pathogenic Bacteria of Clinical Source

R.Priyadharshini¹, D.Sangeetha¹, V. Udhaya², M.R. Suseela² and HarishThummala³

¹Department of Microbiology, Faculty of Science, Annamalai University, Tamil Nadu, India

²Department of Microbiology, Faculty of Medicine, Annamalai University, Tamil Nadu, India

³Katuri Medical College Guntur, Andrapradesh, India

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ABSTRACT

Three different south Indian fruits' seeds have been selected and subjected to test their synergistic antibacterial activity against certain human pathogenic bacteria of clinical source which includes both gram positive and gram negative bacteria. Both sensitive and MDR bacterial strains have been included in this study. Ethanol and water were used as solvents to extract the seeds. The crude extracts were prepared by the ordinary method. Among that, the ethanolic seed extracts had shown its maximum synergistic antibacterial activity towards the tested bacterial strains and recorded as the most effective extractant. Terminalia chebula, Vitis vinifera and Punica granatum seed extracts were tested singly for their antibacterial activity and also tested for its synergistic antibacterial activity with their paired extract combinations. Among the tested combinations, Terminalia chebula seed extract found to shown its maximum synergistic antibacterial activity with Vitis vinifera seed extract. The least level antibacterial and synergistic antibacterial activity was recorded with the single as well as paired extract with Punica granatum seed extract combinations. From this we conclude that the bioactive compounds present in the seed extracts of Terminalia chebula, effectively acted against the human pathogenic sensitive and MDR bacterial strains, than Vitis vinifera and Punica granatum seed extracts. Further this can be prescribed along with other medicinal choice for treating the bacterial infections, including the drug resistant infections.

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Introduction

The traditional use of plant materials to treat various types of ailments is well known fact, which has been unanimously accepted by the various systems of medicine. The World Health Organization (WHO 2006), estimated 80% of the world population rely chiefly on the plant based medicine for their primary health care. The medicinal plants are the best source to bringout different type of drugs (Anjana et al 2009). For the purpose of safety and efficacy such plants and plant products must be examined and to be studied in view of understanding their properties such as toxicity, allergic properties, antimicrobial efficacy and others.

The antimicrobial properties of many herbal plants have been reported from different parts of the world (Rabi et al 2009, Braun et al 2010). At global level significant percentage of the human population showing interest of using the medicinal plants to control microbial infections due to the awareness of the side effects caused by the synthetic antimicrobials (Martins Ekor, 2013). The problems related to the drug resistant pathogens and its negative impacts on the health is increasingly documented, which in turn leads to the search of new antimicrobials from plants sources. People living in the under developed and developing countries, the poor and socio economically weaker section, who are not affordable for the costly drugs, mainly rely on plants, and plant based treatment.

The use of countless medicinal plants used to treat and cure the different types of human diseases is one of the traditional practices of different countries in the world for centuries together (Duraipandiyani et al 2006). Hence it is essential to screen the antimicrobial bioactivities of the discrete and combinations, of the plant extracts to create the scientific records for their antimicrobial qualities and to translate these plant products as curative agents for the drug resistant microbial infections.

Terminalia chebula (Tamil name- Kadukkai) is one of the most familiar and important medicinal plant fruits traditionally used in the Indian medicine system to treat different types of diseases. It 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 uniqueness of the different parts of Terminalia chebula and their medicinal properties had been documented by many authors and the antimicrobial activity is one among that (Sato et al 1997, Malckzadeh et al 2001, Rani et al 2004, Kim et al 2006, Kannan et al 2009,).

Terminalia chebula found to have many aptitudes and having a wide spectrum of pharmacological and medicinal bioactivities. It has been recorded that the various chemical structures of the different types of its bioactive compounds (Tariq et al 2013).

Though *T.chebula* has been traditionally used to treat different types of infections, very few research works had been performed on the possible medicinal applications of this plant against the diseases particularly on multidrug resistant bacterial pathogens and related infections. Hence extensive research is required to exploit their therapeutic ability to combat diseases including drug resistant infections (Anwesa et al 2013).

