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Plant Pathology

In vitro Antibacterial and Synergistic Effects of Plant Extracts and Synthetic Antibiotic 'Aztreonam' Against Extended Bacterial Spectrum

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Abstract

The present study was carried out to assess efficacies of the various plant extracts for their pharmacological potential and synergistic effect in limiting the bacterial growth for formulating new cost effective antimicrobial agent(s) for multi drug resistant organisms. The antimicrobial activities of plant extract of *Pterocarpus santalinus*, *Tectona grandis*, *Gloriosa superba* and its synergistic effects among them as well as with a synthetic antibiotic 'Aztreonam' were assessed against Gram positive bacteria viz. *Staphylococcus aureus* ATCC 25923, *Bacillus subtilis* MTCC 441; Gram negative bacteria viz. *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853. Acetone extract of *Tectona grandis* and *Gloriosa superba*, isopropyl alcohol (IPA) extract of *Pterocarpus santalinus* were found most effective in restricting the growth of bacteria. The efficacies of the various extract combinations in each plant sample varied and the mminimum inhibition concentrations (MIC) of acetone extract in comparison with 'aztreonam' using different Gram-positive and Gram-negative bacteria were found to be around 0.312 – 0.50 mg/ml for *Pterocarpus santalinus*, 0.62 – 1.10 mg/ml for *Tectona grandis* and 0.7 – 2.9 mg/ml for *Gloriosa superba*. The combination of plant extract of *Gloriosa superba* + *Pterocarpus santalinus (2:1)* showed the maximum inhibition on *Pseudomonas aeruginosa* with the strongest synergistic effect. Similarly the maximum inhibition on *Bacillus subtilis was* observed by the combination of plant extract of *Tectona grandis* + *Gloriosa superba (2:1)* whereas, the plant extract of *Tectona grandis* + *Gloriosa superba* inhibited the growth of *Escherichia coli* and *Staphylococcus aureus* to the maximum extent.

Highlights

- Plant extracts alone or in combination with syntheic antibiotic successfully limit bacterial growth
- New formulation antimicrobial agent effective for multi drug resistant organisms.

Keywords: Antimicrobial activity, *Aztreonam, Gloriosa superba, Pterocarpus santalinus, Tectona grandis,*

Traditional systems of medicine continue to be widely practised by common people as a whole and tribal in particular despite the use of modern medicine in the 21st century. Inadequate supply of allopathic drugs for growing population, its high cost, side effects and development of resistance to currently used drugs for infectious diseases have led to increased emphasis on the use of plant materials as a source of medicines for a wide variety of human

ailments (Thatoi *et al*., 2008; Dewanjee *et al*., 2007; Sharma *et al*., 2011). Three quarters of the world's population can't afford allopathic drugs and have to rely upon the use of traditional medicines which are mainly derived from plant materials. Indian subcontinent has been known to be rich repository of medicinal and aromatic plants, which are largely collected as raw materials for manufacture of drugs and perfumery products. About 8000 species of medicinal plants are used in tribal healthcare needs, and only about 1500 plants are in use in Indian Ayurveda, Unani and Siddho systems, largely for elite mass (Essawi *et al*., 2010). Unfortunately, much of the ancient knowledge and many valuable plants are lost due to rapid depletion of forest cover. Many valuable medicinal plants are under the verge of extinction. These medicinal plants synthesise and preserve a variety of biochemical products, many of which are extractable and used as medicine. Secondary metabolites extracted from plants are used in a number of pharmaceutical compounds. However, a sustained supply of the source material often becomes difficult due to the factors like environmental changes, cultural practices, diverse geographical distribution, labour cost, selection of the superior plant stock and over exploitation by pharmaceutical industry (Srivastav *et al*., 2013). Plants, especially used in Ayurveda can provide biologically active molecules and lead structures for the development of modified derivatives with enhanced activity and /or reduced toxicity. Some of the useful plant drugs include vinblastine, vincristine, taxol, podophyllotoxin, camptothecin, digitoxigenin, gitoxigenin, digoxigenin, tubocurarine, morphine, codeine, aspirin, atropine, pilocarpine, capscicine, allicin, curcumin, artemesinin and ephedrine among others (Dwivedi *et al.*, 2011; Ujjwal *et al*., 2011). In some cases, the crude extract of medicinal plants may be used as medicaments (Sharma *et al*., 2011). On the other hand, the isolation and identification of the active principles and elucidation of the mechanism of action of a drug is of paramount importance. Hence, works in both mixture of traditional medicine and single active compounds are very important. Where the active molecule cannot be synthesised economically, the product must be obtained from the cultivation of plant material. Plants extracts having antimicrobial activity represent a vast untapped area of research and it requires further exploration for treatment of different diseases. It has enormous therapeutic potential. In recent years, there has been a lot of interest in the investigation of natural materials as sources of new therapeutic agents.

