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ARTICLE

Antimicrobial effect of ethyl acetate extract and eluted fraction of ethyl acetate extract of *Emilia sonchifolia* with standard antibiotics on different bacteria

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Abstract

The present study was aimed to compare the antimicrobial effect of ethyl acetate extract and eluted fraction of ethyl acetate extract of *Emilia sonchifolia* with standard antibiotics on different bacteria such as *Pseudomonas, Streptococcus, Staphylococcus* and *Enterococcus*. Dried powdered plant material was extracted with ethyl acetate as solvent using soxhlet apparatus. Column chromatography was used for the elution of ethyl acetate extract of *Emilia sonchifolia*. The antimicrobial activity was evaluated by agar well diffusion method. The study revealed that the extract had a stronger antimicrobial effect than the standard antibiotics. It confirms the efficacy of the extract as antibacterial agent. The anti microbial assay of the extract of medicinal plant *Emilia sonchifolia* which is traditionally used in Ayurveda or in other herbal medical practices have scientific basics and can be modified to produce specific medicines against *Pseudomonas, Streptococcus, Staphylococcus* and *Enterococcus.*

Keywords: Antimicrobial, *Emilia sonchifolia*, *Pseudomonas, Streptococcus, Staphylococcus* and *Enterococcus*]

Introduction

The word antibiotic comes from the Greek; anti meaning 'against' and bios meaning 'life' (a bacterium is a life form). The term "antibiotic" was coined by Selman Waksman in 1942. Although there are a number of different types of antibiotic they all work in one of two ways. 1. A bactericidal antibiotic kills the bacteria. Penicillin is a bactericidal antibiotic usually either interferes with the formation of the bacterium's cell wall or its cell contents. 2. A bacteriostatic stops from multiplying. The antibiotics are well known for their pace of action but at the same time, they produce a lot of side effects in the user. If antibiotics are over used or used incorrectly there is a chance that the bacteria will become resistant – the antibiotic becomes less effective against that type of bacterium. Some bacteria develop genes for drug resistance in plasmids, they are able to spread drug resistance to other strains and species during genetic exchange the permeability of the microbial cell wall and membranes and thus prohibiting the passage of antibiotics to the interior. The emergence of new diseases and resistance of bacteria against prevailing antibiotics (Darokar et.al., 1998) becomes major problems for Modern medicine.

Many treatments for infections prior to the beginning of the twentieth century were based on medicinal folklore. A major part of the total population in developing countries still uses traditional folklore medicine obtained from plant resources (Farnsworth 1994, Srivastava et.al., 1996). Green plants synthesise and preserve a variety of biochemical products, many of which are extractable and used as chemical feed stocks or as raw material for various scientific investigations. Herbals are seen as potential medicines for a variety of diseases often viewed to supercede the pharmacological efficacy of allopathic drugs (Chirangini, et.al., 2006).

Emilia sonchifolia belongs to the family Asteraceae. It is commonly called as lilac tassel flower. It is among the "ten sacred flowers of Kerala state in India collectively called as Desapushpam" (Raj M 2012). *Emilia sonchifolia* has been reported to have anti-microbial activity (Shylesh and Padikkala, 1999; Shylesh et.al., 2005; Srinivasan KK and Subramanian SS, 1980). Ariel parts of the plant were studied against 20 bacterial species, 3 yeast species and 12 filamentous fungi (Yoga Latha et.al.,2009). Anti-cancer activity (Raj M, 2012), anti-inflammatory (Muko and Ohiri,1999) and anti oxidant activity (Gayathri Devi et al., 2006; Shylesh and Padikkala, 1999) were also documented. Phytochemical studies indicated that the ariel parts of *Emilia sonchifolia* contain alkaloids, flavanoids, terpenes and tannins (Fu et al.,2002; Lija et al., 2006; Chen X et al., 2009; Srinivasan and Subramaniyan, 1980; Chopra and Major, 2006). Hence, in this present study, an attempt was made for extraction, elution of phytochemicals from the ethyl acetate extract of selected medicinal plant *Emilia sonchifolia* on various bacteria and thereby providing a ray of hope to find alternative solutions for antibiotics to prevent the emergence of resistant strains of bacteria.

