IJPSR (2016), Vol. 7, Issue 11 (Research Article)

INTERNATIONAL JOURNAL OF PHARMACEUTICAL SCIENCES AND RESEARCH

Received on 08 June, 2016; received in revised form, 21 September, 2016; accepted, 03 October, 2016; published 01 November, 2016

EFFECT OF MEDICINAL PLANT EXTRACTS ON THE ANTIMICROBIAL ACTIVITY OF AMOXYCLAVE AND ERYTHROMYCIN AGAINST *E. COLI* AND *S. AUREUS*

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Keywords:

Medicinal plants, Amoxyclave, Erythromycin, Antimicrobial and Synergistic Effect

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ABSTRACT: Development of microbial resistance leads new drug discovery, modification of antibiotics, administration of two or more antibiotics, use of traditional medicinal plants and their combinations with antibiotics. In the present study, 72 medicinal plants extracts were screened to potentiate the antimicrobial with erythromycin and amoxyclave. Among all the medicinal plant, *Colebrookea oppositifolia* was selected to analyze the synergistic activity with erythromycin and amoxyclave. The class I synergism (increase in the zone of inhibition) was observed in petroleum ether extract of leaves and inflorescence of *C. oppositifolia* in combination with amoxyclave with the increased zone from 6±0.2 mm to 8±0.2 mm and 6±0.2 mm to 10±0.2 mm, respectively. The class I synergisms also exhibited in methanol leaf extract of *C. oppositifolia* in combination with erythromycin and amoxyclave against *S. aureus*. Interestingly, the methanolic leaf extract (alone) did not show any antibacterial effect against *S. aureus*, but in combination with erythromycin/amoxyclave enhanced the zone of inhibition from 3±0.1mm to 9±0.2 mm and 5±0.2mm to 9±0.2mm, respectively. Class II synergism (making drug bactericidal) was exhibited by methanol leaf extract against *S. aureus*. The sequential fractionation of the methanolic extract using solvent extraction (n- butanol, ethyl acetate) showed the class I synergism in combination with amoxyclave and erythromycin. The solvent fraction of ethyl acetate has increased the zone of inhibition of erythromycin from 4±0.3 to 11±0.2 mm and amoxyclave from 8±0.1 to 12±0.1 mm against *S. aureus*. The n- butanol fraction increases the zone of inhibition of erythromycin from 4±0.1 to 12±0.2 mm, whereas the zone of inhibition of amoxyclave was increased from 8±0.1mm to 13±0.1mm by against *S. aureus*. The solvent fractions did not show synergism against *E. coli*. Class II synergism was shown by n- butanol and ethyl acetate fraction, which enhanced the potency of erythromycin by making it bactericidal.

INTRODUCTION: The use of antibiotics for the treatment of infectious diseases was the first choice since the discovery of Penicillin by Alexander Fleming in 1928, but still infectious diseases have remained a major cause of death in the history of mankind.

QUICK RESPONSE CODE

DOI: 10.13040/IJPSR.0975-8232.7(11).4615-25

Article can be accessed online on: www.ijpsr.com

DOI link: http://dx.doi.org/10.13040/IJPSR.0975-8232.7 (11).4615-25

Infectious diseases like cholera, tuberculosis, influenza, cryptosporidiosis, hepatitis, HIV/AIDS, meningitis is caused by bacteria, fungi and viruses that account for almost 1/3 of all the deaths in the World [1].

Meanwhile, the extensive use of antibiotics in healthcare has led to the co-evolutionary emergence of dangerous pathogens that are resistant to traditional antibiotics, as well as the re-emergence of some old infectious diseases. One of the major breakthroughs was the discovery of methicillin-resistant *Staphylococcus aureus* (MRSA) and became a problem for nosocomial

infections in 1990. The other observation was vancomycin-resistant *S. aureus* (VRSA). The resistance of *Psuedomonas aeruginosa* to many antimicrobial agents such as carbapenems, quinolones and aminoglycosides, is the topic of worry nowdays [2]. Therefore, increased antimicrobial resistance in pathogens has led scientists to think for other alternatives.

Since, medicinal plants have been used in many forms over the years to treat, manage or control infections [3] for centuries and their use in combination with drugs has led new scope. It has been observed that secondary metabolites of medicinal plants such as alkaloids, essential oils, fatty oils, resins, mucilages, tannins, gums etc. possess the antimicrobial activity [4]. For example, Taxol, derived from the bark of *Taxus brevifolia*, has been used to cure cancer of various types [5]. Picroliv, isolated from rhizome of *Picrorhiza kurroa*, showed hepatoprotective activity [6] and phenol glycoside androsin isolated from this plant was observed to be antiasthmatic [7]. But in contrasts to them, the combination of active biomolecules with known antimicrobial agents has proven to be beneficial. The compound (piperine) isolated from *P. nigrum*, in combination with rifampicin and isoniazid showed synergistic effect against *M. tuberculosis*, the new drug formulation against tuberculosis named as 'resorine' which contains reduced dose (200mg) of rifampicin + isoniazid (300mg) + piperine (10mg) [8].

However, the mechanism of plant extracts to potentiate antibiotics is not understood yet. In addition, the chemical diversity in plants with potential in improving the clinical efficacy of antibiotics still remains largely un-investigated. Thus, the aim of present study is to explore the medicinal plant diversity of Himachal Pradesh to potentiate the antimicrobial activity of traditional antibiotics, against resistant microbes. The screening of 71 medicinal plants of this region was performed to get best synergistic combinations with erythromycin and amoxyclave against *S. aureus* and *E. coli*.

MATERIAL AND METHODS:

Microbial strains: The strain of *Escherichia coli* (MTCC-739) was procured from Institute of Microbial Technology (IMTECH), Chandigarh. Erythromycin resistant strain of *Staphylococcus aureus* (ATCC-43300) was obtained from Post Graduate Institute of Medical Education and Research (PGIMER), Chandigarh and stored on nutrient agar plates and slants at 4°C. Bacterial strains were grown for 12 h in nutrient broth (NB) for 12 h at 37° C and 250 rpm. Muller Hinton (MH) agar medium [9] was used to study the antimicrobial activity by agar well diffusion assay [10] by incubating assay plates at 37°C for 14 h. The concentration of agar was 0.7 % for soft agar and 2% for hard agar. The culture media used in this study were purchased from HiMedia Labs, India. Erythromycin powder was purchased from MP Biomedicals, Inc. USA. Erythromycin (15 µg) and Amoxyclave (30 µg) discs (HiMedia Labs, India) were used as positive control.

Collection of Plant Material and extracts preparation: The plant material used in this study was collected during September- December, 2012 from Solan (altitude 1350 m, temp 20-30°C, humidity 55-68%) and Shimla (altitude 2202 m, temp 12-25°C, humidity 62-80%) district of Himachal Pradesh. The identity of medicinal plants was verified from the herbarium of Dr Y.S Parmar University of Horticulture and Forestry, Nauni, Himachal Pradesh. Plants and their parts used in the study are listed in the **Table 1**. The collected plant material was thoroughly washed under the running tap water, followed by surface sterilization with 1% H_2O_2 and then washed with autoclaved distilled water. The surface sterilized plant material were dried in oven at 40° C for 5 days or until they were dried completely. Dried plant material was ground in mixer grinder to make fine powder. The fine powder (5g) was extracted in Soxhlet apparatus [11] using ethanol as solvent. The extracts were evaporated in rota- evaporator at 40° C. The dried extract was stored at -20° C refrigerator until further use. Antimicrobial assay was performed by dissolving the dried extract in ethanol to a final concentration of 1mg/ml prior to use.