Teak (*Tectona grandis* L.; Family - Lamiaceae) is commonly found in India and other South-East Asian countries (Kumar *et al*., 2009). It is one of the best timbers in the world. It provides major constituents of folklore medicines. Extracts from various parts of teak shows expectorant, anti-inflammatory, antihelmintic properties and is also used against biliousness, bronchitis, hyperacidity, dysentery and diabetes. In traditional medicine, a wood powder paste has been used against bilious headache and swellings. They are also used for treating inflammatory swelling (Bhattacharjee *et al*., 2004). Similarly, Red Sandalwood (*Pterocarpus santalinus* L.; Family *Fabaceae*) is one of the most valuable medicinal plant species. It is used as an external application for curing inflammations of skin diseases, treating bone fracture, leprosy, spider poisoning, and scorpion sting (Bhattacharjee, 2004). *Gloriosa superba* L. is one of the endangered species among the medicinal plants (Badola, 2002) . The tuberous root boiled with sesamum oil is applied twice a day on the joints, affected with arthritis reduces pain (Singh, 1993). It is also used to treat intestinal worms, bruises, infertility, skin problem and impotence. The sap from the leaf tip is used as a smoothening agent for pimples and skin eruptions. The tuberous roots are useful in curing inflammation, ulcers, scrofula, bleeding piles, white discharge, skin diseases, leprosy, indigestion, snake bites, baldness, intermittent fever and debility. It is also considered useful in promoting labor and expulsion of placenta. Seeds are used for relieving rheumatic pain and as a muscle relaxant (Nadkarni, 2002).

As majority of bacteria strains are found exhibiting resistance to many antibiotics that necessitate the discovery of new therapeutic agents having antimicrobial activity (Sharma *et al.*, 2011). Many of the currently used anti-infective and antineoplastic agents are natural products, initially isolated from plants (Poonkothai , 2005a, 2005b; Geetha *et al*., 2011). The synergistic effect from the association of antibiotics with plant extracts against resistant bacteria leads to new choice for the treatment of infectious diseases (Ujjwal *et al.*, 2011). This effect enables the use of the respective antibiotic when it is no longer effective by itself during therapeutic treatment. Therefore, the present investigation was undertaken to investigate synergistic activity of extracts of *Pterocarpus santalinus, Tectona grandis, and Gloriosa superba* with a standard antibiotic 'aztreonam' for combating antimicrobial activities.

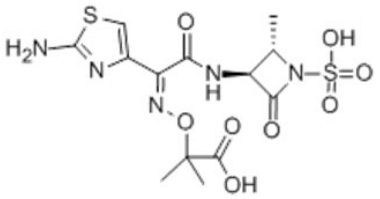
Materials and Methods

Microorganisms

Gram positive bacteria such as *Staphylococcus aureus* ATCC 25923, *Bacillus subtilis* MTCC 441, Gram negative bacteria such as *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853 were obtained from Microbial Type Culture Collection and Gene Bank, Institute of Microbial Technology, Sector 39-A, Chandigarh-160036, India and used in the present study.