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Materials and Methods

Chemicals

The chemicals such as Hexane, Chloroform, Ethyl acetate, DMSO, and other chemicals (analytical or equivalent grade of high purity) used for the experiment were purchased from SRL, Bombay.

Plant material

Emilia sonchifolia were locally collected from Thiruvananthapuram district, Kerala and botanically authenticated. The whole plants collected were washed thoroughly 2 – 3 times with running water and dried in 40 – 60 °C in hot air oven. The dried materials were finely powdered and stored in air tight bottle and used for soxhlet extraction.

Extraction

A quantity of 500 gm of hot air dried powdered plant material was extracted in 2.5 L of ethyl acetate using soxhlet. The soxhletion with ethyl acetate was done for one week to obtain extract. The final extract of *Emilia sonchifolia (E. sonchifolia)* collected was concentrated under rotary evaporator and preserved at 4°C in air tight bottles.

Elution of ethyl acetate extract in hexane : chloroform (9:1)

A part of the concentrated extract obtained from ethyl acetate, which is to be analyzed was used as study material – I for conducting the antimicrobial studies. Column chromatography is used for the separation and purification of organic compounds especially when they are available in small quantities. The remaining part of concentrated extract obtained from ethyl acetate, which is to be separated was applied to the column at the top. Some compounds of the mixture are adsorbed strongly, while others less strongly. The more strongly a substance is adsorbed, the more slowly it moves down the column. The elution was carried out using the mixture of hexane and chloroform (9:1 i.e. 90 ml Hexane : 10 ml Chloroform). Each 50.0 ml fraction was separately collected for a period of 30 days. Identical fractions were pooled together (Bobbit 1963).

The eluted pooled fractions (9:1 of hexane, chloroform) were concentrated under rotary evaporator. The concentrated material was crystallized and re-crystallized using chloroform and preserved in the desiccators at 4°C in air tight bottles as study material II for conducting antimicrobial studies.

Study material

The ethyl acetate extract was treated as study material I and made into a suspension using 10% dimethylsulphoxide (DMSO) in distilled water. The concentration of the material was made in 1mg/ml (1000µg/ml). The eluted crystals from ethyl acetate extract were treated as study material II and made into a suspension using 10% dimethylsulphoxide (DMSO) in distilled water. The concentration of the material was made in 1mg/ml (1000µg/ml). The standard antibiotic filter paper discs purchased from Pathoteq Biological Laboratories, Gujarat were used for the analysis.

Experimental organism

Pure slant cultures of different bacterial strains such as *Pseudomonas, Staphylococcus, Streptococcus* and *Enterococcus* were collected from the Research laboratory of South Travancore Hindu College, Nagercoil, Kanyakumari District, Tamilnadu. The slant cultures were brought to the laboratory within a short time to avoid any possible contamination. It was stored at 4°C in refrigerator for future preparation of sub culture in nutrient agar.

Preparation of selective culture medium

6.6 gm of Muller Hinton agar was added to 175 ml distilled water in a sterilized conical Flask. This mixture was boiled until the agar completely dissolved. The prepared culture medium was plugged with sterilized cotton and autoclaved at 121°C. The cooled sterilized medium was then kept in refrigerator for further use. The prepared culture medium was poured into 18 sterilized culture plates at 40°C and kept undisturbed for solidifying the medium.

Preparation of culture plates

An inoculation loop was touched to 4 to 5 isolated colonies of pathogen *Pseudomonas* growing on slant culture and was inoculated into 5 ml of sterilized distilled water in a test tube. A sterile cotton swab was dipped into the standardized bacterial test suspension and was used to evenly inoculate the entire surface of Muller Hinton agar plate. All the six inoculated plates were allowed to dry for 15-30 min. The similar procedure was followed for *Staphylococcus, Steptococcus* and *Enterococcus* individually.