Antimicrobial assay: Antimicrobial activity was measured by using agar well diffusion method method [10]. Antibacterial activity was tested against drug resistant *S. aureus* and *E. coli*. Inoculums of each bacterium i.e. 2×10^8 cells (measuring the A_{600} = 1) were mixed with the 0.7 % Muller Hinton agar (soft agar), which was overlaid on the 2% M.H

agar (hard agar). The wells were punched with the cork borer (8 mm) in the soft agar and the plant extract each of 40 µg was loaded in the wells. After the incubation of 12 h at 37º C, the zone of inhibition was measured. In the case of disc diffusion assay, discs of Whatman filter no.1 were punched and autoclaved filter discs were loaded with the extracts and placed in the wells.

The zone of inhibition (mm) for three independent experiments of each plant extract and the antibiotic was measured. Erythromycin (30µg) was used as a positive control. The amount of extract used for the antibacterial assay alone and in combination with the erythromycin was 40µg and ethanol as a solvent control in the antibacterial assay. To perform the antibacterial assay for synergistic activity of *C. oppositifolia*, discs of erythromycin (15 µg) and amoxyclave (30 µg) were used alone and in combination with 40 µg extracts against *S. aureus* and *E. coli* strains. The activity was considered as synergistic, when the zone of clearance was increased as compared to the zone of individual extracts or antibiotics.

To assay the bactericidal or bacteriostatic activity of extracts/ antibiotics, cells were carefully taken from the zone of clearance around the well and streaked on nutrient agar plates and observed for the growth after 24 h of incubation at 37°C. Appearance of growth was considered as bacteriostatic, whereas no growth was considered as bactericidal effect.

Analysis of phytochemicals and their derivatives by biophysical techniques: 50 mg of methanolic leaf extract of *C. oppositifolia* was re- suspended in 5 ml luke warm water to make suspension. The mixture was transferred into a separating funnel. 100 ml chloroform was added to the separating funnel, followed by gentle shaking and chloroform fraction was collected. Same procedure of fractionation was repeated for n-butanol and ethyl acetate solvents to obtain fractions of different polarity.

RESULTS: The selected 72 medicinal plants (**Table 1**) were studied for the antimicrobial activity or synergistic effect either in the combination with erythromycin or amoxyclave. The dried powders of plant extracts were suspended in ethanol in the ratio of 1 mg/ml and 40 µl of the plant extract was loaded for antimicrobial activity against *S. aureus* and *E. coli*. Where, erythromycin (50 µg) was used as positive control. The synergistic effect was studied by the combination of 40 µg of plant extract and 50 µg of erythromycin. After the incubation of 12 h, zone of inhibition was measured (mm). In which erythromycin (50 µg) showed bacteriostatic effect against *S. aureus* and *E. coli*. From the list of 73 medicinal plants (**Table 1**), 17 plants were categorized under three classes of synergism as; 1) Class I- increased zone of inhibition; 2) Class II- zone of inhibition same but making drug bactericidal; 3) Class III- zone of inhibition was decreased in combination, but making drug bactericidal (**Table 2**).

TABLE 1: ANTIMICROBIAL ASSAY OF MEDICINAL PLANT EXTRACTS AGAINST *S. AUREUS* AND *E. COLI*

S. no	Name of the medicinal plant	Plant parts used	Zone of inhibition (mm)									
			Erythromycin (50µg)		Extract (40 µg)		Combination		Bacteriostatic /Bactericidal Extract		Combination	
			S. aureus	*E. coli*	*S. aureus*	*E. coli*	*S. aureus*	*E. coli*	*S. aureus*	*E. coli*	*S. aureus*	*E. coli*
1.	*Terminalia arjuna*	Bark	5±0.2	3±0.1	12 ±0.2	7±0.1	10±0.2	9±0.2	Bc	Bc	Bc	Bc
2.	*Eugenia Jambolana*	Leaves	5±0.2	3±0.2	9±0.2	6 ±0.2	9±0.1	6±0.2	Bs	Bc	Bc	Bc
3.	*Rhus cotinus*	Leaves	5±0.2	3±0.2	5±0.1	5 ±0.1	7±0.2	7±0.1	Bc	Bc	Bc	Bc
4.	*Allium cepa*	Bulb	5±0.2	3±0.2	6±0.2	5±0.2	6±0.1	6±0.2	Bs	Bs	Bc	Bc
5.	*Berberis aristata*	Leaves	5±0.1	3±0.1	6±0.1	5±0.1	8±0.2	6±0.2	Bc	Bc	Bc	Bc
		Fruits	5 ±0.2	3±0.2	3±0.2	2±0.2	5±0.1	4±0.2	Bc	Bc	Bc	Bc
6.	*Juglans regia*	Leaves	5±0.2	3±0.2	6±0.2	4±0.2	6±0.3	5±0.2	Bc	Bc	Bc	Bc
7.	*Pinus roxburghii*	Bark	5±0.1	3 ±0.1	6±0.2	6±0.2	7±0.2	7±0.1	Bc	Bc	Bc	Bc
		Leaves	5±0.2	3±0.2	10±0.1	6±0.2	10±0.2	9±0.3	Bc	Bc	Bc	Bc
8.	*Rhododendron viscosum*	Flower	5±0.1	3±0.1	7±0.2	6±0.2	7±0.2	7±0.2	Bs	Bs	Bs	Bs
9.	*Piper nigrum*	Fruit	5±0.2	3±0.2	4±0.2	8±0.2	9±0.2	12±0.2	Bs	Bc	Bc	Bc
10	*Withania somnifera*	Fruit	5±0.2	3±0.2	2±0.2	3±0.1	5±0.2	5±0.1	Bc	Bc	Bc	Bc
		Leaves	5±0.3	3±0.1	12±0.2	3±0.2	15±0.2	6±0.2	Bc	Bc	Bc	Bc
11	*Datura metel*	Fruit	5±0.2	3±0.2	2±0.3	2±0.2	5±0.2	3±0.2	Bs	Bs	Bs	Bc
12	*Capsicum annum*	Fruit	5±0.2	3±0.2	5±0.2	2±0.2	5±0.2	3±0.3	Bs	Bs	Bs	Bs
13	*Punica granatum*	Flower	5±0.2	3±0.3	9±0.3	8±0.2	11±0.2	9±0.1	Bs	Bc	Bc	Bc
		Leaves	5±0.2	3±0.2	5±0.2	6±0.3	5±0.2	6±0.2	Bs	Bc	Bs	Bc
14	*Lawsonia alba*	Leaves	5±0.2	3±0.2	6±0.2	7±0.2	8±0.1	9±0.2	Bc	Bc	Bc	Bc

