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Study on the synergistic effect of ethanolic leaf extracts against some pathogenic microorganisms

Vijayarekha P[1]*, Sengottaiyan N[2]

[1] Research Scholar, Anna University, Chennai
[2] Hindustan College of Engineering and Technology, Coimbatore, Tamilnadu, India.

ABSTRACT

The ethanolic leaf extracts of six medicinal and aromatic plants, namely *Aegle marmelos, Asteracantha longifolia, Tinospora cardifolia*, *Adathoda vasica, Sebania grandiflora* and *Centella asiatica* were prepared and the sensitivity of the ethanolic leaf extracts was tested with selected Gram positive and Gram negative microorganisms and are determined. The possible combination of six ethanolic leaf extracts shows effective antimicrobial activity in both Gram positive and gram negative bacteria. The antibacterial activity is more effective in combinational ethanolic leaf extracts than when it is used alone. The synergistic activity of the combined extracts of *Tinospora cardifolia, Asteracantha longifolia* and *Aegle marmelos* shows remarkable antimicrobial activity than the other combinational extracts against the selected microbes.

Keywords: Leaf extracts, ethanol, synergistic activity, microbes and antimicrobial effect.

***Corresponding author**
Email: kar.sudha@gmail.com

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INTRODUCTION

The medicinal plants are the main resources and promising safe drugs for many infections and diseases, the complete screening of many medicinal plants for its biological and pharmacological properties is still unknown and it is under research. Many ongoing researches world-wide, is on medicinal plants for to study their pharmacological active compounds. Phytoconstituents such as flavonoids, alkaloids, phenols, etc. are having safe biological action. For example, studying about medicinal plants for their properties such as, antioxidant, anticancer and antimicrobial activities are the prime concern for finding out an effective active principle. Drug resistance is a growing public health threat with pathogenic organisms is a quick beginning to cope with the challenges posed by the therapeutic agents [2]. When the antibiotic penicillin was discovered some fifty years ago, it was considered the miracle drug of the century, but the development was suddenly changed with the resistance among the bacteria and studies were made on penicillin resistant types of bacteria [14]. The resistance to antibiotic treatment is a common phenomenon among the bacteria causative of every possible infection. Hence, attempts are being made to produce more effective antibiotics. The medicinal and aromatic plants constitute a major source of natural organic compounds widely used in medicine [4]. The rationale behind this approach is simple, that is, instead of testing the plants at random those plants which are being used by traditional societies and also those are aromatic in nature are being tested for their antimicrobial effect. The pharmacologically active products derived from medicinal plants may contribute to the search for new drugs.

The control of pathogenic microorganisms is a big question because they have developed resistance for synthetic and semi-synthetic drugs. The different plant species of medicinal claims have been screened for their antimicrobial activity against different groups of microorganisms. In search of new drugs several plant species have been explored for finding out activity compounds of natural origin. A study showed that the phytomedicinal formulations used by the street herbal vendors of Tamilnadu having remarkable and effective antimicrobial activities. Plants have aromatic phytochemical which constitutes a major source of natural organic compounds which are widely used in medicine [11]. Two anticancer drugs of vincristine and vinblastine are extracted from *Catharan thusroseus* and have been prescribed for leukemia and Hodgkins disease respectively [3]. The chemical known as 'Taxol' made headlines as a breakthrough treatment for ovarian cancer and it was extracted from the bark of *Taxus brevifolia* [12] and several species of fungi are sensitive to oils of *Pimentara cemosa* [1].

Phenols, quinones, flavononles, flavonoids, tannins, terpenoids, alkaloids and polyphenols are the major groups of antimicrobial compounds present in plants. Phenols in the highest oxidation state are effective against viruses, bacteria and fungi [11, 13 & 4]. Quinones are highly reactive and render substrates unavailable to the microorganisms. Plant phytoconstituent Flavonones having phenolic structures with one carbonyl group and are responsible for many microbial infections. Tannins are other polymeric phenolic substances shown in their antibiotic activity in methanolic extracts of the bark of *Teriminalia alata* [14]. The present study was aimed to determine the synergistic effect of ethanolic leaf extracts derived from six various species of against Gram positive and Gram negative bacteria [5].