Antibiotic

'Aztreonam' ($C_{13}H_{17}N_5O_8S_2$) is a synthetic bactericidal antibiotic containing a monocyclic â-lactam (monobactam). The monobactam has a unique monocyclic beta-lactam nucleus and is structurally different from other beta-lactam antibiotics (eg, penicillins, cephalosporins, cephamycins). The sulfonic acid substituent in the 1-position of the ring activates the beta-lactam moiety; an aminothiazolyl oxime side chain in the 3-position and a methyl group in the 4-position confer the specific antibacterial spectrum and beta-lactamase stability. It is designated chemically as (Z)-2-[[[(2-amino-4- thiazolyl) [[(2S,3S) -2-methyl- 4-oxo-1-sulfo -3-azetidinyl] carbamoyl] methylene] amino]oxy]-2-methylpropionic acid. It has a molecular weight of 435.44 and structural formula is as follows



Preparation of crude extract

The plant samples were collected from *Pterocarpus santalinus, Tectona grandis,* and *Gloriosa superba* grown in the orchard of Banaglore, India. The collected plant samples were first thoroughly washed with water and soaked in detergent to remove the microbial load on the surface of plant samples. These were then shade dried and finely powdered in mortar and pestle and was sieved with fine muslin cloth. This fine powder (20 g each) of *Pterocarpus santalinus* bark, *Tectona grandis* leaves and *Gloriosa superba* tubers were sequentially extracted with isopropyl alcohol and acetone at room temperature for 48 hours and stored separately (Singh *et al*., 2012). The extracts were filtered and concentrated under reduced pressure using rotary evaporator to get completely dried extracts. The yield of the crude extracts obtained from *Pterocarpus santalinus, Tectona grandis* and *Gloriosa superba* were about 20 mg, 16 mg and 15 mg respectively. The extracts were then dissolved (5 mg/ml of solvent) separately in dimethyl sulfoxide (DMSO) as a solvent.

Antibacterial activity screening

Minimum Inhibitory Concentration (MIC) Test

Antibacterial activity of all extracts from *Pterocarpus santalinus, Tectona grandis* and *Gloriosa superba* were carried out against Gram positive bacteria such as *Staphylococcus aureus* (ATCC 25923), *Bacillus subtilis* (MTCC 441) and Gram negative bacteria such as *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853). The Minimum Inhibitory Concentration (MIC) for different bacterial cultures was performed following the standard method (Ferreira *et al*., 2003). The extract was dissolved in dimethyl sulfoxide (DMSO). The initial concentration of the extract was 5 mg/ml and it was further diluted to 0.039 mg/ml. A series of culture tubes were prepared all containing the same volume of the medium inoculated with test microorganisms. The lowest concentration of sample at which the subculture from test dilution yielded no viable organisms was recorded as minimum Inhibitory concentration (Nathan *et al*., 2001; Mishra and Mishra, 2011. Decreasing concentration of drug was added to the tubes usually a step wise dilution (2-fold serial dilutions) was used starting from highest to lowest concentrations. One tube was left without drug to serve as positive control and other without drug and inoculums to serve as negative control. The cultures were incubated for 24 hrs at 37 °C. The tubes were inspected visually to determine the growth of organisms by the presence of turbidity and the tubes in which antibiotic is present in minimum concentration sufficient to inhibit the microbial growth (tubes without turbidity) was noted as Minimum Inhibitory Concentration (MIC) of the extract. All the experiments were repeated thrice.

Estimation of Antimicrobial activity

Antibacterial activity of all extracts from *Tectona grandis* were checked against Gram positive bacteria such as *Staphylococcus aureus* ATCC 25923, *Bacillus subtilis* MTCC 441 and Gram negative bacteria such as *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853. The extracts were tested at 5mg/ml of DMSO. The 5 mm of wells were loaded with 5µl of extracts per petridish. The diameter of zone inhibited was measured in mm. The

experiment was performed in triplicate and the average zone of inhibition was recorded. Result expressed as diameter of inhibition zone and compared with standard antibiotic 'aztreonam'

