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## Certain innovative techniques and methods used in the screening of antibacterial and synergistic bioactivities of the mangrove plant extracts and antibiotics

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## ABSTRACT

The mile stone of the scientific technologies and methodologies, shown its well improved evidence based proof and created a striking landmark at global level. The various sectors, including Government and the private sectors involved in introducing new improved technologies cum methodologies day today in competitive manner. Though many sophisticated rapid technologies such as PCR etc., created a land mark in current scientific world, still if seems, the scientists, researchers, those who involved in the initial step of their research rely on the golden traditional techniques and methodologies even today. Innumerous research publications indicating the interests of the scientists and researchers towards the drug discovery especially from the plant sources. The initial step for the drug discovery involves the primary screening of the plant extracts or any other testing agents for their antimicrobial activities pertain to the antibacterial activities. The disc diffusion and the agar well diffusion methods are the traditional choice to perform the antibacterial activity. In this study we have presented an innovative plate technique and certain innovative methods in the screening of antibacterial and synergistic bioactivities of the mangrove plant extracts and antibiotics which is time and energy saving, economic and easy to perform.

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## Introduction

Agar disk-diffusion testing developed in 1940 (Mounyr et al 2015) and it is the traditional golden method used in many clinical microbiology laboratories to perform the routine antimicrobial susceptibility testing. Currently, many standards are published by the Clinical and Laboratory Standards Institute (CLSI) for bacteria and yeasts testing (CLSI- 2012) and (CLSI- 2004) by using specific culture media, various incubation period with different conditions and interpretive criteria for inhibition zones (CLSI -2012).

In this well-known technique, the agar containing plates, (usually 90 mm) are inoculated with a standardized inoculum (Mac Farland opacity 0.5) (Harish.T et al., 2016) of the test microorganism. Then, sterile whatmann filter paper discs (about 6 mm in diameter), containing the test compound at a desired concentration, are placed on the agar surface. The Petri dishes are incubated under suitable conditions either aerobically or anaerobically at 37°C. In this dics diffusion method, the antimicrobial agent diffuses into the agar and inhibits the germination and growth of the test microorganism. The diameters of the zone of inhibition formed around the discs are measured and that is usually considered as antibacterial activity of the tested agents. The disc diffusion method have advantages mainly due to its simplicity and low cost, easy to perform. Because of its advantages, it is used by many research authors to test and to screen the plant extracts, essential oils and other drugs for their antibacterial activity (L.

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Fourati *et al.*, 2005, K.Konate *et al.*, 2012, V.G.Billerbeck, 2007, & K.Das *et al.*, 2010).

The increased and remarkable percentage of the morbidity and mortality has been recorded with microbial drug resistance associated infections. This attracted the researchers interest at global level and created a land mark in the search of plant based new drugs which could be effectively act against the drug resistant microbial pathogens. Therefore evolutionary steps in the techniques and methods used in the antimicrobial susceptibility testing is felt essential to have the betterment with the existing techniques and methods. Though some of the techniques subjected to the Clinical and Laboratory Standards Institute standardisation (CLSI), and European Committee on Antimicrobial Susceptibility Testing (EUCAST) procedures (CLSI- 2004 &CLSI - 2012), some types of modifications often essential in the Invitro anti-microbial testing of the natural products. So it is necessary to be cautious not to change the basics of the standardised procedures, instead lesser methodological adaptations or minor modifications to the standardised protocols can be welcome to fortify accurate experimental approach which can help the other research authors to compare the results (Mounyr et al., 2015).

Different types of the bioassays are so far used to test or to study the antimicrobial activity of the plant products. Among that, disk-diffusion, well diffusion and broth or agar dilution method are the familiar and routinely used by the authors. The antimicrobial testing is the one of the initial steps can be used in the drug discovery, epidemiology and prediction of therapeutic outcome. In this paper we have focused on certain innovative techniques and presented some refined innovative methods and procedures in the use of studying the antimicrobial bioactivities such as antimicrobial activity, synergism, antagonism and additive bioactivities of the mangrove plant leaf extracts and antibiotics. It is very better much important to develop and introduce understanding, simple innovative techniques and methods for the preliminary screening of the antimicrobial activity of any agents for their applications not only in human health but also to the agriculture and environment.

It was aimed to explore possible future use of the innovative techniques / methodologies presented in this study which is simple time and energy saving and economic and can be used in the screening of the bio medicinal values of the mangrove plants extracts, which can also be applicable to test the mangrove plant extracts in multidimensional research views. And these techniques and methodologies may be useful to perform experimental studies in the inter-connected fields of life science.