Experimental Procedure

The following plants were selected, based on the easy availability and the presence of phytochemicals present in plants selected for the study.

Preparation of the Extract

The plants selected for the extract preparation are *Aegle marmelos, Centella asiatica, Asteracantha longifolia, Sebania grandiflora, Tinospora cardifolia* and *Adathoda vasica.* The leaves of the above plants collected and were individually washed with tap water, blotted with filter paper and spread over news paper for air drying under shade. After complete drying, the leaves of the individual plants were powdered using a mixer grinder. 50 gm of the leaf powder of each plant was taken in a 250 ml of conical flask to that added 100 ml of ethanol (85%). Ethanol was used for the extraction of phytochemicals because it has the ability to dissolve the phytochemical compounds like tannins, polyphenols, flavonoids, terpenoids and alkaloids [6]. The ethanol leaf powder mixtures were kept at room temperature for 48 hours and rapidly stirred using a glass rod every 8 hours. After 48 hours, the extract of each plant was filtered through the Whatmann No.1 filter paper

to exclude the leaf powder. Then each filtrate was kept in a beaker in a water bath at 45°C until the solvent got evaporated. The greasy final material (crude extract) obtained from each plant was transferred to a screw cap bottles and stored under refrigerated conditions till use [10].

Preparation of Stock and Test Solution

200 mg of each crude extract was carefully taken in a standard measuring flask and 5ml of ethanol was added to dissolve the extract and one or two drops of emulsifier (triton-X100) was added to completely dissolve the extract. Then it was made up to 200 ml by adding distilled water. This was made up to 200 ml by adding distilled water. This formed the stock solution of 1000 ppm. For studying the combined anti-microbial test micro-organisms, the test concentrations were prepared based on the number of extracts of the selected plants to be combined, i.e., combinations of extracts of any two, three plants, four plants, five plants and six plants in a separate container, care was taken to maintain 1000 ppm concentration of combined extract solutions by adding volumes of respective stock solutions of crude extracts.

Microorganisms used

Four selected bacterial species, namely *Bacillus Cereus*, *Streptococcus pyrogens, Pseudomonas aeruginosa and Proteus mirabilis* obtained from the Microbial Type Culture Collection (MTCC) of the Institute of Microbial Technology (IMTECH), Chandigarh, were used in the present study. These species were maintained by subculturing in nutrient agar slants. The Kirby-Bauer disc diffusion technique was used to test the sensitivity of selected test microorganisms to the ethanolic leaf extracts [1].

Preparation of Antimicrobial disc using Crude Extracts

Discs of 5 mm in diameter from a sheet of filter paper were punched out, placed in Petri dishes allowing a distance of 2 to 4 mm between each disc and sterilized in a hot air oven at 160°C for 1 hour. After allowing the disc to cool, 0.2 ml of each test solution was added on to each disc and then the discs were treated with distilled water (200 ml) containing 5 ml ethanol +2 drops of emulsifier at 0.02 ml/disc. The petri plates of 100 mm diameter with nutrient agar were swabbed with broth culture of each test bacteria in separate plates by using a sterile swab. Over this, prepared antimicrobial discs were placed under aseptic conditions. Three discs of each extract were placed in triangles. Control sets with standard antibiotic penicillin (30 gm/disc) were simultaneously maintained. Also the disc without plant extract (disc prepared using 200 ml distilled water + 5ml ethanol + one or two drops of emulsifier) were also maintained as another set of control for each test organism. The plates were then incubated at 37°C for 24 hours and the Zone of Inhibition (IZ) was measured and recorded. From the results, activity index was calculated by comparing the Zone of Inhibition (IZ) of leaf extracts with standard antibiotic.