15	*Zingiber officinale*	Bulb	5±0.3	3±0.2	5±0.2	4±0.2	6±0.2	6±0.1	Bs	Bs	Bc	Bc
16	*Curcuma longa*	Bulb	5±0.4	3±0.2	2±0.2	5±0.3	6±0.1	7±0.2	Bc	Bc	Bc	Bc
17	*Emblica officinalis*	Leaves	5±0.1	3±0.2	8±0.2	4±0.2	9±0.2	4±0.1	Bc	Bc	Bc	Bc
18	*Nigella sativa*	Seed	5±0.2	3±0.2	4±0.2	4±0.3	5±0.2	6±0.1	Bc	Bc	Bc	Bc
19	*Zanthoxyllum armatum*	Leaves	5±0.1	3±0.2	5±0.1	6±0.2	6±0.2	7±0.2	Bs	Bc	Bc	Bc
20	*Ruta gravelons*	Fruit	5±0.2	3±0.2	5±0.2	6±0.1	7±0.2	9±0.2	Bs	Bc	Bc	Bc
21	*Aegle marmelos*	Fruit	5±0.2	3±0.1	6 ±0.1	6±0.2	7±0.2	7±0.1	Bs	Bs	Bc	Bs
22	*Cannabis sativa*	Leaves	5±0.1	3±0.2	5±0.2	8±0.1	5±0.2	12±0.2	Bc	Bc	Bc	Bc
23	*Myristica fragrans*	Fruit	5±0.2	3±0.2	5±0.2	4±0.1	6±0.2	7±0.2	Bc	Bc	Bc	Bc
		Flower	5±0.1	3±0.2	3±0.2	3±0.2	6±0.1	3±0.2	Bc	Bc	Bc	Bc
24	*Populus Canadensis*	Leaves	5±0.2	3±0.3	7±0.1	2±0.2	7±0.2	3±0.2	Bc	Bs	Bc	Bc
25	*Cinnamonum tamala*	Bark	5 ±0.2	3±0.4	Nd	2±0.2	7±0.3	4±0.2	Bs	Bs	Bs	Bs
26	*Azardica indica*	Leaves	5 ±0.2	3±0.2	6±0.2	5±0.2	6±0.2	5±0.6	Bs	Bc	Bc	Bc
27	*Ferula asafoetida*	Latex	5 ±0.2	3±0.2	5±0.1	Nd	5±0.1	3±0.3	Bc	Bs	Bc	Bs
28	*Allium sativum*	Bulb	5 ±0.2	3±0.2	1±0.2	1±0.2	6±0.2	3±0.2	Bs	Bs	Bs	Bs
29	*Callistemon citrinus*	leaves	5 ±0.2	3±0.2	5±0.2	4±0.2	7±0.2	5±0.2	Bc	Bc	Bc	Bc
30	*Syzygium aromaticum*	Flower bud	5 ±0.2	3±0.1	7±0.2	5±0.2	7±0.4	7±0.2	Bs	Bc	Bc	Bc
31	*Ajuga reptans*	Flower	5 ±0.2	3±0.2	4±0.2	5±0.2	4±0.2	5±0.2	Bc	Bs	Bc	Bs
		Leaves	5 ±0.2	3±0.1	9±0.2	7±0.2	9±0.3	9±0.2	Bs	Bc	Bc	Bc
32	*Colebrookea oppositifolia*	Inflorescence	5 ±0.3	3±0.2	Nd	5±0.2	7±0.2	6±0.2	Bc	Bc	Bc	Bc
		Bark	5 ±0.2	3±0.2	5±0.2	5±0.2	5±0.3	6±0.2	Bc	Bc	Bc	Bc
33	*Thymus vulgaris*	Whole plant	5±0.3	3±0.2	5±0.3	4±0.2	5±0.2	6±0.2	Bs	Bc	Bs	Bc
34	*Euphorbia hirta*	Fruit	5±0.3	3±0.2	5±0.2	3±0.2	5±0.3	3±0.2	Bs	Bs	Bs	Bs
35	*Taraxacum officinale*	Leaves	5±0.3	3±0.4	7±0.2	Nd	7±0.2	3±0.2	Bc	Bs	Bc	Bs
36	*Camellia sinensis*	Leaves	5±0.1	3±0.1	2±0.2	Nd	2±0.2	3±0.2	Bs	Bs	Bs	Bs
37	*Hypericum perforatum*	Flower	5 ±0.2	3 ±0.2	19±0.2	8 ±0.2	20±0.2	10±0.2	Bc	Bc	Bc	Bc
38	*Abrus precatorius*	Seeds	5±0.1	3 ±0.2	7±0.2	2 ±0.2	7±0.2	3±0.2	Bs	Bs	Bc	Bc
39	*Cymbopogon citrates*	Leaves	5 ±0.2	3±0.2	15±0.2	16±0.2	17±0.1	20±0.2	Bc	Bc	Bc	Bs
40	*Bombax ceiba*	Flower	5 ±0.2	3±0.2	3±0.4	Nd	5±0.4	3±0.2	Bs	Nd	Bs	Bs
41	*Cuscuta reflexa*	Stem	5 ±0.2	3±0.2	Nd	Nd	5±0.2	3±0.2	Bs	Nd	Bs	Bs
42	*Tinospora cordifolia*	Stem	5±0.2	3±0.2	5±0.2	4±0.2	7±0.3	7±0.2	Bc	Bc	Bc	Bc
43	*Chrysanthemum indicum*	Leaves	5 ±0.1	3±0.2	4±0.3	5±0.2	6±0.2	7±0.3	Bc	Bc	Bc	Bc
44	*Mentha viridis*	Leaves	5 ±0.2	3 ±0.1	1±0.5	1±0.2	5±0.2	3±0.1	Bs	Bs	Bs	Bs
45	*Berginia ciliate*	Leaves	5 ±0.2	3±0.2	2±0.2	4 ±0.2	6±0.2	4±0.1	Bs	Bc	Bc	Bc
46	*Urtica dioica*	Leaves	5±0.2	3±0.2	Nd	Nd	5±0.2	3±0.2	Bs	Nd	Bs	Bs
47	*Trigonellia foenum-graecum*	Leaves	5±0.5	3±0.1	2±0.2	Nd	5±0.2	3±0.3	Bs	Nd	Bs	Bs
48	*Prunus amygdalus*	Oil	5±0.2	3±0.2	Nd	Nd	5±0.2	3±0.2	Bs	Nd	Bs	Bs
49	*Trachyspermum ammi*	Seeds	5±0.2	3±0.1	1±0.2	Nd	5±0.1	3±0.2	Bs	Nd	Bs	Bs
50	*Bauhinia variegate*	Flower	5±0.1	3±0.2	1±0.2	2±0.2	5±0.2	3±0.2	Bs	Bs	Bs	Bs
51	*Musa paradisiacal*	leaves	5±0.1	3±0.2	6±0.1	8±0.1	8±0.3	6 ±0.2	Bc	Bc	Bc	Bc
52	*Mimosa pudica*	Se eds	5±0.2	3±0.2	Nd	Nd	7±0.3	3±0.2	Bs	Bs	Bs	Bs
53	*Brassica nigra*	Seeds	5±0.2	3 ±0.1	2±0.4	2±0.2	5±0.2	3±0.2	Bc	Bc	Bc	Bc
54	*Rumex hastatus*	Leaves	5±0.2	3±0.2	3±0.3	Nd	5±0.1	3±0.2	Bs	Nd	Bs	Bs
55	*Colocasia esculenta*	Tuber	5±0.2	3 ±0.2	Nd	Nd	5±0.1	3±0.2	Nd	Nd	Bs	Bs
56	*Asclepias curassavica*	Leaves	5 ±0.2	3 ±0.2	1±0.2	2±0.2	5±0.2	3±0.2	Bc	Bc	Bc	Bc
		Flower	5 ±0.2	3 ±0.2	2±0.2	2±0.1	5±0.2	3±0.2	Bc	Bc	Bc	Bc
		Stem	5 ±0.2	3 ±0.3	2±0.3	5±0.3	3±0.3	3±0.2	Bs	Bs	Bs	Bs
57	*Cocos nucifera*	Oil	5 ±0.2	3 ±0.3	Nd	Nd	5±0.2	3±0.3	Nd	Nd	Bs	Bs
58	*Podophyllum hexandrum*	Seeds	5 ±0.2	3 ±0.1	Nd	Nd	5±0.2	3±0.2	Bs	Nd.	Bs	Bs
59	*Vitex negundo*	Leaves	5 ±0.2	3 ±0.2	5 ±0.3	6±0.2	7±0.2	7±0.2	Bc	Bs	Bc	Bs
60	*Ageratum conyzoides*	Flower	5 ±0.3	3±0.1	2±0.2	4±0.2	5±0.2	3±0.1	Bc	Bs	Bc	Bs
61	*Cichorium intybus*	Leaves	5 ±0.2	3 ±0.2	Nd	2±0.3	5±0.2	3±0.2	Bs	Bs	Bs	Bs
62	*Pelargonium hortorum*	Leaves	5±0.1	3 ±0.1	2±0.2	2±0.2	5±0.3	3±0.2	Bc	Bs	Bc	Bc
63	*Cinnamomum camphora*	Leaves	5 ±0.2	3 ±0.2	3±0.3	2±0.2	5±0.1	6±0.1	Bc	Bc	Bc	Bc
64	*Parthenium hysterophorus*	Whole plant	5 ±0.1	3 ±0.2	1±0.2	2±0.3	5±0.2	3±0.2	Bc	Bc	Bc	Bc
65	*Brassica oleracea*	Flower	5 ±0.2	3 ±0.1	2±0.3	Nd	5±0.1	3±0.2	Bs	Nd	Bs	Bs
67	*Lavandula angustifolia*	Leaves	5 ±0.1	3 ±0.1	5±0.2	4±0.1	7±0.2	4±0.1	Bc	Bc	Bc	Bc
68	*Chenopodium album*	Leaves	5 ±0.3	3 ±0.3	1±0.2	Nd	5±0.2	3±0.2	Bs	Nd	Bs	Bs
69	*Oenothera biennis*	Flower	5 ±0.2	3 ±0.1	1±0.2	2±0.2	5±0.2	3±0.2	Bs	Bs	Bs	Bs
70	*Carissa spinarum*	Leaves	5 ±0.2	3 ±0.1	1±0.4	1±0.2	5±0.1	3±0.3	Bs	Bs	Bs	Bs
71	*Santalum album*	Bark	5±0.2	3 ±0.5	2±0.2	2±0.2	6±0.2	5±0.2	Bc	Bc	Bc	Bc
72	*Cathrenthus rosens*	Leaves	5±0.1	3 ±0.1	2±0.2	1±0.2	5±0.3	3±0.2	Bc	Bs	Bc	Bc
73	*Momordica charntia*	leaves	5 ±0.4	3 ±0.3	2±0.4	1 ±0.3	5 ±0.3	3 ±0.4	Bs	Bs	Bs	Bs