#### **Materials and Methods**

The practical experimental work was carried out in the Department of Biotechnology, Karpaga Vinayaga of Engineering and Technology, Chinna kollambakam, Padalam, Kanchipuram District, Tamil Nadu and some of the experiments were conducted in the C.A.S in marine biology, Parangipettai, Annamalai University, Chidambaram, Tamil Nadu, India. Throughout the study period, the work was constantly supervised by the qualified research supervisors.

## Mangrove plant source and cleaning innovative procedures

The mangrove plant Rhizophora apiculata and Avicenna marina was chosen to perform the Invitro experimental study. The mangrove plants were harvested from Pichavaram sea shore. The harvested mangroves were placed in a cleaned plastic bag and carefully transferred to the laboratory after properly labelled. The healthy fresh leaves were plucked and removed from unhealthy leaves and dipped in tap water added with few drops of detergent gel (Vim gel – Indian product) and washed in such a way to rotate clockwise and anticlockwise movement for 3 to 5 minutes and the water was drained. The further washing process without the detergent was carried out till the detergent bubbles disappeared. Then the final wash was continued for three times with sterile distilled water and finally drained.

## Innovative method used in mangrove leaves drying process Re - use of discarded Aavin milk/Ponlait milk bottles & its source

The thrown out discarded Aavin milk/Ponlait milk bottles (A Indian Government products) were reused in our study. After drinking the milk, the glass containers (bottles) which were discarded by the consumers. The bottles were collected and thoroughly washed with soap water and the cotton plugged bottles were sterilised at hot air oven at 180°C for one hour and used.

The washed mangrove leaves were immediately crushed with the help of electronic mixer and the crushed leaves were transferred to a sterile Aavin milk bottles and exposed to the sunlight for one day and it was further kept in the oven at 55°C for 3hrs. Again the glass containers were exposed to the sun light for another one more day and finally once again at 55°C at hot air oven and it was stored in the room temperature and processed for the extract preparation.



Discarded Aavin milk bottles reused in our innovative study process to prepare the mangrove leaves for extract preparation.

Fig.1a: crushed mangrove leaves inside the sterile Aavin milk bottles exposed to drying under sun light. Fig.1b: After exposed to 55°C at hot air oven for 1 hrs. **Extract Preparation** 

Ethanol is the extracting of our choice used to get extract from the chosen mangrove plant leaves. The ethanolic crude extract of the mangrove leaves were prepared as follows; briefly 300 grams of these crushed leaf material was placed in a glass container added with 500 ml of ethanol. The content was mixed thoroughly and kept in the oven at 55°C for one hour then left at room temperature for 3hrs. Then it was filtered through sterile filter paper. The filtrate was reduced to 50ml by keeping it in the oven at 45°C or till it get reduced to the expected volume, considered as the master extract solution. The reduced and concentrated extract was stored in refrigerator till the Invitro assay was performed.

## **Invitro Synergistic Antibacterial Test**

## **Bacterial Source 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 obtained from the department of microbiology, Karpaga Vinayaga Institute of Medical Sciences, Chinnakolambakkam, Kanchipuram district, Tamilnadu, India. The bacterial isolates were identified at species level by the qualified medical microbiologists.

#### **Bacterial Stock Culture Maintenance**

The obtained human bacterial pathogens were maintained as stock cultures and stored in the refrigerator till it get used. The gram positive bacteria, S.aureus, Lactobacilli, Bacillus Sp., and the gram negative bacteria E.coli, Klebsiella, and Pseudomonas had been included in our study .The bacterial isolates were maintained in nutrient agar slopes. All the bacterial cultures were labelled and maintained in triplicates, one set is maintained as Mother Culture, stored in the freezer. The second and the third sets of the bacterial cultures were treated as running cultures and used in the Invitro tests (Fig.2a).

## Recheck viability and Purity Check of the Bacterial Isolate

In order to obtain the discrete bacterial isolates and to check the purity the primary culture, which were obtained from different sources were inoculated by streak plate method. The purity of the bacteria was assessed by examining the uniformity of the typical characteristic bacterial colonies on the respective media. If any subculture plates shown more than one type of bacterial colonies, they were discarded and further the same bacterial culture from the mother culture was sub-cultured and used. If occational numbers of the contaminant bacterial growth appeared out of the culture

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streak (**Fig.2b**) that was ignored and the bacterial growth from the streak sites were used for the teat assays.





