



Synergistic Antiadherence Bioactivity of *Terminalia Chebula* and *Catharanthus Roseus* Ethanol Extracts against Human Pathogenic Bacteria of Clinical Source

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ARTICLE INFO

Article history:

Received: 11 November 2017;

Received in revised form:

6 December 2017;

Accepted: 15 December 2017;

Keywords

Microbial adhesion,
Innovative Dual Invitro Model
System.

ABSTRACT

Microbial adhesion is an initial and essential step for the attachment of microbes to the host tissues. Adhesion is considered as one of the microbial virulence markers. In view of finding plant based solution to prevent the bacterial adhesion, the present study was undertaken. Invitro experimental study was conducted to screen the antiadherence bioactivity of *T. chebula* and *C. roseus* extracts. Both gram positive and gram negative bacterial strains were included in this study. Tube from urine collection bag was used as substratum in the invitro study. Innovative Dual Invitro Model System (IDIMS) was used to perform the experiments. Tested specimens were collected and screened under light microscope. Specimens were also subjected to culture and CFUs were counted. The reduced number of bacterial cells in the specimen was recorded from the light microscopic study, and from specimen culture. Decreased number of CFUs were noted. From this we conclude that the *T. chebula* and *C. roseus* extracts (singly) as well as extract combination possess antiadherence property. Hence we suggest these two plant extracts, singly or their combination can be used as antimicrobial agent to prevent the adhesion of the human pathogenic bacteria to the objects. However further established research in this field is felt essential to bring out new informations and to support our study reports.

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Introduction

Bacterial adhesion

Adhesion is the process that helps bacterial attachment to any surface. Adhesion is an important and initial step for the colonization of the microbes to the host or other nonbiological environments, which can contribute to the bacterial or microbial pathogenesis and related outcome. Microbial adhesion, coadhesion and anti adhesion studies would carry significant clinical implication. Interms of preventive measures should be taken to avoid the initial steps involved in the pathogenesis of the microbes.

The World Health Organization realized the importance of the medicinal herbs and their use in traditional medicine. Therefore, WHO actively creating strategies, guidelines and standards for the medicines of botanical source (WHO 2002). The positive qualities of the medicinal plants have been acknowledged by many authors (Kaushik et al 2002, Aliyu et al., 2007) and they are highly appreciated.

Based on the different types of mechanism and their function, the organic chemicals known as plants secondary metabolites, classified as chemotherapeutic agents and antimicrobials (bacteriostatic and bactericidal agents). Therefore the scientific community at global level leads to develop new better drugs against these microbial infections, especially against the drug resistant infections (Parekh and Chana, 2007). In this situation it is felt essential to search and discover new antimicrobials which are the most effective to treat the drug resistant pathogens.

The different types of medicinal properties of the *C.roseus* had been documented. The antibacterial (Muhammad et al., 2009) antifungal (Jaleel et al., 2007) and the antiviral (Farnsworth et al., 1968) had been already studied. *Terminalia chebula* (Retz.) is a south Indian medicinal plant, called the "King of Medicine" in Tibet and is always listed first in the list of Ayurvedic Materia Medica because of its extraordinary healing power. Anwesa et al (2011) found out the combination effect of *T. chebula* fruit extract with some selected routinely used antibiotics against multidrug-resistant uropathogenic *E. coli* in a view to elucidate their possible synergistic activity.

The involvement and the interaction of the gram positive and the gram negative bacteria for their synergistic colonization mechanism was extensively studied (Holmes et al 1996, Jenkins et al 1990, Hogan & Kolter 2002). Keeping all these points in view, in this present study, we included drug resistant poly pathogenic bacteria to test the antiadherence property of the *T. chebula* and *C. roseus* ethanolic extracts.

Material and Method

Plant extract preparation

The method already we have used and published earlier (Priyadharshini et al 2016) has been used to prepare the extracts from *T. chebula* seed and *C. roseus* leaves. Each individual concentrated extracts (30 ml) were taken and mixed with sterile distilled water (30 ml) separately and used

in the test. For extract combination each diluted (30 ml) individual extracts had been mixed and it was used.