Though innumerable studies had been performed with the medicinal plants at global level, still there is a lack of scientific experimental studies in different views at different angle. Compared to the available scientific literatures on the antimicrobial activity of the discrete plant extracts, the study on the synergistic antimicrobial activity of the different plant extracts combinations seems to be less in number. Especially the synergistic, antagonistic, and additive antibacterial activities of the south Indian medicinal plants are yet to be studied in detailed manner. To add up the informations to the detailed profile of the seed extracts of *Terminalia chebula*, *Vitis vinifera*, and *Punica granatum*, to the aspects of the synergistic antibacterial activity, it is felt essential to perform research on the present study topic.

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). Study reports shown the anti *H. pylori*, activities of *Vitis vinifera* seed extracts (Brown et al 2009).

P.granatum, commonly known as pomegranate, in Tamil it is called as Maadullai and have its historical and traditional values. It has been used in several systems of medicine for a wide variety of ailments. The therapeutic and medicinal properties and its values had been extensively studied and published in the past decades. The synergistic bioactivities of *P.granatum* has also been studied (Jurenka 2008). Many research publications indicating the amazing potential bioeffects of the 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).

Compared to the scientific research literatures published on the antimicrobial activity of the individual seed part of *Terminalia chebula*, *Vitis vinifera*, and *Punica granatum*, the research publications related to the synergistic antibacterial activity of these plant seed extracts seems to be rare. In this situation we aimed to study the synergistic antibacterial activity of the south Indian medicinal plants *T.chebula*, *V.vinifera*, and *P.granatum* seed extracts against the human bacterial pathogens of clinical source in view of bringing the hidden medicinal qualities of these plants' seed extract combinations.

Material and methods

Sources of the Medicinal Plants

The three different fruits species named *Terminalia chebula*, *Vitis vinifera*, and *Punica granatum* traditionally used in the Indian medicine systems and folkloric medicine, were purchased from the local fruit shops and from the native herbal shops. In order to improve the efficacy of the plant extracts, the fruits were collected from different places in and

around Cuddalore district, Tamil nadu, pooled and processed further.

Extract Preparation and solvents used

Totally two different solvents ie; ethanol and water were used to prepare the plant extracts. The method of Udhaya 2012 was followed. Briefly each 30 grams of the three different medicinal plants species' dried seed powder were placed a separate glass container, contained 150 ml of ethanol and 150 ml of water. For water extract, the mixed content was boiled for 30 minutes at low flame and then kept for overnight at 4°C, then it was filtered by sterile whatman no.1 filter paper. The filtrate was finally reduced to 3 ml by evaporation, like wise for ethanol extract the mixed content in the glass container was placed in hot air oven at 50°C for 1 hr then it was placed at room temperature for further 2 hrs. In between intermittent shaking and thorough mixing was carried out with sterile glass rod. Then it was filtered through sterile whatman no.1 filter paper and the filtrate was bringdown to 3ml, and this was considered as **Master extract solution** and stored in a sterile container and stored in the refrigerator.

Extracts preparation for invitro synergistic antibacterial activity test

Every time, just before to perform 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 of the Master extract solution and used in the invitro test.

Bacteria tested and inoculum preparation

The sensitive and MDR human pathogenic bacteria isolated from different clinical specimens were included in the invitro experimental study to screen the antibacterial synergistic bioactivity of the seed extracts of *Terminalia chebula*, *Vitis vinifera* and *Punica granatum*. The multidrug resistant (MDR) bacterial strains (shown resistance to 5 or more than five individual drugs) and the sensitive bacterial strains were selected and included. Both gram positive and gram negative bacterial strains were included in this study. Totally (12) seven different bacterial species, (*S.aureus*, *Str. pyogens*, *Pneumococcus*, *Actinomycetes*, *Lactobacilli*, *B.cereus*, *E.coli*, *Klebsiella pneumoniae*, *Proteus*, *V.cholerae*, *Pseudomonas aeruginosa*, *P.fluorescence*), and (120) one hundred and twenty bacterial strains (10 strains from each species) were tested for the synergistic anti bacterial activity. Bacterial isolates were maintained as stock cultures on a nutrient agar slopes and blood agar slopes, in case of fastidious organism and stored in the refrigerator till it used, in the experimental assays.