Synergistic activity

The synergistic activity study was calculated by combining different extracts with the standard antibiotic 'aztreonam' by means of agar gel well method. The plates were incubated for 24 hours at 37 °C and the diameters of inhibitory zones were measured on the second day. The study was also carried out by mixing different extracts in different concentration. The plant extract (50 µg/ml) were combined with antibiotic (5µg/ml). The concentration and combinations yielded seven different solutions (GGP, PPT, GGT, PPT, TTP, GTP, TTG) where T stands for *Tectona grandis*, G stands for *Gloriosa superba* and P stands for *Pterocarpus santalinius*. The number of time the representative letter shows the ratio in sample taken (eg: in GGP *Gloriosa superba* and *Pterocarpus santalinius* is in 2:1 ratio respectively, in GTP all three extracts are in 1:1:1 ratio). The plates were incubated for 24 hours at 37 °C and the diameters of inhibitory zones were measured on the second day. The diameter of zone inhibited was measured in mm. The experiment was performed in triplicate and the average zone of inhibition was recorded.

Results and Discussion

Estimation of Anti-Microbial Activity and its synergistic effects

In vitro antibacterial activities of the dried plant extract *Tectona grandis, Pterocarpus santalinus, Gloriosa superba* were shown in Table 1 and Fig.1. The test organisms *Staphylococcus aureus*, *Bacillus subtilis, Escherichia coli, Pseudomonas aeruginosa* used in the present study are associated with various forms of human infections. The extracts obtained using the solvent Isopropyl alcohol **(**IPA) and acetone were compared. Acetone extracts of *Gloriosa* and *Tectona* were found effective whereas extract for *Pterocarpus* was the most effective on the above test organisms. The extracts tested showed effective inhibition towards the growth of bacteria. The extracts were also found more effective in inhibiting the growth of test microorganisms as compared to the synthetic antibiotic 'Aztreonam'.

The synergism of *P. santalinus* with antibiotic was proved to be most effective against *Escherichia coli* whereas synergistic effect *T. grandis* with antibiotic was found best against *Staphylococcus aureus* (Table 1). The combination

Table 1: Antimicrobial activity of plant extracts and antibiotic 'Aztreonam' against pathogenic test organisms *Bacillus subtilis, Pseudomonas aeruginosa, Escherichia coli* and *Staphylococcus aureus*

Treatment combination (Plant extract & antibiotic)	Inhibition zones in diameter (mm)			
	Bacillus subtilis	Pseudomonas aeruginosa	Escherichia coli	Staphylococcus aureus
G	26	29	42	35
P	30	20	39	35
T	34	31	36	37
GGP	27	48	51	46
PPT	24	24	32	21
GGT	25	34	51	50
PPG	21	31	42	38
TTP	16	21	31	29
GPT	18	25	43	43
TTG	49	36	44	33
G+Aztreonam(AZ)	29	41	42	35
P+ Aztreonam(AZ)	35	20	44	43
T+ Aztreonam(AZ)	35	33	32	40
Aztreonam(AZ)	28	30	20	20

N.B. T stands for alcoholic extract of *Tectona grandis*, G stands for *Gloriosa superba* and P stands for *Pterocarpus santalinius*. Seven different combination of plant extracts (GGP, PPT, GGT, PPT, TTP, GTP, TTG) where the number of time the representative letter shows the ratio in sample taken (eg: in GGP *Gloriosa superba* and *Pterocarpus santalinius* is in 2:1 ratio respectively, in GTP all three extracts are in 1:1:1 ratio).