Nd- no zone of inhibition detected, Bc- bactericidal, Bs- bacteriostatic

TABLE 2: LIST OF MEDICINAL PLANTS CATEGORIZED UNDER THREE CLASSES OF SYNERGISM AGAINST *S. AUREUS* AND *E. COLI*

S. no	Name of the plant	Plant parts used	Bacteria	Class I	Class II	Class III
1	*Aegle marmelos*	Fruit	*S. aureus*	+	-	-
			E. coli	+	-	-
2	*Curcuma longa*	Tuber	*S. aureus*	-	-	-
			E. coli	+	-	-
3	*Tinospora cordifolia*	Stem	*S. aureus*	+	-	-
			E. coli	+	-	-

4	*Cymbopogon citrates*	Leaves	*S. aureus*	+	-	-
			E. coli	+	-	-
5	*Allium cepa*	Bulb	*S. aureus*	+	-	-
			E. coli	+	-	-
6	*Zingiber officinalis*	Tuber	*S. aureus*	+	-	-
			E. coli	+	-	-
7	*Cinnamonum tamala*	Bark	*S. aureus*	+	-	-
			E. coli	+	-	-
8	*Pinus roxburghii*	Leaves	*S. aureus*	+	-	-
			E. coli	+	-	-
9	*Piper nigrum*	Seeds	*S. aureus*	-	-	-
			E. coli	+	-	-
10	*Withania somnifera*	Leaves	*S. aureus*	+	-	-
			E. coli	+	-	-
11	*Punica granatum*	Flower	*S. aureus*	+	-	-
			E. coli	+	-	-
12	*Lawsonia alba*	Leaves	*S. aureus*	+	-	-
			E. coli	+	-	-
13	*C. oppositifolia*	Inflorescence	*S. aureus*	+	-	-
			E. coli	+	-	-
		Leaves	*S. aureus*	-	-	-
			E. coli	-	+	-
14	*Zanthoxyllum armatum*	Leaves	*S. aureus*	-	+	-
			E. coli	-	+	-
15	*Terminalia arjuna*	Bark	*S. aureus*	-	-	+
			E. coli	-	-	-
16	*Syzygium aromaticum*	Flower bud	*S. aureus*	-	+	-
			E. coli	+	-	-
17	*Musa paradisiaca*	Leaves	*S. aureus*	-	-	-
			E. coli	-	-	+

Class I- extract alone showed zone of inhibition, but in combination with erythromycin become bactericidal; Class II- zone of inhibitions remains the same with the extract alone and combination of plant extract with erythromycin, but making drug bactericidal; Class III- zone of inhibition was decreased in combination with erythromycin as compare to extract alone, but combination with erythromycin become bactericidal.

Minus (-) indicates absence and plus (+) indicates presence of synergistic activity

TABLE 3: ANTIMICROBIAL ASSAY OF POLAR AND NON POLAR EXTRACTS OF *C. OPPOSITIFOLIA* AGAINST *S. AUREUS*

S. no	Plant parts	Solvent used for extraction	Zone of inhibition (mm)					Bs/ Bc (AE)	Bs/Bc (BE)
			A	B	E	AE	BE		
1.	Leaves	Petroleum ether	6±0.2	3±0.1	6±0.1	8±0.2	3±0.2	Bc	Bs
		Methanol	5 ±0.2	3±0.1	Nd	7±0.2	9±0.2	Bc	Bc
2.	Inflorescence	Petroleum ether	6 ±0.2	3±0.2	8±0.1	10±0.2	7±0.2	Bc	Bc
		Methanol	6±0.1	3±0.1	6±0.2	6±0.1	4±0.2	Bc	Bc
5.	Bark	Petroleum ether	6±0.3	3±0.2	6±0.3	6±0.2	8±0.2	Bc	Bc
		Methanol	6±0.2	3±0.2	5±0.2	6±0.1	7±0.2	Bc	Bs

Nd- no zone of inhibition detected; Bs- bacteriostatic; Bc-bactericidal; A- Amoxyclave; B- Erythromycin; E- Extract alone; AE- amoxyclave and plant extract together; BE- erythromycin and plant extract together