Experimental Design

All the *Pseudomonas* inoculated plates were divided into three groups. (Each group contains two *Pseudomonas* inoculated plates respectively). Group I has 4 sterilized well received 10 µl, 20 µl, 30 µl and 40 µl of study material I respectively, Group II has three sterilized wells received 40 µl of study material I, 40 µl of study material II and 40 µL of DMSO alone. Group III has only standard antibiotics discs

All the *Staphylococcus, streptococcus, enterococcus* inoculated plates were divided into two groups. (Each group contains two bacterial inoculated plates respectively). Group I has 4 sterilized well received 10 µl, 20 µl, 30 µl and 40 µl of study material I respectively, Group II has 3 sterilized well received 40 µl of study material I, 40 µl of study material II and 40µl DMSO alone.

Antibacterial Susceptibility Test

Agar well diffusion method is widely used to evaluate the antimicrobial activity. The agar plate surface is inoculated by spreading a volume of the microbial inoculums over the entire agar surface. Then, a hole with a diameter of 8 mm is punched aseptically with a sterile cork borer or a tip, and a volume (10–100 µL) of the antimicrobial agent or extract

Table 1. Experimental Design

Groups	Study Material I (µl)				Study Material II (µl)	Control (DMSO ALONE) (µl)	Std antibiotics
	10	20	30	40	40	40	
Pseudo -1	+	+	+	+	-	-	-
Pseudo -2	-	-	-	+	-	-	-
Pseudo -3	-	-	-	-	-	-	+
Staphy -1	+	+	+	+	-	-	-
Staphy -2	-	-	-	+	+	+	-
Strepto- 1	+	+	+	+	-	-	-
Strepto – 2	-	-	-	+	+	+	-
Entero -1	+	+	+	+	-	-	-
Entero – 2	-	-	-	+	+	+	-

solution at desired concentration is introduced into the well. 10 µL, 20 µL, 30 µL and 40 µL of study material I and 40 µL of study material II and 40 µL of DMSO were added to each well (8 mm diameter holes cut in the agar gel, 20 mm apart from one another) separately. The systems were incubated for 24 h at 36°C ± 1°C, under aerobic conditions. After incubation, confluent bacterial growth was observed. Inhibition of the bacterial growth was measured in mm.

Results

Evaluation of dose dependent antimicrobial activity of four different concentration of ethyl acetate extract of Emilia sonchifolia (study material – I) on Pseudomonas, Staphylococcus, Streptococcus and Enterococcus.

Results are given in Table no: 2

Administration of four different concentrations of 10µg, 20µg, 30µg and 40 µg of ethyl acetate extract of *Emilia sonchifolia* on the separate wells made on the *Pseudomonas Streptococcus, Enterococcus* and *Staphylococcus* bacterial culture individually, showed a well-marked inhibition zone in dose dependent manner. The increased inhibitory zone was observed in well received 40 µg (40 µl) of ethyl acetate extract on all four different bacteria.

Evaluation of comparative effect of antimicrobial activity of study material I and study material II of Emilia sonchifolia on Pseudomonas, Enterococcus, Streptococcus and Staphylococcus.

Results are given in Table no: 3

The wells treated with the concentrations of 40µg ethyl acetate extract and 40µg eluted fraction (hexane : Chloroform (9:1)) of ethyl acetate extract of *Emilia sonchifolia* individually on *Pseudomonas, Enterococcus, Streptococcus* and *Staphylococcus* inoculated plates showed well marked inhibition zone in wells treated with crude extract alone. The better inhibitory activity was noted in the well administered with crude extract alone. Treatment of eluted fraction did not show any inhibitory activity on Enterococcus inoculated plates whereas the crude extract showed a well-marked inhibition zone in *Enterococcus* inoculated plates.

Antimicrobial activity of standard antibiotics on Pseudomonas.

Results are given in Table no: 4

In Pseudomonas inoculated plates the maximum inhibitory activity expressed by Levofloxacin (QB),Ciprofloxacin (RC), Ampicillin / Sulbactum (AS) and Roxythromycin (AT). The other antibiotics like Cephalexin (PR), Linezolid (LZ), Cloxacillin (CX), Gentamycin (GM), Co-trimoxazole (BA), Tetracycline (TE), Cefotaxime (CF) and Lincomycin (LM) showed only very little inhibition zone diameters on *Pseudomonas* inoculated plates.

Comparative study on the antimicrobial effects of study material –I, study material – II and standard antibiotics.