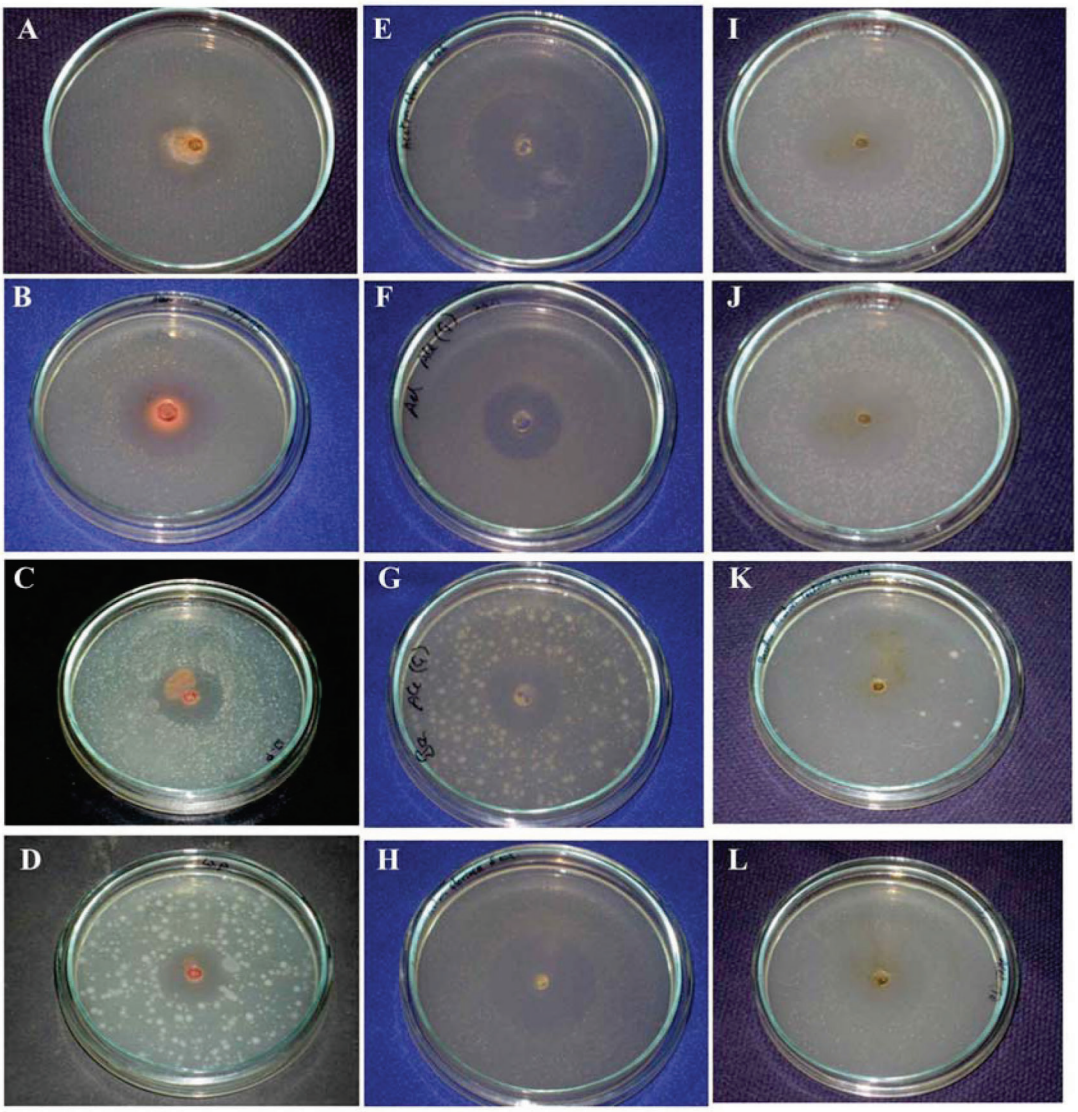


Fig. 1: Antibacterial activity of Pterocarpus santalinious on E. coli, P. aeruginosa, B. subtilis, S. aurous (A-D) Gloriosa superba on E. coli, P. aeruginosa, B. subtilis, S. aurous, (E-H); Tectona grandis on E. coli, P. aeruginosa, B. subtilis, S. aurous (I-L)

of antibiotic 'aztreonam' and plant extract of *P. santalinus* inhibited the growth of *Pseudomonas aeruginosa* miniumum.

The synergistic effect *Gloriosa superba* with antibiotic was effective against *Staphylococcus aureus* and *Bacillus subtilis*). The synergism of each extract in seven different combinations was carried out (Table 1, Figure 2). The combination of plant extract of *Gloriosa superb*a + *Pterocarpus santalinus (2:1)* showed maximum inhibition on *Pseudomonas aeruginosa.* Similarly, the combination of plant extract of *Tectona grandis* + *Gloriosa superb*a (2:1) was proved most effective for inhibition of growth of *Bacillus subtilis* whereas on *Escherichia coli and Staphylococcus aureus*, *Tectona grandis* + *Gloriosa superb*a showed maximum inhibition. These studies showed all the extract are broad spectrum and can be more effective through synergism.