Under these classes, 17 plant extracts have shown synergistic effect (**Table 3)** against *E. coli* and *S. aureus.* In class I category, the fruit extract of *A. marmelos* was observed synergistic against *S. aureus* with the increase of zone size of erythromycin from 5±0.2mm to 7±0.2mm, whereas extract alone has shown zone of 6±0.1 mm against *S. aureus* (**Fig 2.S**). Similarly *T. cordifolia* (stem) (**Fig 1.A**), *C. citrates* (oil), *A. cepa* (bulb), *Z. officinale* (tuber) (**Fig 1.D**), *C. tamala* (bark) (**Fig 1.J**), *M. fragrans* (fruits) (**Fig 1.K**), *P. nigrum* (fruit), *W. somnifera* (leaves), *P. granatum* (flower) and *L. alba* (leaves) (**Fig 1.F**) has shown the class I synergism with the increase in zone of inhibition of erythromycin (5±0.2) to 6±0.3, 17±0.1, 6±0.1, 7±0.2, 7±0.3, 9±0.2, 9±0.2, 15±0.2, 11±0.2 and 8±0.1, respectively against *S. aureus.* Zone of inhibition shown by these plants extracts against *S. aureus* was 3±0.2 (*T. cordifolia*), 15±0.2 (*C. citrates*), 6±0.2 (*A. cepa*), 4±0.2 (*Z. officinalis*), 3±0.2 (*C. tamala*), 6±0.2 (*M. fragrans*), 4±0.2 (*P. nigrum*), 12±0.2 (*W. somnifera*), 9±0.3 (*P. granatum*) and 6±0.2 (*L. alba*).

The *C. oppositifolia* (inflorescence) extract shows class I synergism as it did not show any antibacterial activity alone but enhanced the zone of inhibition of erythromycin from 5±0.3 to 7±0.2 against *S. aureus.* Ethanolic extract of *C. longa* (tuber) showed class I synergism against *E. coli*, with increase in the zone of inhibition of erythromycin from 3±0.4mm to 7±0.1mm, whereas *C. longa* (tuber) extract showed 5±0.2 mm zone of inhibition against *E. coli* **(Fig 1.M).** The zone of inhibition of erythromycin (3±0.2) was enhanced by extract of *T. cordifolia* stem (7±0.2) (**Fig 1.M),** *C. citrates* oil (20±0.2), *A. cepa* bulb (6±0.2), *Z. officinalis* tuber (6±0.1) (**Fig 1.P**), *C. tamala* bark (4±0.2) (**Fig 1.V**), *M. fragrans* fruits (7±0.2) (**Fig 1.W**), *P. roxburghii* bark (7±0.1), *P. nigrum* fruit (12±0.2), *W. somnifera* leaves (6±0.2), *P. granatum* flower (6±0.2), *L. alba* leaves (9±0.2) (**Fig 1.R**) and *C. oppositifolia* inflorescence (6±0.2) with class I synergism against *E. coli*. The zone of inhibition shown by these plants alone was 5±0.3, 4±0.2, 16±0.2, 5±0.2, 5±0.2, 2±0.2, 3±0.1, 6±0.2, 8±0.2, 3±0.2, 8±0.2, 5±0.2 and 5±0.2, respectively against *E. coli.*

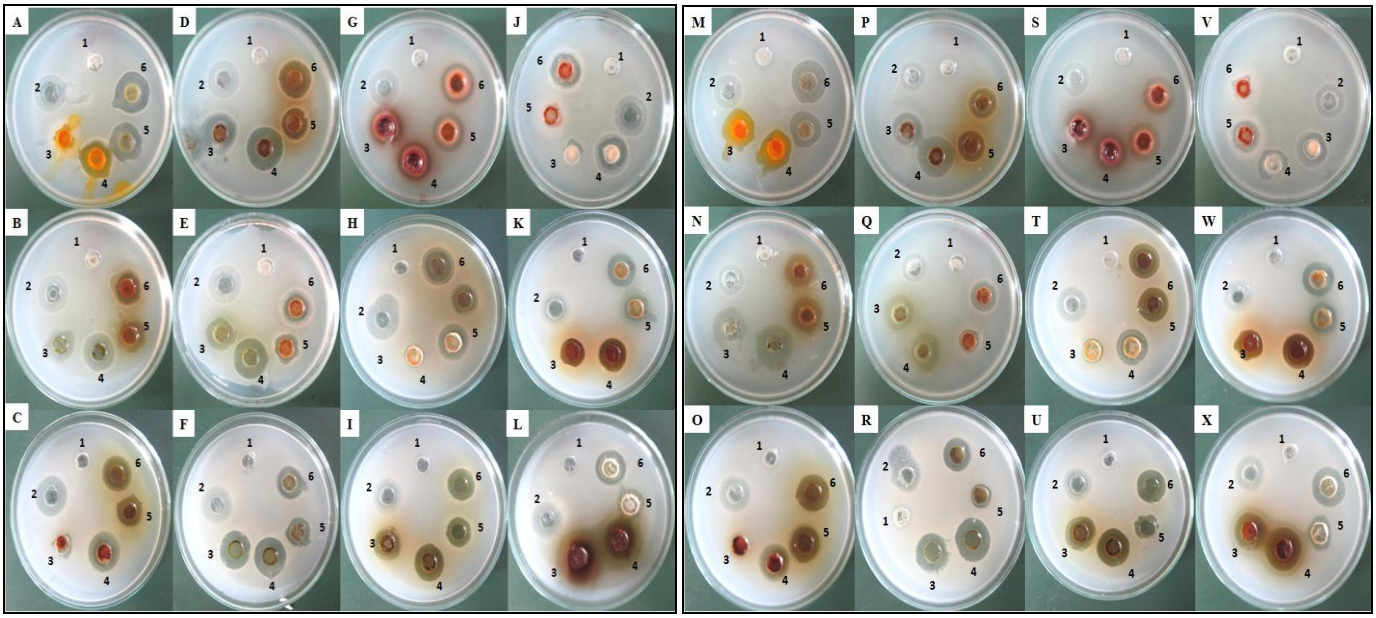

FIG. 1: ANTIMICROBIAL ASSAY OF MEDICINAL PLANTS EXTRACTS AGAINST *S. AUREUS* (A-L) AND *E. COLI* (M-X): 1- ethanol, 2- erythromycin; (A, M) 3- *Curcuma longa* (tuber); 4- erythromycin and *Curcuma longa;* 5- *Tinospora cordifolia* (stem); 6- erythromycin and *Tinospora cordifolia (*B, N) 3- *Zanthoxyllum armatum* (leaves)*;* 4- erythromycin and *Zanthoxyllum armatum;* 5-*Ajuja reptans* (flower); 6- erythromycin and *Ajuja reptans* (C, O) 3- *Chenopodium album* (leaves)*;* 4- erythromycin and *Chenopodium album;* 5- *Colebrookea oppositifolia* (bark); 6- erythromycin and *Colebrookea oppositifolia* (D, P) 3- *Zingiber officinale* (tuber)*;* 4- erythromycin and *Zingiber officinale;* 5- *Myristica fragrans* (flower); 6- erythromycin and *Myristica fragrance* (E, Q) 3- *Callistemon citrinus* (leaves)*;* 4- erythromycin and *Callistemon citrinus;* 5- *Berberis aristata* (fruit); 6- erythromycin and *Berberis aristata* (F, R) 3- *Lawsonia alba* (leaves)*;* 4- erythromycin and *Lawsonia alba;* 5- *Datura metel* (fruit); 6- erythromycin and *Datura metel* (G, S) 3- *Pinus roxburghii* (leaves)*;* 4- erythromycin and *Pinus roxburghii;* 5- *Aegle marmelos* (fruit); 6- erythromycin and *Aegle marmelos* (H, T) 3- *Santalum album* (bark)*;* 4- erythromycin and *Santalum album;* 5- *Allium sativum* (bulb); 6- erythromycin and *Allium sativum* (I, U) 3- *Chrysanthemum indicum* (leaves)*;* 4- erythromycin and *Chrysanthemum indicum;* 5- *Cinnamomum camphora* (leaves); 6- erythromycin and *Cinnamomum camphora* (J, V) 3- *Cinnamonum tamala* (bark)*;* 4- erythromycin and *Cinnamonum tamala;* 5-*Oenothera biennis* (flower); 6- erythromycin and *Oenothera biennis* (K, X) 3- *Myristica fragrans* (fruit)*;* 4- erythromycin and *Myristica fragrans* (fruit)*;* 5- *Withania somnifera* (fruit); 6- erythromycin and *Withania somnifera* (L,X) 3- *Syzygium aromaticum* (flower bud)*;* 4- erythromycin and *Syzygium aromaticum;* 5- *Nigella sativa* (seed); 6- erythromycin and *Nigella sativa*