Activity Index= Inhibition area of test sample/ Inhibition area of standard antibiotic.

Experimental Results

The crude ethanolic extracts of the leaves of selected plants were individually tested for antimicrobial activity against Gram negative and Gram positive bacteria.

Table 1 Effect of extraction Gram positive and Gram negative bacteria (24 hours)

S. No.	Ethanolic extracts of plant leaves used	Zone of Inhibition (ZI) in cm			
		SP	BC	PA	PM
A	*Aegle marmelos*	0.8	0.9	0.6	0.7
B	*Centella asiatica*	AD	AD	-	-
C	*Asteracantha longifolia*	1.3	1.1	1.0	0.9
D	*Sebania grandiflora*	-	-	-	0.1

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E	*Tinospora cardifolia*	1.3	1.2	1.0	0.9
F	*Adathoda vasica*	-	0.2	0.1	-

SP = *Streptococcus Pyrogens*, BC= *Bacillus Cereus*, PA = *Pseudomonas Aeruginosa,*
PM= *Proteus Mirabilis*, AD = Around Disc.

Table 2 Antibacterial reference standard

Antibiotic	Concentration	Test Microorganisms	Zone of Inhibition (cm)
Penicillin	30 µg	*Bacillus cereus*	2.1
Penicillin		*Pseudomonas aeruginosa*	1.8
Penicillin		*Proteus mirabilis*	1.9
Penicillin		*Streptococcus pyrogens*	1.8

Table 3: Activity index showed by the extracts against Gram positive and Gram negative bacteria (Reference standard: Penicillin)

S. No.	Ethanolic extracts of the plant species	Activity Index			
		SP	BC	PA	PM
A	*Aegle marmelos*	0.4	0.5	0.4	0.5
B	*Centel laasiatica*	-	-	0.2	0.1
C	*Asteracantha longifolia*	0.7	0.6	0.7	0.6
D	*Sebania grandiflora*	0.4	0.1	-	-
E	*Tinospora cardifolia*	0.6	0.5	0.6	0.5
F	*Adathoda vasica*	0.1	-	-	0.1

Table 4 Synergistic activity of ethanolic extracts in combination of three of the selected plants against Gram Positive and Gram Negative bacteria (24 hours)

S. No.	Combination of Plant extracts tested	Zone of Inhibition (IZ) in cm			
		SP	BC	PA	PM
1.	A+B+C	O.7	0.8	1.1	0.7
2	A+B+D	AD	AD	-	-
3	A+B+E	0.9	1.3	0.6	-
4	A+B+F	-	-	0.5	0.7
5	B+C+D	1.1	1.6	1.3	0.9
6	B+C+E	-	AD	-	-
7	B+C+F	1.7	1.6	1.7	1.3
8	C+D+E	1.2	1.2	1.6	1.1

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9	C+D+A	-	-	AD	-
10	C+D+F	1.2	1.1	1.6	0.9
11	D+E+A	-	1.4	1.4	-
12	D+E+B	1.3	1.4	1.4	1.2
13	D+E+F	1.7	1.7	1.6	1.3
14	E+F+A	-	0.3	0.2	-
15	E+F+B	1.6	1.6	1.8	1.3
16	E+F+C	1.4	1.7	1.7	1.5
17	F+A+C	-	0.6	0.8	AD
18	F+A+D	AD	-	-	0.1
19	F+B+D	AD	AD	-	-
20	A+C+E	1.7	1.7	1.8	1.7

Table 5 Activity index showed by the plant extracts in combination of three against Gram positive and Gram negative bacteria (Reference standard Penicillin)