Bacterial inoculum standardization

Prior to the invitro test, each time, all the bacterial stock cultures were sub cultured 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 MacFarland opacity 0.5 which equals to 10^8 cells /ml and used in the in vitro synergistic antibacterial test.

Synergistic antibacterial activity – assessment

The method of Harish Thummala et al 2016 was used in our study to assess the synergistic antibacterial activity of the seed extracts of *Terminalia chebula*, *Vitis vinifera*, and *Punica granatum*. It was carried out by comparing the zone of inhibition (ZOI) formed around the discs / wells which contains the paired seed extract combination and the disc / wells contains with single extracts.

If the measure the zone of inhibition formed around the paired seed extracts combination exceeding the measure of the diameter of the zone of inhibition formed around the single seed extracts, then it was considered as positive for the synergism.

Antagonistic antibacterial activity - assessment - Harish Thummala et al 2016

Either absence of zone of inhibition, or the decreased measure of the zone of inhibition formed around the discs or wells contains paired extracts, (when compared to the zone of inhibition formed around the single extracts) was assessed as antagonism.

Additive activity – assessment – Udhaya-2016

When there was no change in the original (or) previous measure of zone of inhibition, without adding or decreasing their antibacterial efficacy, then it was considered as additive activity.

Agar well diffusion method

For the primary synergistic antibacterial activity screening, to test both discrete plant extracts and combinations of two discrete seed extracts, the innovative technique introduced by Meenakshi et al 2015, was followed. Briefly, 124 mm, large 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.

Disc diffusion method -The sterile whatman no.1 filter paper disc immersed in discrete plant seed extract and placed on the inoculated plate. And for synergistic antibacterial activity, the paper disc was dipped in the paired extract combination and placed near to the respective single seed extracts. The inoculated plates were incubated at 37°C for 24 hrs under aerobic incubation and the synergistic, antagonistic and additive antibacterial activity was assessed by measuring and comparing the ZOI. **well diffusion method** - With the help of sterile micro tip, 4 mm wells were made. The cut wells were labeled at back side of the petri plate. The known quantity (30 micro liter) 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, antagonistic and additive antibacterial activity was assessed by measuring and comparing the ZOI.

Result and discussion

The random use and misuse of the antibiotics leads to drug resistance, and it was unanimously accepted by all research communities including the medical professionals.

The fast increase of microbial drug resistance lead us to search of new remedies and to develop new innovative and novel antimicrobials, (Harish et al 2016). The increased use of antibiotics results in the emergence of drug resistance and interferes in treating the infectious diseases. For the past decades the researchers at global level involved in the study of microbial drug resistance and related plant based remedies (Bonjar 2004).

Although the plants were used from the earliest time by the people, without the knowledge of their bioactive compounds, the current research world extraction, fractionation, purification, concentration, or the physical and the biological process are to be studied and carried out for any herbal products for the human consumption. 80% of the human population at global level, rely on the plant based drugs for their primary health care either to prevent or to cure the different types of their ailments.

Increased attention has been paid by the researchers to bringout the positive eco-friendly aspects of these plant and plant products for the human use (Manoharan Sivananthan 2013).

Our precious observations and study results proving the synergistic antibacterial activity of the seed extracts which is the most appreciable and can be applicable in the system of medicine to treat the drug resistant bacterial infections. Based on the currently available research publications, we could say that very limited researchers involved in the synergistic, antagonistic and additive antibacterial activity studies of the medicinal plants, that too not extensively involved. Hence it is much more essential to study in detail about the positive qualities of the medicinal plants in view of add up and delete the negative qualities that will helps to carryout the proper application of these plants for medical use and to produce plant based products.

All the three plants seed extracts had found to shown their antibacterial activity individually and with their combinations. It has been already recorded the ethanolic extract of *T.chebula* found to have antibacterial activity against the MDR uropathogenic *E.coli* and the phenolics were found to be responsible for that (Bag et al 2014). *Terminalia chebula*, *Vitis vinifera*, and *Punica granatum* seed extracts expressed their antibacterial bioactivity at different level towards the chosen sensitive and drug resistant/ multi drug resistant, gram positive and gram negative bacterial strains (table 1&2). From this we could understand that, each plant species possess, bioactive compounds which varies in their potency as well as in their phytochemistry.