Antibacterial or synergistic activity of methanolic and petroleum ether extracts of *C. oppositifolia* were analyzed against *S. aureus*. The class I synergistic effect was observed in leaf extract (petroleum ether) against *S. aureus* with the increase in zone of amoxyclave from 6±0.2 to 8±0.2 mm (**Fig 2.B**). Inflorescence extract (petroleum ether) of *C. oppositifolia* showed class I synergistic effect with the increase in the zone of inhibition of amoxyclave from 6±0.2 to 10±0.2 mm (**Fig 2.A**). The zone of inhibition of petroleum ether extracts of leaves and inflorescence alone was 6±0.1 and 8±0.1 mm respectively. The methanolic leaves extract of *C. oppositifolia* showed class I synergism in combination with erythromycin and amoxyclave. Interestingly, the methanolic leaf extract have not shown any antibacterial effect against *S. aureus*, but enhances the zone of

inhibition of erythromycin from 3±0.1 to 9±0.2 mm (**Fig 2.E**) and amoxyclave from 5±0.2 to 9±0.2 mm (**Fig 2.B**).

Class II synergism was exhibited by methanolic leaf extract against *S. aureus*, by making erythromycin bactericidal. The methanolic leaf extract of *C. oppositifolia* and erythromycin, both were bacteriostatic (**Fig 2.e**). The synergistic effect was more pronounced in leaf extracts as compared to inflorescence and bark, as methanolic leaves extract was not antibacterial alone against *S. aureus* but enhances the effect of erythromycin and amoxyclave (**Table 4**). The methanolic extract was further subjected to the sequential fractionation using n- butanol, ethyl acetate and chloroform solvent fractions. n- butanol and ethyl acetate fractions exhibited potent synergistic effect (class I) in combination with amoxyclave and erythromycin (**Fig 2.A** and **B**) against *S. aureus*. The zone of inhibition of erythromycin was increased from 4±0.3 to 11±0.2 mm, whereas the zone of inhibition of amoxyclave was increased from 8±0.1 to 12±0.1 mm by ethyl acetate fraction respectively against *S. aureus*. The zone of inhibition of erythromycin was increased from 4±0.1 to 12±0.2, whereas the zone of inhibition of amoxyclave was increased from 8±0.1 to 13±0.1 by n- butanol fraction against *S. aureus*. The ethyl acetate and n- butanol fractions were not antibacterial alone against *S. aureus* but showed the zone of inhibition of 1±0.1 and 2±0.3mm respectively against *E. coli* (**Fig 2.A** and **B)**.

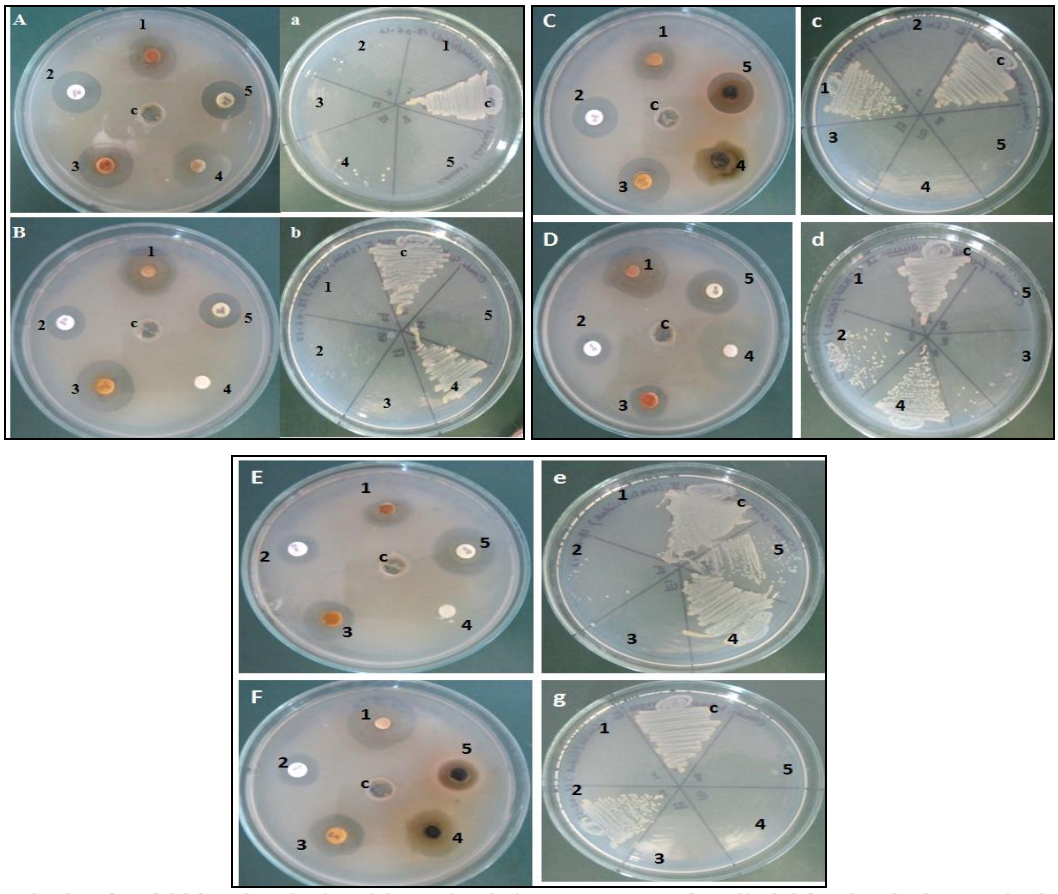

FIG. 2: ANTIMICROBIAL ASSAY OF *C. OPPOSITIFOLIA* (DIFFERENT PARTS) AGAINST *S. AUREUS*: A, B, C, D, E, F SHOWED ANTIBACTERIAL ASSAY AND a, b, c, d, e AND f SHOWED BACTERICIDAL AND BACTERIOSTATIC ASSAY:

c- ethanol; 2- amoxyclave (Fig A and a) 1- inflorescence extract of petroleum ether; 3- amoxyclave and inflorescence extract (petroleum ether); 4- bark extract of petroleum ether; 5- bark (petroleum ether) and amoxyclave (Fig B and b) 1- leaf extract (petroleum ether); 3- amoxyclave and leaf extract (petroleum ether); 4- leaf extract of methanol; 5- leaf extract (methanol) and amoxyclave (Fig C and c) 1- bark extract of methanol; 3- amoxyclave and bark extract (methanol); 4- inflorescence extract of methanol; 5- inflorescence extract (methanol) and amoxyclave.