Bacterial isolates used in this study

The gram positive bacteria *Staphylococcus aureus*, *Lactobacillus species*, and the gram negative bacteria *E.coli*, *Klebsiella*, has been included in our study. They all were isolated from different types of clinical specimens such as sputum, pus, vaginal swabs, urine, faces oral swabs, and indwelling medical devices such as drain tip, endotracheal tubes and urinary catheters. Three different bacterial strains from each bacterial species (totally 12 isolates) were included. A loopfull of 24 hrs fresh culture of all these bacterial strains were mixed with 10 ml of sterile distilled water and adjusted to Mac Farland opacity no 0.5. This standardized bacterial inoculum was used in the experimental study.

The invitro anti adherence test – static method

The plastic tube from the urine collection bag was cut into 1 inch pieces with multiple numbers. Each three pieces of these tubes were placed in large sterile test tube contained 15 ml of sterile Brain Heart Infusion Broth (BHIA). According to the need, multiple test tube sets were used to test the multiple bacterial strains and plant extracts. Three ml of the (test extract) South Indian medicinal plant extract (either single or paired combination) was added to the conical flask. The known quantity (10 micro litre) of the standardized bacterial inoculum was added to the BHIB. The control sets were also tested the poly bacterial suspension strains without the addition of plant extracts. Then it was incubated for 3 hrs at 37 °C.

After 3 hrs the fluid in the test tubes were aspirated by sterile Pasteur pipette and discarded. Further the 1 inch cut tubes were washed three times with Phosphate Buffered Saline (PBS). With the help of sterile forceps and scalpel, the internal content in the cut tubes were scraped gently and tested for the presence of poly bacterial pathogens. All these steps were carried out in the laminar flow chamber. Smears were prepared from each specimen. The smears were stained with crystal violet and examined under light microscope for the adherent bacterial cells.

Invitro antiadherence – Interpretation

Static method

Light microscopic study

Both the control specimens and plant extracts tested specimens were examined for the number of the adhered bacterial cells. When compare to the control specimens, if reduced number of the adhered bacterial cells found in the test specimens added with the single plant extract / or paired extract combinations of the *T. chebula* and *C. roseus* plants then it was considered as antiadherence bioactivity of that particular extract / extract combinations.

The number of adhered bacterial cells were graded as occasional (1-5 bacterial cells/100x), few bacterial cells (10-30/100x), many bacterial cells (50-100/ 100x), plenty / numerous, and crowded bacterial cells (bacterial cells not able to count). It was assessed as absent when there was no appreciable bacterial cells in the microscopic field. In addition to this the disintegrated bacterial cells broken cells or denatured cell morphology has also been noted and recorded.

Invitro antiadherence -Dynamic Flow Method

Innovative Dual Invitro Model System (IDIMS) – used in this study

The Innovative Dual Invitro Model System (IDIMS) was constructed by Meenakshi 2013, Ph.D scholar,

Department of Biotechnology and the Department of Electrical and Electronic Engineering (EEE), Karpaga Vinayaga College of Engineering and Technology, Chinna Kollampakkam, Tamilnadu, India. IDIMS is a innovative dual experimental model system which was used to study both antibiofilm antiadherence bioactivity of the plant extracts. Static and flow method was carried out with this single experimental model. Hence it was named as IDIMS by the Ph.D. scholar Meenakshi 2013. This IDIMS experimental model was used in our study to perform dynamic flow antiadherence bioactivity of the test extracts.

The invitro dynamic flow method was performed by using IDIMS to study the anti adherence bioactivity of the *T.chebula* and *C.roseus* extracts. Sterile 250 ml conical flask contained 100 ml of BHIB was taken. The standardized poly bacterial inoculum (50 microliter) and 20 ml of the prepared plant extract was added to the content in the conical flask. Finally 3 pieces of the 1 inch cut tubes were placed to the content. The mouth of the conical flask was closed by the plastic crock which contains two holes. These holes were connected to the inlet and outlet tubes (plastic tubes obtained from the urine collection bag) that was connected to the regulator power supply (fig. 1).