We feel it would be better of the detailed research study could be performed in the allied field of specialization, to bringout the complete informations about each chemical compounds and its bioactivities, that will help us to understand their special qualities which could be highly beneficial for us. From our study we could able to observe the increased level of antibacterial activity of *Terminalia chebula*, seed extract than *Vitis vinifera*, and *Punica granatum* seed extracts. The maximum measure of the zone of inhibition formed against gram negative bacteria was recorded as 36mm, 11mm, and 2mm respectively (with ethanol extracts in well diffusion method) (table .1), and for gram positive bacteria it was documented as 25 mm, 10mm, and 2mm respectively (table 2).

Table.1 Synergistic Antibacterial activity of Terminalia chebula, Vitis vinifera, and Punica granatum seed extracts against gram negative bacteria (average score).

Sl. No.	Seed extracts	Zone of Inhibition			
		Disc diffusion method		Well diffusion method	
		Water	Ethanol	Water	Ethanol
1.	<i>Terminalia chebula</i>	8-21	10-28	10-30	20-36
2.	<i>Vitis vinifera</i>	0-2	0-4	0-4	2-11
3.	<i>Punica granatum</i>	Nil	0-Trace	0-Trace	0-2
4.	T.c + V.vini	5-28*	5-18*	10-36*	18-38*
5.	T.c. + P.gra	8-21•	8-28•	10-28■	10-36•
6.	V.vini + P.gra	0-2•	4-6*	4*-7*	4-18*

* - Synergism, • - Antagonism, ■ - Additive activity

Table 2. Synergistic Antibacterial activity of Terminalia chebula, Vitis vinifera, and Punica granatum seed extracts against gram positive bacteria (average score).

Sl. No.	Seed extracts	Zone of Inhibition			
		Disc diffusion method		Well diffusion method	
		Water	Ethanol	Water	Ethanol
1.	Terminalia chebula	6-18	10-20	13-25	18 -25
2.	Vitis vinifera	Trace	0-1	0-2	1-10
3.	Punica granatum	Nill	Nill	0-Trace	0-2
4.	T.c + V.vini	2-20*	2-11■	5-26*	18-33*
5.	T.c. + P.gra	6-8■	6-20■	8-25■	10-25■
6.	V.vini + P.gra	Nill	0-trace●	0-2■	4-6●

* - Synergism, ● - Antagonism, ■ - Additive activit

However the antibacterial activity of the seed extracts of *Punica granatum* had been recorded (Hemant et al, 2014). From this we can understand that the difference occurred in the results pertain to their antibacterial bioactivity expression is not related to the potency of the extracts but, the probable reason could be the difference in the susceptible pattern of the bacterial pathogens to these extracts. In this situation it is advisable to perform extensive and elloborative research study in this field of specialization, to conclude the actual qualities/properties of these plant seed extracts.

According to Tariq 2012, the seeds of *Terminalia chebula*, seeds possess phenol, reducing sugar, flavinoides, tanic acid, terpenoid, protein. *Punica granatum* seed and its phytochemistry was studied by Satheesh Kumar et al 2012. Though the phytochemicals of these seed extracts have been studied at satisfactory level, still, it lacks in the multidimensional aspects especially medical aspects. The least and even nill activity was recorded with ethanol extract of *Punica granatum* seed extracts.