Minimum Inhibitory Concentration

Minimum Inhibition Concentration (MIC) values ranged from 0.152 to 2.5 mg/ml, while and *E. coli* was found to be the most susceptible to the extracts of *Gloriosa superba*. The MIC analysis of plant extracts showed the optimum bacteriostatic and bacteriocidal concentration for crude

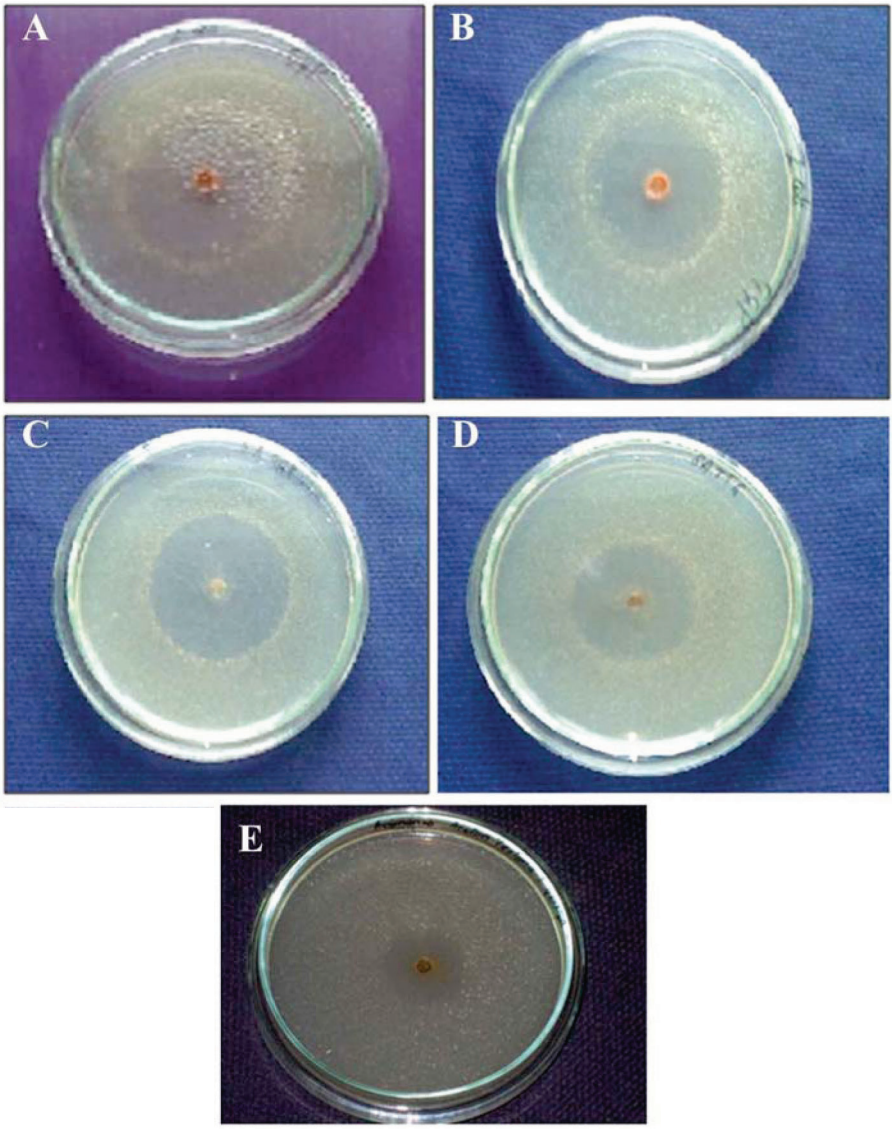


Fig. 2: Inhibitory zone of GGP (A) and GGt (B) on E. coli; GGT (C) on S. aurous; TTTG (D) on B. subtilis; TTG (E) on B. subtilis