The IDIMS was allowed to run by given connection to the regulator power supply adjusting the speed level 9.8. The fluid content in the conical flask was allowed to run through the outlet and inlet tubes for 3 hours. After that, the electrical connection was disconnected. Then the cork was carefully removed from the conical flask. The 1 inch cut tubes in the fluid was taken out and placed in the sterile test tube contained 30 ml of PBS. It was washed for three times by adding fresh PBS each time. After washing, the internal content in the 1 inch cut tube was scraped. One loop full of the collected specimen was placed in a tube contained 0.5 ml BHIB. It was vortexed for 3 minutes to get homogenised. Smears were made from homogenised specimen and was subjected to simple stain with crystal violet and examined under light microscopy. 10 microliters of the homogenised specimen was cultured on Brain Heart Infusion Blood agar, under aerobic incubation for 24 hrs. The CFUs were counted and the antiadherence bio activity of the plant extracts was evaluated.

Dynamic flow method–specimen culture - CFUs and Interpretation

The invitro dynamic flow antiadherence test interpretation was carried out by counting and evaluated the bacterial Colony Forming Units (CFUs) formed by the tested specimen culture (which were collected from the specimens added with and without the test plant extracts). When reduced number of the colony forming units formed (CFUs) by the test specimens, than the control specimens it was interpreted as positive for the antiadherence bio activity of that particular plant extracts.

Results and Discussion

T. chebula seed extract treated specimens showed bacterial cells ranged between nil to 5 – bacterial cells / 100x. About 80 % of the tested specimens had shown absence of the usual typical morphological forms of the bacterial cells. Whereas the specimens tested with *C. roseus* had shown the bacterial cells ranged between few to many bacterial cells. However only 3% had been recorded as numerous. Overall, about 90 % of the specimens had shown the denatured and broken bacterial cells along with least number of bacterial structures.

All together both *T. chebula* and *C. roseus* had expressed their high level antiadherence bioactivity to both gram positive and gram negative bacteria. Comparatively *T. chebula* seed extract found as the best than *C. roseus* extract. The paired extract combination of these plant extracts found to possess the best antiadherence bioactivity than the single extracts. The average score of the bacterial cells presence and its score ranged between 3 to 7 /100x. 100% of the specimens shown disintegrated cells. From this we came to understand the best antiadherence synergistic bioactivity of the *T.chebula* and *C.roseus* extract combinations. Whereas the contrôle specimens average score was recorded as numerous and plenty/ 100x. The maximum CFUs formed by the contrôle specimen was noted as confluent growth where the individual CFUs were not formed. While the CFUs recorded with the individual extracts of *T.chebula* and *C.roseus* was noticed as 80 and 70, 275 and 180 for gram positive and gram negative bacteria. But the paired extract combination of these plant extracts treated specimen yielded the maximum score of 18 and 3 respectively.

From this we could able to understand that the biochemical or the biomolecules of the different bioactive compounds of the medicinal plants are varied with their bioactivities. The different biological activities of numerous medicinal plants have been extensively studied by many authors world wide. Comparatively the antiadherence property of *T. chebula* and *C. roseus* plants have been poorly studied and understood. Hence this study focused on the synergistic anti adherence activity of two different plant species that is *Terminalia chebula* seed ethanolic extracts and *Catharanthus roseus* leaf ethanolic extracts and their combination.

Conclusion

Since these plant extracts shown their synergistic anti adherence property, surely they will act on the human pathogens and could prevent the first step that is the attachment/ adhesion to the host cells. This antiadherence property may prevent and block the further entry and establishment of the pathogens to the human host, while they infect with single species or multiple.



Fig.1. Innovative Dual Invitro Model System-(IDIMS)- Newly by Meenakshi, Ph.D. Scholar, C.A.S.in Marine Biology, Annamalai University, was used in our study in the invitro anti adherence experiments.

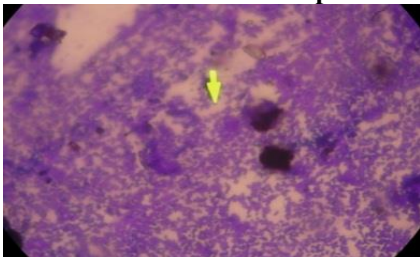


Fig. 2. Control specimen untreated with the plant extract. - screened by light microscope showing crowded bacterial cells.