c- ethanol; 2- erythromycin (Fig D and d) 1- leaf extract of petroleum ether; 3- erythromycin and leaf extract (petroleum ether); 4- bark extract of petroleum ether; 5- bark extract (petroleum ether) and erythromycin (Fig E and e) 1- inflorescence extract of petroleum ether; 3- erythromycin and inflorescence extract (petroleum ether); 4- leaf extract of methanol; 5- leaf extract (methanol) and erythromycin (Fig F and f) 1- bark extract of methanol; 3- erythromycin and bark extract (methanol); 4- inflorescence extract of methanol; 5- inflorescence extract (methanol) and erythromycin

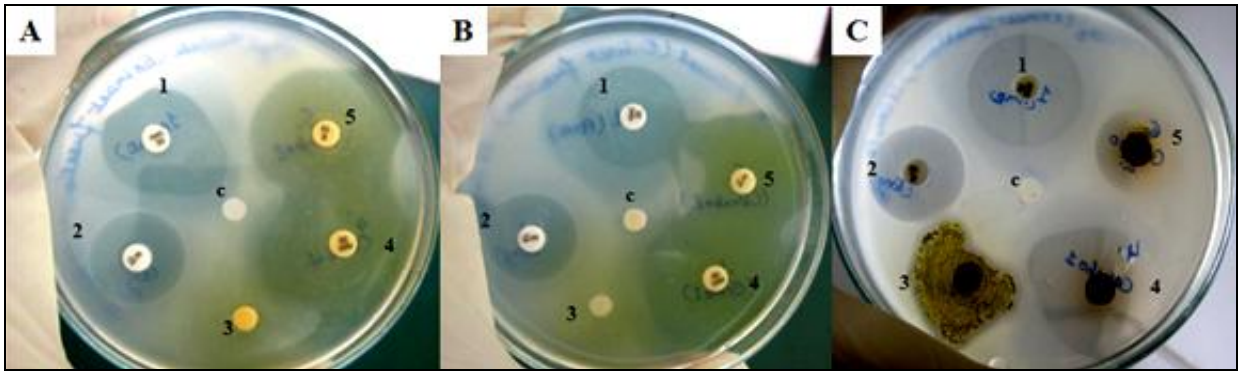

FIG. 3: ANTIMICROBIAL ASSAY OF POLAR AND NON POLAR FRACTIONS OF *C. OPPOSITIFOLIA* AGAINST *S. AUREUS:* A) Ethyl acetate B) n- butanol C) chloroform: c- ethanol; 1- amoxyclave; 2- erythromycin; 3- extract fraction; 4-combination of amoxyclave and extract fraction; 5- combination of erythromycin and extract fraction

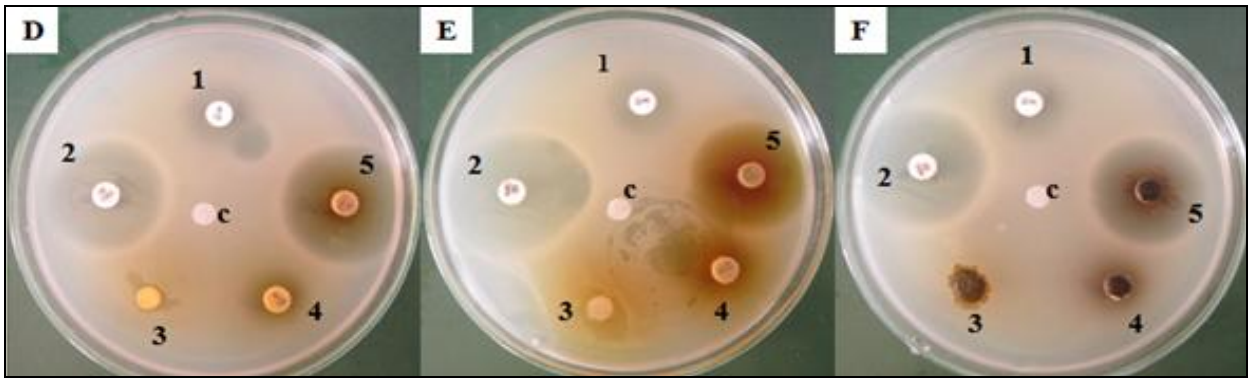

FIG. 4: ANTIMICROBIAL ASSAY OF POLAR AND NON POLAR FRACTIONS OF *C. OPPOSITIFOLIA* AGAINST *E. COLI*: D -Ethyl acetate; E- n- butanol; F – chloroform; c- ethanol; 1- amoxyclave; 2- erythromycin; 3- extract fraction; 4-combination of amoxyclave and extract fraction; 5- combination of erythromycin and extract fraction

Though the chloroform fraction showed antimicrobial activity with the zone of inhibition of 8±0.1 mm but no further increase in the zone of inhibition was observed when combined with erythromycin and amoxyclave against *S. aureus*. Chloroform fraction did not show any antimicrobial activity against *E. coli*. Class II synergism was shown by n- butanol and ethyl acetate fraction, in which both of these fractions enhances the toxicity of erythromycin by making it bactericidal against both the tested pathogens (**Tables 4** and **5**).

TABLE 4: ANTIMICROBIAL ACTIVITY OF POLAR AND NON-POLAR FRACTIONS OBTAINED OF *C. OPPOSITIFOLIA* EXTRACT AGAINST *S. AUREUS*

Extract (plant part)	Fraction	Zone of inhibition (mm)					Bs/Bc (3+1)	Bs/ Bc (3+2)
		1	2	3	4	5		
Leaves (Methanol)	Ethyl acetate	8±0.1	4±0.3	Nd	12±0.1	11±0.2	Bc	Bc
	n-butanol	8±0.1	4±0.1	Nd	13±0.3	12±0.1	Bc	Bc
	Chloroform	8±0.2	5±0.2	8±0.2	8±0.1	4±0.1	Bc	Bs

Nd - no zone of inhibition detected, Bs- bacteriostatic, Bc-bactericidal; 1- amoxyclave; 2- erythromycin; 3- extract fraction; 4-combination of amoxyclave and extract fraction; 5- combination of erythromycin and extract fraction

TABLE 5: ANTIMICROBIAL ACTIVITY OF POLAR AND NON-POLAR FRACTIONS OBTAINED FROM *C. OPPOSITIFOLIA* EXTRACT AGAINST *E. COLI*

Extract (plant part)	Fractionation Solvent	Zone of inhibition (mm)					Bs/Bc (3+1)	Bs/ Bc (3+2)
		1	2	3	4	5		
Leaves (Methanol)	Ethyl acetate	3±0.1	7±0.2	1±0.1	2±0.2	8±0.2	Bc	Bc
	n-butanol	3±0.2	8±0.1	2±0.3	3±0.1	7±0.1	Bc	Bc
	Chloroform	3±0.1	7±0.3	Nd	3±0.1	7±0.1	Bs	Bs

Nd - no zone of inhibition detected, Bs- bacteriostatic, Bc-bactericidal; 1- amoxyclave; 2- erythromycin; 3- extract fraction; 4-combination of amoxyclave and extract fraction; 5- combination of erythromycin and extract fraction

DISCUSSION: The mode of action of antibiotics comprise of inhibiton of crucial life sustaining processes in the organism i.e synthesis of cell wall material, DNA, RNA, ribosomes and proteins. Herbal medicine and plant-derived therapeutic products in various forms have been available since years for the treatment of diseases in both Eastern and Western cultures. Herbs have been used in under traditional systems of medicine like Ayurveda, Sidha and Unani. In the traditional background of medicine, India needs to increase its share in the world market [12].