Fig.2b

Fig.2a: Pure culture maintained as stock cultures in the BHIA slopes

Fig.2b: Re-check of the pure culture to check viability & purity. Culture showing uniform bacterial growth assuring the purity

#### **Bacterial Inoculum Standardization**

Prior to the Invitro synergistic antibacterial assay, the bacterial stock cultures were subjected to subculture in the Brain Heart Infusion Broth (BHIB). For this a loopful of bacterial stock culture from the stock culture was taken and inoculated into the tube containing 1ml of BHIB, incubated at  $37^{\circ}$ C for one hour and the suspension was adjusted to Mac Farland opacity no . 0.5 equals to 10<sup>8</sup> cells / ml and the fresh cultures were used in this study to perform the Invitro synergistic antibacterial bioactivity of the mangrove plants species and antibiotics.

#### **Extracts Preparation for Invitro Synergistic Antibacterial** Activity Test

Every time just before do the Invitro synergistic antibacterial test, the desired plant extract combinations had been prepared by mixing equal quantity (1 ml each) of the two individual mangrove plant extracts which was already stored in the refrigerator (Master extract solution) and used in the Invitro test. In case of testing the synergistic antibacterial bioactivity of the mangrove plant extract with antibiotics, the desired concentration of the individual plant extract was prepared and used.

## Invitro synergistic antibacterial assay- Innovative technique

## Antibiotics used to check the synergistic effect

The standard antibiotic discs routinely used for the gram positive and gram negative bacteria were purchased from the Himedia (Mumbai, India) company and used in this study to screen the synergistic antibacterial and antifungal activities of the mangrove plant leaf extracts. All the antibiotic discs containing vials were kept in the refrigerator till it get used in the Invitro test assays.

## Synergism screening innovative disc diffusion method

The sterile Muller Hinton Agar (30 ml) was poured into the large 124 mm sterile Petri dishes, after solidify the media, the antibiotic discs were placed on the culture plate as shown in figure(**Fig.4a**).

The plain sterile filter paper discs (Whatmann No-1 Filter paper) soaked only with the mangrove plant leaf extract was placed and the antibiotic disc soaked with the mangrove plant extracts was placed near to that followed with corresponding antibiotic disc.(eg., mangrove extract alone, next – Ampicillin disc soaked with mangrove leaf extracts followed with Ampicillin disc). The multiple number of the mangrove extract with different concentration soaked in sterile filter paper disc also can be placed and checked (**Fig.4a**).The inoculated plates were incubated at  $37^{\circ}$ C for 24 hrs incubation and the synergistic, antagonistic and the additive antibacterial bioactivities of the mangrove plant leaf extracts along with the antibiotics was studied assessed

# Agar Well Diffusion Method – To study the synergism of two discrete mangrove extracts

For the primary synergistic antibacterial activity screening, to test both mangrove plants and antibiotics, we have introduced the innovative technique. Briefly 124 mm sterile petri plate was used instead 90 mm petri plate. 30 ml sterile Muller Hinton agar was poured. After solidify, 25 microliters of the standardized bacterial inoculum was delivered on the culture plate and it was uniformly spread on the agar plate to form a lawn culture. With the help of sterile micro tips, 4 mm wells were made. The cut wells were labelled at back side of the petri plate. The known quantity (30 microliters) of the individual as well as the desired combination of the mangrove plant leaf 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 the additive antibacterial bio activity was assessed.

#### Innovative agar diffusion method

After delivered the desired quantity (30 microliters) of the mangrove plant extracts to the cut wells, the individual antibiotic discs were placed separately to the wells already contained the plant extracts (**Fig.4b**). Then the plates were incubated at  $37^{\circ}$ C for 24 hrs or overnight then the plates were read for zone of inhibition formed around the well.

#### Dual comparative agar diffusion method

Both disc diffusion and well diffusion method was used in this dual comparative agar diffusion method, was used to study or to assess the synergistic antibacterial activity of the mangrove plant leaf extracts and antibiotics discs. The antibacterial activity of the individual antibiotics as individual mangrove plant extracts also studied. The media containing 124 mm plate was divided into two half, right side was used to test the synergistic antibacterial activity of *R. apiculata* leave extract and antibiotics in the same way the left side was used to test A.marina leaf extract. The antibiotic disc immersed with the A. marina leaf extract was placed (left side of 124 mm plate) next to that cut well was made and loaded with 30 microliter of the A. marina leaf extract in which the desired antibiotic to be tested for the synergism was placed. Very next to that the same antibiotic disc alone was placed (fig.4c). The same method was followed to test the R.apiculata leaf extract, and then the plate was incubated at 37<sup>°</sup>C aerobically for 24 hrs or, after it was assessed for synergistic, antagonistic and additive antibacterial activity.