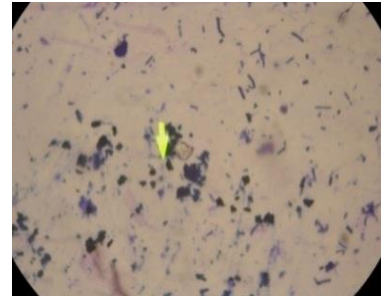


Fig.3. Specimen treated with the ethanolic extract combination of *T.chebula* and *C.roseus*, - reduced numbers of bacterial cells and disintegrated bacterial cells.



Fig. 4. Contrôle, untreated with plant extract showing crowded confluent bacterial growth.



Fig.5



Fig.6

Fig. 5& 6. Showing the reduced numbers of the bacterial CFUs formed by the specimens treated with *C.roseus* extract and combination of *T.chebula* & *C.roseus* extract. Better antiadherence bioactivity expressed by extract combination.

Table 1. Antiadherence bioactivity of the south Indian medicinal plant extracts test results.

S. No.	Plant extracts	CFUs formed by Specimen Culture		
		Gram positive bacteria	Gram negative bacteria	Control untreated specimens
1	<i>T. chebula</i> seed extract	15 – 80	30 – 70	Crowded growth
2	<i>Catharanthus roseus</i> leave extract	80 – 275	40-180	Confluent growth
3.	<i>T. chebula</i> and <i>C. roseus</i> extract combination	09 – 18	0-03	Confluent growth

Note ;Based on the typical colony morphology on BHIA the gram positive and gram negative bacteria was identified

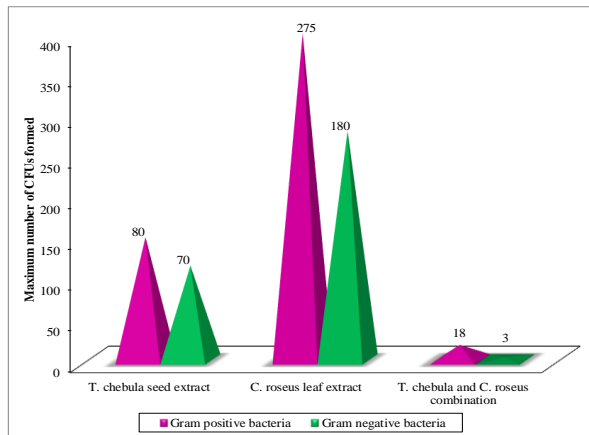


Figure 7. The best bacterial antiadherence bioactivity against poly pathogens.

Minimum number of CFUs indicating the high level antiadherence bioactivity of the plant extract. Compare to the single extract the paired extract combination of *T. chebula* seed and *C. roseus* leaf extract shown its best antiadherence bioactivity against gram positive and gram negative bacterial strains

Countless publications mentioning about the use of medicinal plants to treat various type of diseases. The authors Kamba and Hassan 2000 quoting that the modern pharmaceutical products contains plant and plant based products as its base. Since many reports says and acknowledging the medicinal and nutritive properties of the medicinal plants Jaleel et al 1996 *T. chebula* and *C.roseus* plant based products can be routinely taken up by the human to prevent their entry and further establishment within the host. Likewise, the plant extract combinations which emerge with the synergism could be positively used in the traditional medicine to treat the chronic infections. However the invitro experiments such as animal experiments and cytotoxicity test should be performed to rule out the toxicity of these extracts.

From our study results we can suggest that the extracts of *T. chebula* seed and leaf extract of *C. roseus* may be used either as a therapeutic or prophylactic agent to manage the microbial infections. And we also suggest the future scientists and students to involve in the research with the isolation and identification of the effective bioactive compounds / biomolecules responsible for the antiadherence property of *T. chebula* seed extracts and *C. roseus* leaf extracts. Further scientific studies related to the synergistic antibacterial adherence studies of the bioactive compound combinations that would give better solution for the future development of the effective drug. This may be highly helpful in the prevention of the microbial infections.

Acknowledgement

The Department of microbiology, Faculty of science, Annamalai University, highly acknowledging Mrs.Meenakshi, PhD scholar, Karpaga Vinayaga Institute to help us to use IDIMS experimental model in this study. We also acknowledge,Our special thanks goes to the VISVA AROKYA TRUST- Chidambaram for provided financial assistance and assisted through the research person C.Sudan Suhaa,Biotechnologist, to successfully carry out this study.

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