Synergistic and antagonistic – antibacterial activity

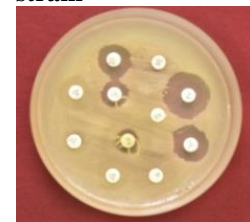
It was possible for us to document the increased level antibacterial activity of these seed extracts, when they combined together and acted against the subjected human bacterial. The reason is nothing but the synergism exists with these seed extracts. However the antagonistic as well as the additive antibacterial activity of these seed extracts also had been documented with the combination of these three seed extracts (table.1 & table. 2). On the whole the best synergism was expressed by the *Terminalia chebula*, and *Vitis vinifera* seed extracts combination and the maximum measure of the zone of inhibition was recorded as 38mm and 33mm with respective to the gram negative and gram positive bacteria. These particular extract combination did not shown antagonism towards its antibacterial bioactivity to gram negative and gram positive bacteria. From this we suggest that the detailed study pertain to the positive and negative aspects of the plant based bioactive compounds should be performed with increased numbers of the plant extracts with increased number of bacterial strains. The antibacterial activity of the ripened seeds of *T. chebula* had been recorded against *Staphylococcus aureus* (Bonjar et al 2004). *T. chebula*, *V. vinifera*, and *P.granatum* are the famous fruits and the most versatile south Indian medicinal plants possess a wide spectrum of pharmacological and medicinal properties.

Figure. 1
Sensitive strain



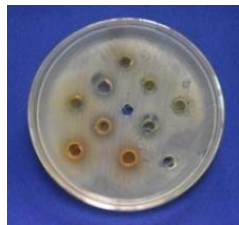
Staphylococcus aureus
isolated from pus specimen

Figure . 2
Multi drug resistant strain



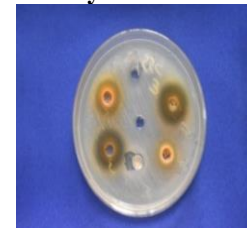
Staphylococcus aureus
isolated from pus specimen

Figure . 3
Discrete Ethanolic extracts



T.chebula, P.granatum & V.vinifera seed extracts - antibacterial activity against E. Coli isolated from Urine specimen

Figure . 4
Synergistic antibacterial activity



T. chebula, P.granatum & V.vinifera ethonolic seed extracts, combinations and its synergistic antibacterial activity against Klebsiella isolated from sputum specimen

These fruits and fruit parts are the unique sources of different kinds of bioactive compounds, having diverse chemical structures. Although they have a number of medicinal and pharmacological properties (Khan et al, 2009), so far, very little research work has been performed on their medicinal applications, especially the synergistic, antagonistic, and additive activity of these three plant seed extracts combination against the sensitive and MDR bacterial pathogens (Anwesa et al 2013).

We proud to state here our study is the first study in which, we could able to explore some valuable informations about the synergistic antagonistic and additive antibacterial bioactivities of the seed extract combinations of *T. chebula*, *V. vinifera* and *P. granatum*. Whereas the *Terminalia chebula*, and *Punica granatum* seed extracts combination expressed only antagonism and additive antibacterial activity where as no additive activity but both antagonistic and synergistic activity was observed from the combination of the *Vitis vinifera* and *Punica granatum* seed extracts (table .1, Fig. 1 & 2). Overall the high level synergistic antibacterial activity was recorded from the *T. chebula*, and *V. vinifera* seed extracts combination and low level antagonism and additive antibacterial activity was expressed by the seed extracts of *P.granatum*, and *V. vinifera*. While no antagonism was expressed by *T.chebula* and *P.granatum* seed extracts.

Conclusion

From our three years research study observations, we conclude that the seed extracts of *T. chebula*, *V.vinifera*, and *P. granatum* had shown their antibacterial activity against the

sensitive and drug resistant, even against the multi drug resistant (MDR) human pathogenic bacterial strains.

The increased antibacterial efficacy of these plant seed extracts had been noted with the combination of paired discrete extracts, due to the synergistic activity of the bioactive compounds of the seed extracts of *T. chebula*, *V. vinifera*, and *P.granatum*. This will help us to bring out the good quality plant based products for human consumption to treat or to prevent the bacterial infectious diseases, includes drug resistant infections.

However the antagonism and additive antibacterial activities have also been recorded with these plants seed extracts and the bioactive compounds that express these negative aspects should be further studied in detail and that may be avoided while preparing the plant based products as remedies for human consumption. Hence the researchers are welcome to perform such studies in future.

From our study results we can suggest to utilize the *T.chebula* as good co- remedy and can be given along with other plant remedies to treat the bacterial infections including the drug resistant infections and make practical and effective use of its synergism.

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