# Synergistic Antibacterial Activity assessment - ref. Udhaya (2012)

The synergistic antibacterial activity of the two different mangrove plant leaf extracts, and with antibiotics and

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antifungals were assessed by measuring the diameter of the Zone of Inhibition (ZOI) formed around wells which contains single mangrove plant leaf extract (alone) and, the diameter of the zone of inhibition formed around the wells which contains both mangrove plant species leaf extracts. If the measure of the diameter of the zone of inhibition formed around the well which contains the mixture of the two discrete mangrove extracts, exceeded than the diameter of the zone of inhibition formed by the individual mangrove plant leaf extracts, and individual antibiotic discs, then, it was considered as positive for the synergistic antibacterial activity.

### Antagonistic Antibacterial Activity - Assessment - ref. Udhaya (2012)

If 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 antibacterial activity.

## Additive Activity – Assessment – ref. Udhaya (2012)

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.

## **Results and discussion**

The main aim of the present study was to present a simple time and energy saving innovative technique cum methodology which can be used in the testing or preliminary screening of the antibacterial activity and synergistic antibacterial activity of the mangrove plant extracts with antibiotics. In this study we have presented certain innovative process related to the chosen current topic. All these techniques and methods can be beneficially applicable for the microbiological study where the aseptic precautionary methods are felt essential to follow.

## Mangrove Species Selected & Included

The mangrove species distributed in and around the Pichavaram seashore, Tamil Nadu, India.Each and every steps involved starting from the mangrove species collection to the extract preparation, it was processed in the systematic way. After harvesting the mangrove species, it was labelled in order to avoid the confusion while processing. The healthy mangrove leaves were separated from the unhealthy leaves to provide the good quality materials for the microbiological proceedings. The healthy leaves were thoroughly washed by using the detergent with given clockwise and anticlockwise movement while washing process because to remove the strongly adhered dust particles on the leaf material, especially the dust particles hidden in the pores of the leaves (pores at back side of the mangrove leaves).



Fig.3

From the short survey conducted by our co-research scholar,

(Harish Thummala - 2013, Dept. of microbiology, Faculty of medicine, Annamalai University), among the different mangrove species, the mangrove species *Rhizophora* and

Avicenna species found to be familiar among the people residing in and around Pichavaram seashore and used as medicinal herb to treat different types of infectious and non-infectious diseases. So these two mangrove species were included in our study.

In view of adopting certain innovative methods in our study, we have tried to reuse the thrown out milk bottles and successfully it was possible for us to proceed with that and the bottles were able to withstand the temperature at 180 degree Celsius for 1 hour at hot air oven. From this we can suggest that this bottles can be used for any other experimental purposes instead of using the usual glass containers used in the laboratory which are costlier than this bottles.

Since our study was related to the microbiology, to follow the aseptic precautionary method, we have used these bottles to load the crushed mangrove leaves for drying. By reviewing the literatures the plant materials drying process usually done in the open environments that may allow the air contamination which may interfere in the experiments. Aiming at the complete exclusion of the microbes and to provide the complete sterile condition we have followed these method of drying process.

To fasten the drying process, in addition to the sunlight exposure, the crushed mangrove leaves in the bottles were kept in the hot air oven at  $55^{\circ}$ C. Successfully we could able to obtain the dried mangrove leaf materials at the end of third day. Since the mangrove leaves are thick, and contains mucoid contents, usually the period require for drying at sunlight exposure ( $45^{\circ}$ C) was recorded as 8 to 10 days. If the leaves crushed and subjected to the same drying process, it took at least one week period for drying. But by our innovative process it was possible for us to complete the drying process within three days.

#### Mangrove extract preparation

Different authors used different ratio of mangrove plant powder and the respective solvents to get extracts (Kathiresan et al 2012), some authors used 30 grams plant powder plus 500 ml solvents, but in our study in order to use certain innovative methods and to bring out the refinement with existing methods and procedures, we have used comparatively less quantity which was essential to dissolve, we have used 500 ml solvents for 300 grams mangrove leave powder.