S. No.	Combination of Plant extracts tested	Activity Index			
		SP	BC	PA	PM
1.	A+B+C	0.7	0.8	0.9	0.6
2	A+B+D	-	-	0.3	0.4
3	A+B+E	0.6	0.7	0.7	0.8
4	A+B+F	0.8	-	-	0.5
5	B+C+D	-	-	0.4	0.4
6	B+C+E	0.7	0.6	0.7	0.6
7	B+C+F	-	0.3	0.3	0.4
8	C+D+E	0.6	0.7	0.7	0.8
9	C+D+A	-	0.5	-	0.4
10	C+D+F	0.4	-	0.4	0.5
11	D+E+A	0.7	0.7	0.9	0.8
12	D+E+B	0.3	0.5	-	0.1
13	D+E+F	-	-	0.3	0.2
14	E+F+A	0.7	0.8	0.9	0.9

15	E+F+B	-	0.8	0.9	-
16	E+F+C	0.8	0.9	0.8	0.8
17	F+A+C	0.6	0.7	0.7	0.6
18	F+A+D	0.4	0.3	-	-
19	F+B+D	0.2	-	-	0.1
20	A+C+E	0.9	0.8	0.9	0.7

RESULTS

The crude ethanolic leaf extracts of the selected six plants *Aeglemarmelos, Centella asiatica, Asteracantha longifolia, Sebania grandiflora, Tinospora cardifolia* and *Adathoda vasica* were individually tested for its antimicrobial activity against gram negative and Gram positive bacteria. The ethanolic leaf extracts of *Centella asiatica, Sesbania grandiflora* showed no activity against all the bacterial species tested. The extracts of *Adathoda vasica* showed very minimum activity against *Bacillus cereus* and *Pseudomonas aeruginosa* only. The maximum activity was shown by *Aegle marmelos Astracantha longifolia, Tincospora cordifolia,* respectively (Table 1). The antimicrobial reference standard, Penicillin had an equal effect on all the bacterial strains tested (Table 2). No zone formation was observed in all the bacteria tested with control, i.e. without plant extract.

The synergistic activity of ethanolic extracts in 20 possible different combinations of the selected plants was performed and shown in Table.4. The maximum activity was shown by the ethanolic leaf extract (combinations of *Aegle marmelos + Astracantha longifolia + Tincospora cordifolia*). The other combinations showed minimum or on antimicrobial activity. The extract combination of the plants, namely *Centella asiatica*, *Sesbania grandiflora* and *Adathoda vasica* had no effect on any of the bacteria tested. The activity index recorded against different bacteria tested in is shown in Table 3. The experiment with the combination of the different leaf extracts for the activity index were determined in (Table 5). However, no significant synergistic effects were obtained against the selected Gram positive and Gram negative bacteria for these combinations.

DISCUSSION

In the present study, ethanolic extracts of the leaves of the plants, namely, *Aegle marmelos, Centella asiatica, Astracantha longifolia, Tincospora cordifolia, Sesbania grandiflora* and *Adathoda vasica* were tested individually and in different combinations against the gram positive bacteria *Bacillus cereus* and *Streptococcus pyrogens,* and Gram negative bacteria, *Pseudomonas aeruginosa* and *Proteus mirabilis*. The results on the effect of the extracts tested individually against the selected bacteria reveals that the crude ethanolic extracts of *Aegle marmelos***,** *Astracantha longifolia, Tincospora cordifolia* had a maximum zone of inhibition and activity index. The extracts of *Centella asiatica*, *Sesbania grandiflora* and *Adathoda vasica* had no effect on the test bacteria and this clearly indicates that the crude ethanolic extracts of these plants are not so effective. Since almost all the identified components extracted from the plants were active against microorganisms and are aromatic or saturated organic compounds in the present study, the use of ethanol for the extraction of phytochemicals was justified.