Results are given in Graph: 1

Comparative analysis of antimicrobial effect of study material I, study material II with standard antibiotics clearly exhibited the antimicrobial competency. The statistical analysis clearly revealed that the crude extract has significant antibacterial activity against the tested microbe.

Table 2. Dose dependent effect of antimicrobial activity of four different concentrations ethyl acetate extract of *Emilia sonchifolia* on *Pseudomonas, Enterococcus, streptococcus* and *Staphylococcus.*

Inhibition Zone (IZD) in mm IZD in mm	Name of the bacteria	Different concentration of ethyl acetate extract as Study material I				Control (DMSO alone)
		10µl (10µg/ml)	20µl (20µg/ml)	30µl (30µg/ml)	40µl (40µg/ml)	10µl diluted (10µg/ml)
	Pseudomonas	10 ± 0.10	14 ± 0.20	15 ± 0.20	16 ± 0.30	8 ± 0.01
	Enterococcus	8 ± 0.01	14 ± 0.20	15 ± 0.20	16 ± 0.30	8 ± 0.01
	Streptococcus	12 ± 0.20	14 ± 0.20	16 ± 0.30	20 ± 0.40	8 ± 0.01
	Staphylococcus	14 ± 0.20	16 ± 0.30	18 ± 0.40	20 ± 0.40	8 ± 0.01

Table 2A. Two way ANOVA – showing significance of IZDs.

Sources of variation	Sum of Square	Degree of freedom	Mean Square	F value	P value	F critic
Between groups	55.11875	3	18.37292	142.0953	1.18E-13	3.098391
With in groups	514.525	4	128.6313	994.8279	1.07E-22	2.866081

Table 3. Comparative effect of antimicrobial activity of study material I and study material II of *Emilia sonchifolia* on *Pseudomonas, Enterococcus, Streptococcus* and *Staphylococcus.*

Inhibition Zone	Sl.No.	Name of the bacteria	Study Material I Ethyl acetate extract	Study Material II Eluted fraction (90:10 hexane : Chloroform)	Control (DMSO alone)
			40µl (40µg/ml)	40µl (40µg/ml)	40µl (40µg/ml)
IZD in mm	1.	Pseudomonas	16 ± 0.30	8 ± 0.01	8 ± 0.01
	2.	Enterococcus	16 ± 0.30	8 ± 0.01	8 ± 0.01
	3.	Streptococcus	20 ± 0.40	14 ± 0.20	8 ± 0.01
	4.	Staphylococcus	20 ± 0.40	18 ± 0.30	8 ± 0.01

Table 3A. Two way ANOVA – showing significance of IZDs.

Sources of variation	Sum of Square	Degree of freedom	Mean Square	F value	P value	F critic
Between groups	101.3333	3	33.77778	321.387	1.01E-11	3.490295
With in groups	405.3333	2	202.6667	1928.322	8.91E-16	3.885294

Discussion

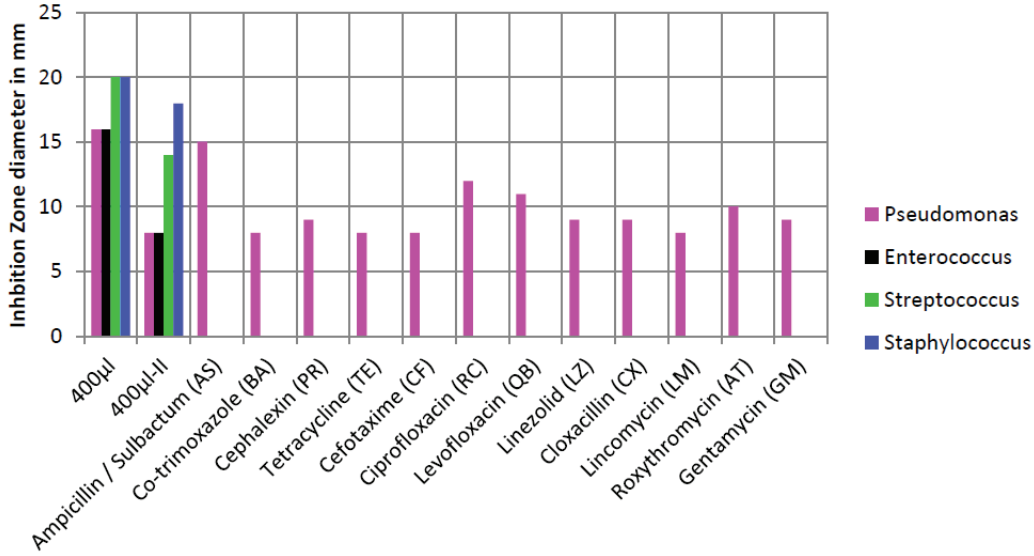
The emergence of antibiotic resistance is an evolutionary process that is based on selection for organisms that have enhanced ability to survive doses of antibiotics that would have previously been lethal. Antibiotics like Penicillin and Erythromycin, which used to be one-time miracle cures are now less effective because bacteria have become more resistant. Inappropriate antibiotic treatment and overuse of antibiotics have been a contributing factor to the emergence of resistant bacteria. In recent times, due to several intricacies of modern antibiotics, there has been significant