## Advantage of maintaining three sets of the stock cultures

The human pathogenic bacterial isolates were maintained as stock cultures in triplicates, one set was safely maintained as mother culture and the other two sets were used as running cultures in order to maintain and use the same isolates in our study. When particular running cultures get contaminated, that was discarded and the bacterial strains were sub-cultured from the mother culture and used in our study. By maintaining and followed these steps it was possible for us to perform our experimental study in successful way without any loss of the selected bacterial strains.

Periodical re-check of the stock culture was done to check the viability of the stock cultures and to check the purity of the pure stock cultures. This was done to maintain the quality assurance of the microbiological Invitro experimental procedures. From our study, only few cultures were contaminated with fungus and none of the cultures were recorded with loss. 100 % viability was recorded. For the bacterial inoculum preparation fresh cultures were used. By our innovative method we could able to prepare fresh culture within one hour from the stock culture, that was worked out well in our study and was not shown any difference from the 24 hrs culture with the same study. Therefore, we recommend the use of 1 hr old cultures to perform the Invitro antibacterial test assav.

#### Multiple use of the innovative single plate method

By using the same 124 mm plate technique, with add up of the desired methodologies, this technique can be used to test other test materials also. We have used this innovative technique and methods to perform the following Invitro assays in our PhD research work.

1. Invitro Synergistic antibacterial activity of the two different mangrove Extracts.

2. Invitro Synergistic antibacterial activity of the two different mangrove Extracts with antibiotic discs.

3. Invitro Synergistic antifungal activity of the two different mangrove extracts.

4. Invitro Synergistic antifungal activity of the two different mangrove Extracts with antifungal drug discs

#### Advantages of the present innovative technique

Compared to the 90 mm Petri dishes, which are routinely used in the microbiology laboratories to test the antibacterial activity or synergistic antibacterial activity, multiple numbers of the testing agents can be tested by using this 124 mm petri dishes. Maximum 10 to 12 testing agents can be tested by disc diffusion method by using 90 mm petri dishes while 25 to even 30 numbers of the testing agents can be tested by using 124 mm petri dishes and this technique and methodology may be applied to study and or to screen the synergistic effect of any other testing agents also.



Fig.4a

Fig.4c

Fig.4a:Disc diffusion method - antibacterial activity of the individual mangrove plant extracts (brown coloured extract of R.apiculata, green coloured- A.marina leaf extract ) and antibiotic discs as positive and negative controls - showing the positive and negative antibacterial activity.

Fig.4b: Innovative diffusion method – synergistic antibacterial bioactivity of the mangrove plant extracts and antibiotics - antibiotic disc placed in the well containing the mangrove plant extract showing the synergism.

Fig.4c:dual comparative method – antibiotic disc placed in wells containing mangrove plant extracts tested for the synergistic antibacterial bioactivity and antibiotic disc alone tested by disc diffusion method which was placed very near to the wells. Wells with both mangrove extracts and antibiotic disc showing the wider zone of inhibition representing synergism exists with mangrove extracts and antibiotics.

In case of agar well diffusion method maximum 6 to 8 wells can be prepared with 90mm Petri dish but 20 to 25 wells can be created with 124mm petridish. The innovative method which we have introduced in our study i.e., innovative agar well diffusion method (Fig.4b), dual agar diffusion method (Fig.4a) and (Fig.4c) dual comparative agar diffusion method to testing the antibacterial activity and synergistic antibacterial

bioactivity of the mangrove plant extracts and antibiotic discs. It was possible for us to feel the dual agar diffusion method as time and energy saving method by which we could able to use and add the exact quantity of the antibiotics at microliters level of the agar wells which contains the exact quantity (30ml) of the mangrove plant extracts.

The preparative procedures for preparing the particular antibiotic from the original from (as powder or suspension) was restricted which we used the readymade commercially available antibiotic discs which contains the levels of the antibiotics. Likewise by following the innovative dual comparative method, it was possible for us to test and compare the both disc diffusion method testing the commercially available antibiotic discs as well as we were able to test and compare synergistic antibacterial activity with the dual agar well diffusion. And this technique and methodology can be applicable to test the synergistic antibacterial or even the antifungal activity of any other testing agents such as other medicinal plant extracts, chemicals, drugs, nanoparticles etc. Even if some authors clearly mentioned about their techniques and methodologies, they seems to be time consuming and energy wasting process. The purpose of present study is to present the simple, time and energy saving, innovative Invitro technique and methodology used in the screening or study of the synergistic antibacterial effect of the mangrove plant extracts with antibiotic against the human pathogenic bacteria of clinical source . Technology leadership will depends on the ability of the government, Universities and industries to collaborate effectively in the development of the appropriate policies, especially for innovative steps. Innovation will be supported through new granting mechanisms to support interdisciplinary networks and public private partnerships accelerate the pace of technology development. To meet point of success, technological progress should lead to the significant level.