extracts of the plants tested. The Table 2 depicted the MIC of all plant extracts and the zone of inhibition results reflected in MIC. The *E. coli* and *Pseudomonas aeruginosa* appeared to be sensitive to the plant extract of *Gloriosa superba* The gram positive *Staphylococcus aureus* were the moderate sensitive of all against all the extracts except to *Tectona grandis* + Aztreonam combination, From MIC results of present study the extracts of Gloriosa superba and Pterocarpus santalinus showed prominent inhibitory action against all the pathogens tested. The MIC was found to be the least with combination of synthetic antibiotic 'aztreonam' and extract of *Tectona grandis, P. santalinius, G. superba*. Moreover, the therapeutic efficacy was found to be higher even in low concentration. This clearly exhibits the advantages of administering the combinations of synthetic antibiotic 'aztreonam' and extract of *Tectona grandis, P. santalinius, G. superba* over the other two individual forms coupled with enhanced synergistic activity of antibiotic alone.

A better agreement was found between the agar well diffusion and MIC tests that were used to determine the antibacterial activity of *Pterocarpus santalinus, Tectona*

Table 2: Minimum inhibitory concentration (MIC) values of plant extracts and extracts- antibiotic combinations against *Bacillus subtilis, Pseudomonas aeruginosa, Escherichia coli* and *Staphylococcus aureus.*

Name of microorganism	Bacillus subtilis	Pseudomonas aeruginosa	Escherichia coli	Staphylococcus aureus
Treatment combination (Plant extract & antibiotic)				
Pterocarpus santalinus	≤ 0.312	≤0.625	-	≤5.0
Tectona grandis	≤0.625	≤0.312	≤0.625	≤2.5
Gloriosa superba	≤0.152	≤.1.25	≤0.156	≤0.780
Pterocarpus santalinus + Aztreonam	≤0.625	≤0.316	≤2.500	≤0.625
Tectona grandis + Aztreonam	≤0.625	≤0.625	≤0.312	≤0.312
Gloriosa superba + Aztreonam	≤0.312	≤1.250	≤0.312	≤0.625

grandis, *Gloriosa superba*. Gram-negative bacteria are more complex in structure than the Gram-positive bacteria; Still the extracts of these plants showed a higher activity against both Gram-positive bacteria (*Staphylococcus aureus, Bacillus subtilis)* and Gram negative bacteria (*Escherichia coli, Pseudomonas aeruginosa*. The extracts prepared from *Pterocarpus santalinus, Tectona grandis*, *Gloriosa superba* are a source of different secondary metabolites which may act in synergy to produce an increased activity against microbes. The results from the above studies may justify the use of plant in the treatment of certain diseases caused by microorganisms (Aboaba *et al*., 2006; Duraipandiyan *et al*., 2006; Parekh and Chanda; 2007).,. Therefore the results of the present study seems to be promising and may enhance the natural products uses, showing the potentiality of *Pterocarpus santalinus*, *Tectona grandis*, *Gloriosa superba* in the treatment of various infectious diseases caused by bacteria. Further studies on the chemical characteristics of extract and active components should be carried out for the plant and its antimicrobial property (Sharma *et al*., 2011, Geetha *et al*., 2011). The possible activities of substances found in plant extracts on ribosome structure and bacterial enzymes inhibition appear to be related with synergism profile between plant extracts and the inhibitions of protein synthesis, however, the understanding of synergism mechanism is fundamental to development of pharmacological agents to treat disease by various bacteria using medicinal plants .

Conclusion

The present study provides an insight for the use of natural products alongwith syntheic antibiotics for the effective managemnt and contol of different bacterial infection occurred in human and animals. The findings also have fairly good degree of correlation with ethnomedicinal uses of the plant. It aso explores pharmacological potential of different plant extracts and provide an insight to formulate new cost effective antimicrobial agent for multi drug resistant organisms, based on the synergistic activity of 'Aztreonam'.

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