Table 4. Antimicrobial activity of standard antibiotics on *Pseudomonas*.

Antibiotics	Pseudomonas IZD (mm)	Control (DMSO alone)
Ampicillin / Sulbactum (AS)	15±0.20	6±0.01
Co-trimoxazole (BA)	8 ±0.01	6±0.01
Cephalexin (PR)	9 ±0.01	6±0.01
Tetracycline (TE)	8 ±0.01	6±0.01
Cefotaxime (CF)	8 ±0.01	6±0.01
Ciprofloxacin (RC)	12 ±0.20	6±0.01
Levofloxacin (QB)	11±0.20	6±0.01
Linezolid (LZ)	9±0.01	6±0.01
Cloxacillin (CX)	9±0.01	6±0.01
Lincomycin (LM)	8±0.01	6±0.01
Roxythromycin (AT)	10±0.20	6±0.01
Gentamycin (GM)	9±0.01	6±0.01

Table 4A. One way ANOVA.

Sources of variation	Sum of square	Degree of freedom	Mean square	F value	P value	F critic
Between groups	209.8667	14	14.99048	588.6313	6.31E-18	2.424364



Graph 1. Comparative analysis of study material - I, Study material - II and Std antibiotics.

Plate 1. Dosage effect of four different concentration of ethyl acetate extract (study material I) of *Emilia sonchifolia(E. sonchifolia)*

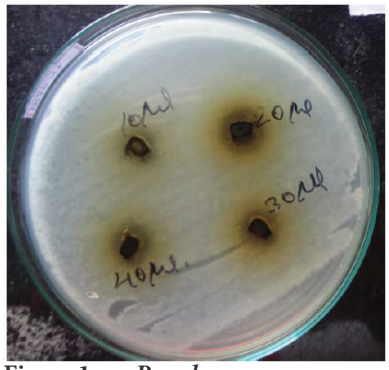


Figure 1. on *Pseudomonas*

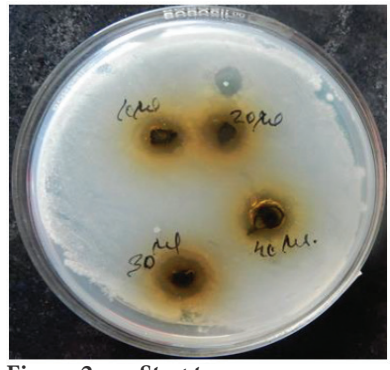


Figure 2. on *Streptococcus*

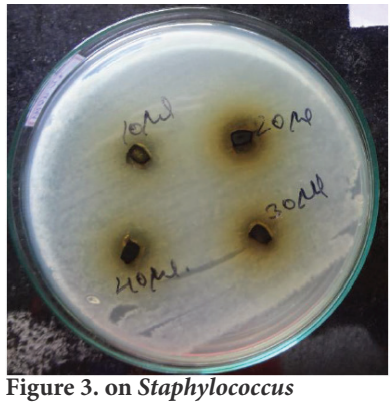


Figure 3. on *Staphylococcus*

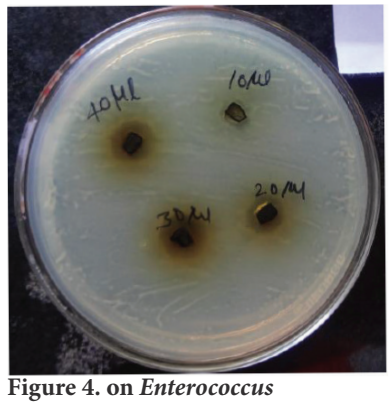


Figure 4. on *Enterococcus*

Plate 2. Comparative effect of extract and eluted fraction of ethyl acetate extract of *Emilia sonchifolia(E. sonchifolia)*