## Conclusion

From our study we conclude, the Need of refinement & innovation technologies for today present and future benefits Innumerous research publications indicating the interests of the scientists and researchers towards the drug discovery especially from the plant sources.

Thus today, it is felt essential to develop much of the basic innovative research techniques and methodologies for the future betterment. The basic research knowledge may be the first/initial point from where one can achieve the advanced research with high performance. In concluding in the field of marine biotechnology research, pertain to the screening of the bio medicinal values of the mangroves and other plants, the use of basic innovative techniques and methodologies is in the stage of infancy.

Refining the existing techniques / methods and developing the new innovative techniques and methodologies are essential and its effective application remains an important source of economic growth.

Overall all these above mentioned innovative techniques and refined innovative methods were experienced and as easy to perform energy and time saving, economic and also have many advantages. These innovative techniques and methods can be also used as innovative techniques / methods to test the antibacterial or synergistic antibacterial activity of any other plant extracts or chemical agents etc. We strongly recommend these innovative techniques and methods for the research authors especially those who are all engaging or plan to have increased multiple numbers of the testing agents in

their study to test the antibacterial, antifungal or the respective synergistic activity.

## References

• Ariaki Nagayama. Author links open the author workspace. Opens the author workspace Opens the author workspace Keizo Yamaguchi. Author links open the author workspace. Kunitomo Watanabe. Author links open the author workspace. Masatoshi Tanaka. Author links open the author workspace. Intetsu Kobayashi. Author links open the author workspace. Zenzo Nagasawa

• Clinical and Laboratory Standards Institute Administrative Procedures (CLSI 2012 & 2004)

• Final report from the Committee on Antimicrobial Susceptibility Testing, Japanese Society of Chemotherapy, on the agar dilution method (2007)

• Harish.T *et al.*, 2013- Bio medicinal values of the mangrove Plants- A short summary Conducted at villages near Pichavaram Mangrove forest-Ph.D work, Depatment of Microbiology, Faculty of Medicine Annamalai University, Tamil Nadu-India.

• Harish.T *et al.*, Synergistic antimicrobial activity of the mangrove plant leaf extract and Non Mangrove medicinal plant seed extracts against the multi drug resistant bacterial human pathogens, *Elixir biosci.* 96 (2016) 41839-41844

• K. Das, R.K.S. Tiwari, D.K. Shrivastava, 2010, Techniques for evaluation of medicinal plant products as antimicrobial agents: current methods and future trends, J. Med. Plants Res.,

4 (2010), pp. 104–111 Journal of Infection and Chemotherapy Volume 14, Issue 5, 2008, Pages 383-392

• K. Konaté, J.F. Mavoungou, A.N. Lepengué, *et al.* Antibacterial activity against  $\beta$ -lactamase producing Methicillin and Ampicillin-resistants *Staphylococcus aureus*: Fractional Inhibitory Concentration Index (FICI) determination Ann. Clin. Microbiol. Antimicrob., 11 (2012), p. 18

• L. Fourati-Ben Fguira, S. Fotso, R. Ben Ameur-Mehdi, *et al.* 2005 Purification and structure elucidation of antifungal and antibacterial activities of newly isolated *Streptomyces* sp. strain US80 -Res. Microbiol., 156, pp. 341–347

• Mounyr Balouiri, Moulay Sadiki, Saad Koraichi Ibnsouda, 2015 Methods for in vitro evaluating antimicrobial activity: A review *Journal of Pharmaceutical Analysis* 6 (2016) 71–79.

• Priyadharshini *et al.*, 2016- Synergistic antibacterial activity of Terminalia chebula, Vitis vinifera and Punica granatum seed extracts against certain Human pathogenic bacteria of clinical source-*Elixir Biosciences* 101 (2016) 44018-44023.

V.G. De Billerbeck, 2007, Huiles Essentielles et Bactéries Résistantes aux Antibiotiques- Phytotherapie, 5, pp. 249–253
V.Udhaya, Professor, Department of Microbiology, Rajah Muthiah Medical college- Annamalai University, Tamil Nadu-India. Ongoing research Methodology.

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