In the modern era the treatment of diseases have been done by different medicinal plant formulations alone and in combination therapy with antibiotics or other plant formulations. The main idea of development of this combination therapy is to formulate less toxic drug with low dosage, classified as synergism. Ethanolic leaf extract of *Vangueria spinosa* in combination with doxycycline and ofloxacin was reported as synergist against *S. aureus, E. coli, K. pneumonia* and *P. aeruginosa* [13]. The extracts of *Psidium guajava, Rosmarinus officinalis, Salvia fruticosa, Majorana syriaca, Ocimum basilucum, Syzygium aromaticum, Laurus nobilis* and *Rosa damascena* were observed to be enhancing the activity of antimicrobial agents of different mechanisms [14]. Betoni *et al* (2006) [15] observed synergism in *Rhus coriaria*, *Psidium guajava*, *Lawsonia inermis*, and *Sacropoterium spinosum* with oxytetracyclin HCl, enrofloxacin, gentamicin sulphate and sulphadimethoxin against *Staphylococcus aureus*. Some formulations are based upon the complete herbal combinations [16].

The synergistic effect of traditional medicinal plants of Solan and Shimla were discussed in combination with erythromycin, amoxyclave against *S. aureus* and *E. coli*. The best synergistic plants of current study are listed in **Table 2.** *C. citrates* (oil) and *Z. officinale* (rhizome) has shown class I synergism against *S. aureus* and *E. coli* with erythromycin. The class I synergistic effect was observed by *S. aromaticum* (flower bud) against *E. coli*, whereas against *S. aureus* the class II synergism was observed. *C. citrates, S. aromaticum* and *Z. officinale* has been reported earlier as synergist for various antimicrobial drugs [15, 17].

T. cordifolia (stem) has shown the class I synergism against *S. aureus* and *E. coli*, whereas in *A. marmelos* (fruit) was observed class I synergist against *S. aureus*. But to the best of our knowledge, no synergistic activity of *A. marmelos* is reported in literature, whereas the synergistic effect of leaf extracts of *Tinospora cordifolia* with nalidixic acid was observed against *K. pneumoniae* and *B. cereus* [18]. The class I synergism was shown by flower extract of *P. granatum* against *E. coli* and *S. aureus* in combination with erythromycin. The compound extracted from *Punica granatum* in combination with fluconazole showed a synergistic interaction against *Candida albicans* and *Candida parapsilosis* [19]. Also synergistic interaction between *Punica granatum* extract and antibiotics (chloramphenicol, gentamicin, ampicillin, tetracycline and oxacillin) against 30 clinical isolates of methicillin-resistant and sensitive *Staphylococcus aureus* was reported by Braga *et al.* (2005) [20]. In present study the class I synergistic activity was also observed in *C. longa* (rhizome) against *S. aureus* and *E. coli*. In previous study *C. longa* (rhizome) was reported synergistic to clotrimoxazole, ampicillin, cloxacillin, chloramphenicol [21].

In contrast to our observations, there are earlier reports which supports our observation e.g. in *T. arjuna* (bark), we observed class III synergism against *S. aureus* and has been reported by Pathania *et al.* (2013). *A. cepa* (bulb), *M. fragrans* (fruit) and *C. tamala* (bark) have shown the class I synergism against *S. aureus* and *E. coli. The synergistic effect of A.* cepa (oil) with ketoconazole against *Trichophyton* species have been studied by Pyun and Shin, (2006) [22].

The class I synergism was also shown by *Z. officinale* (rhizome), *P. roxburghii* (leaves), *P. nigrum* (fruit), *W. somnifera* (leaves), *P. granatum* (flower) and *L. alba* (leaves) against *E. coli* and *S. aureus* except *P. roxberxyhii* (leaves) in which it was observed only against *E. coli.* The essential oils extracted from *Z. officinale* and *M. fragrans* with nisin (bacteriocin of *Lactococcus lactis* subsp), showed synergistic effect against *Listeria monocytogenes* [23]. Arora *et al.* (2004) [24] showed synergistic effect of *W. somnifera* extracts and Das *et al.* (2012) [25] have shown synergism for *L. alba.*

The compound (piperine) isolated from *P. nigrum*, in combination with rifampicin and isoniazid has shown strong synergism against *M. tuberculosis*, the new drug formulation against tuberculosis was named as 'resorine' which contains reduced dose (200mg) of rifampicin + isoniazid (300mg) + piperine (10mg) (Chawla, 2010). Class II synergism was observed in *Z. armatum* (leaves) against *S. aureus* and *E. coli*. No synergism was observed in *M. fragrans* (mace) extract in combination with erythromycin. The synergistic effect of *S. aromaticum* and *C. tamala* was reported by Sukatta *et al.* (2008) [26] against *Aspergillus niger*, *Alternaria alternata*, *Colletotrichum gloeosporioides*, *Lasiodiplodia theobromae*, *Phomopsis viticola* and *Rhizopus stolonifer*. The synergistic effect between pine oil, tetrasodium EDTA and sodium xylene sulfonate, against both gram positive and gram negative bacteria was reported by Spaulding *et al.* (1992) [27].

The plant extracts and solvent fractions of *C. oppositifolia* have shown the potent synergism with erythromycin, amoxyclave, against *S. aureus* and *E. coli* bacteria. The solvent fractions of methanolic extract of *C. oppositifolia* have shown antibacterial and synergistic activity, in which we have observed the strong synergism of ethyl acetate and n- butanol fractions, against *S. aureus* and *E. coli*. The solvent fractions (n- butanol, ethyl acetate) of methanolic leaf extract showed the class I synergism with amoxyclave and erythromycin against *S. aureus*. The ethyl acetate and n- butanol fractions have not shown antibacterial activity alone. The solvent fractions have not shown class I synergism against *E. coli*. Class II synergism was shown by n-butanol and ethyl acetate fraction, in which both of these fractions enhances the potency of erythromycin by making it bactericidal. Ali *et al* (2011) [28] have isolated the acteoside from *C. oppositifolia* which alone was not antifungal, enhances the effect of amphotericin B against *Candida albicans*, *Cryptococcus neoformans* and *Aspergillus fumigates*.

The synergistic effect observed in present study may be due to complex formation of phytocompounds/ extracts with antibiotics which leads to the more effective for particular species of microorganisms by inhibiting the crucial life sustaining processes and causing its lyses or death. Synergistic study in herbal medicine may lead to improved traditional formulations. It would the challenge to formulate new improved drugs with globally demanded drugs of Indian medicinal plants so that India can lead the world market of herbal drugs.

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How to cite this article:
Sharma V, Kumar T and Dev K: Effect of medicinal plant extracts on the antimicrobial activity of amoxyclave and erythromycin against *E. coli* and *S. aureus*. Int J Pharm Sci Res 2016; 7(11): 4615-25.doi: 10.13040/IJPSR.0975-8232.7(11).4615-25.