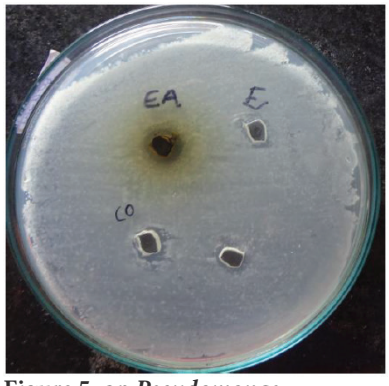


Figure 5. on *Pseudomonas*

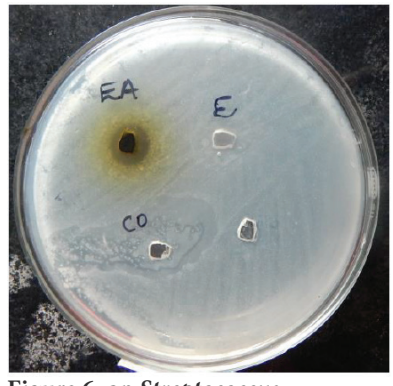


Figure 6. on *Streptococcus*

Figure 7. on *Staphylococcus*

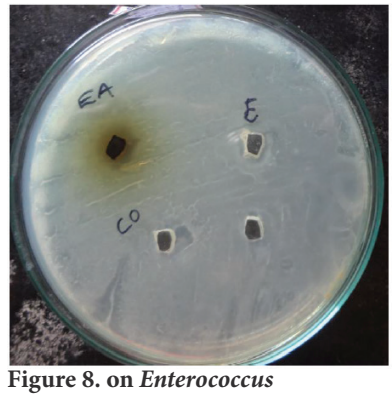


Figure 8. on *Enterococcus*

shift towards alternative treatment and herbal remedies Patwardhan B, Vaidya ADB and Chorghade M (2004). Antibiotic screening of plants and natural products used in alternative systems of medicines like Ayurvedic and unani is a major thrust of R & D, in the Indian pharmaceutical sector today Afaq S h et.al., (2004) and Baijal R, Patel N and Kolhapure S A (2002).

Our study revealed that the wells treated with different concentrations of 10µg, 20µg, 30µg and 40µg of ethyl acetate extract (crude) of *Emilia sonchifolia* on *Pseudomonas, Staphylococcus* and *Streptococcus* innocculated plates showed well marked antibacterial activity in dose dependent manner. These differences could be due to the nature and level of the antimicrobial agents present in the extract and their mode of action on different test microorganisms. Flavanoid fraction showed stronger antibacterial activity against *staphylococcus* aureus in concentration 0.8g/ml (Chen X et al., 2009). The increased inhibitory zone developed in wells received 40 µg of ethyl acetate extract of *Emilia sonchifolia*. But the eluted fraction of ethyl acetate extract of *Emilia sonchifolia* (study material II) did not show any inhibitory activity on *Enteroccocus* inoculated plates.

From the results it is clear that the maximum inhibitory zone was observed in wells received 40 µg of crude extract than the purified compound. The purified compounds (eluted fraction of ethyl acetate extract) had no effect on *Pseudomonas* and *Enterococcus* innoculted plates. This may be due to the eluted fraction may contain inert substance only and not the active component.

Our results are in agreement with Shylesh (2005) and (Chopra and Major,2006). Comparative analysis of antimicrobial effect of study material I, study material II with standard antibiotics clearly exhibited the antimicrobial competency.

This comes to the conclusion that medicinal plant *Emilia sonchifolia* which are traditionally used in Ayurveda or in other herbal medical practices have scientific basics and can be modified to produce specific medicines against each bacterium.

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