Synergistic activity of different combinations of the ethanolic extracts of the plant species used in the present study showed different degrees of antimicrobial activity against Gram positive and Gram negative bacteria and it has also been compared with a standard antibiotic Penicillin. The activity index was almost identical. With the combination of the plant extracts 20 different combinations were tested against the test bacteria, in which, wherever the *Aegle marmelos + Astracantha longifolia + Tincospora cordifolia*. The extract was combined with any of the other plant extract showed maximum activity. Remarkably, in the combination of three plant extracts, *Aegle marmelos + Astracantha longifolia + Tincospora cordifolia* along with any of the other plant extract showed enhanced antibacterial activity which was recorded as the maximum in the this study. This clearly indicates the synergistic effect of the plant extracts of some combination showed some

remarkable activity, the synergistic effect was less than that of the combination of three plants *Aegle marmelos + Astracantha longifolia + Tincospora cordifolia.* So, it is clear that effective synergistic activity of plant extracts relies on the particular combination of different plants, even through any one of the combined plants does not show any activity when used alone. They may act upon the microorganisms in several ways. Plant Phytoconstituents are classified as nitrogen compounds (alkaloids, non-protein amino acids, amines, alcamides, cyanogenic glycosides and glucosinolates) and non-nitrogen compounds (monoterpenes, diterpenes, triterpenes, tetraterpenes, sesquiterpenes, saponins, flavonoids, steroids and coumarins). Lectins another class of phytoconstituents which is carbohydrate-binding proteins and their biological properties include cell-cell interactions. This study reports a combination of plant extracts (mixture of secondary metabolites), with antimicrobial activity against Gram-negative and Gram-positive bacteria activity towards human [9].

Phenolics present in the plants cause the cell membrane disruption, and form complex with the cell wall and inactivate enzymes and cause substrate deprivation [8]. Alkaloids can intercalate into the cell wall. In the present study as the plants were selected based on the phytochemical substances present in them, the mechanism of action in the combined extracts could be due to any of the above mentioned mechanisms or a combined activity of one or more of them.

Summary

The ethanolic leaf extracts of six plant species *Aeglemarmelos, Centellaasiatica, Astracanthalongifolia, Tincosporacordifolia, Sesbaniagrandiflora* and *Adathodavasica* were tested alone and in possible combination of the plant extracts against gram positive and gram negative bacteria, *Bacillus cereus*, *Streptococcus pyrogens,* and *Pseudomonas aeruginosa*, *Proteus mirabilis.* The penicillin was used as standard antibiotic reference. The Zone of Inhibition and activity index for all the extracts for each of the test microorganisms was recorded. The antimicrobial activity of individual plant extract was higher in *Asteracantha longifolia* than the other extracts examine against test microorganisms. Among the different combinations tested, the combination of three plant extracts showed enhanced antimicrobial activity. *Aegle marmelos + Astracantha longifolia + Tincospora cordifolia* combination showed inhibition zones of 0.9, 0.8, 0.9, 0.7 and against *Streptococcus pyrogens, Bacillus cereus, Pseudomonas aeruginosa* and *Proteus mirabilis* respectively and this indicates their synergistic effect. The present study reveals that there is a scope to the ethanolic extracts of the leaves of *Aegle marmelos + Astracantha longifolia + Tincospora cordifolia* with other plant extracts against various bacteria.

CONCLUSION

The antimicrobial activity of ethanolic extracts of *Aegle marmelos, Centella asiatica, Asteracantha longifolia, Sesbania grandiflora, Tinospora cardifolia* and *Adathoda vasica* showed lower inhibition zones when used alone than that of the extract combinations. However, the combination of three plant extracts such as *Tinospora cardifolia, Asteracantha longifolia* and *Aegle marmelos* was enhanced maximum antimicrobial activity against the test microbes. The ZI reached 1.3 cm against certain bacteria by the combination of aqueous extracts of all the six plants used in the study, indicating the high potential of combined use of plant extracts against pathogenic microorganisms. The mechanism of the synergism of the plant extracts yet to be studied. There is a possibility of using plant extracts in combinations against pathogenic bacteria as has been observed from the results.

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