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Comparative Analysis of Antimicrobial Activity of Herbal Extracts Against Pathogenic Microbes

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Abstract

Antimicrobial activities of *Spinacia oleracea* leaf, *Zingiber officinale* rhizome, *Coriandrum sativum* leaf, *Allium sativum* clove, *Aloe vera* gel and leaf was carried out against the multi-drug resistant strains using the minimum inhibitory concentration method. The direct TLC bioautography method is performed to identify the bioactive compounds present in the extracts exhibiting the antimicrobial property. All the plant extracts effectively inhibited the growth of pathogenic strains used in the study at a concentration of 250 to 31.25 mg/ml. These results provide evidence that the tested plant extracts possess antimicrobial properties which can be tested further in the development of novel antimicrobial agents.

Keywords: Antimicrobial Activity; TLC Bioautography Method; Herbal Extracts; Multi-Drug Resistant Strains; Mc Farland

Introduction

The emergence of multi-drug resistant microorganisms with decreased susceptibility to antibiotics due to improper usage of broad spectrum drugs is increasing day by day globally. In developing countries, the increase in the rate of mortality and morbidity is due to the infectious diseases [1,2]. Synthetic drugs which are used to treat infectious diseases are expensive and often cause side effects. Hence, there is a need to develop novel drugs to control the spread of pathogenic microbial strains. Plants with medicinal value are of interest as they are the richest source of secondary metabolites with therapeutic properties considered to be safe and effective compared to synthetic drugs [3]. The different types of plant material like leaves, root, stem, flowers, fruits and vegetables are potential sources of antimicrobial compounds due to the presence of bioactive compounds. Medicinal plants contain large number of secondary phytomedicines like phenols, flavonoids, alkaloids, saponins, tannins, steroids, etc. possesses synergistic effects used for remedial purposes [4]. These secondary bioactive compounds have different structures with a different mode of action used to control microbial growth and survival. Therefore, many dreadful diseases can be cured with the use of plant preparations [5].

The importance of natural products obtained from plants remains unchanged even after the developments in the field of synthetic organic chemistry and pharmacology. They have been used widely as traditional medicine and particularly when synthetic drugs are inaccessible and unaffordable [6]. A large number of plant species were screened for their antimicrobial properties by many researchers till now. Since ancient times, herbs and spices have been used as flavoring agents, food additives and as preservatives. They are effective in eradicating the microbial population, used widely in food industries generally considered as safe. The antimicrobial activity varies depending upon the type of food, type of spice, microorganism, essential oils and type of extracts used [7]. The study of bioactive compounds and the development of plant based antimicrobial drugs have gained importance in current research due to increase in demand for safe and effective drugs. The present study mainly focused on *Aloe vera* gel, *Aloe vera* leaf, *Spinacia oleracea* leaf, *Zingiber officinale* rhizome, *Coriandrum sativum* leaf and on an *Allium sativum* clove to evaluate their antimicrobial properties which could provide valuable information in order to use them as therapeutic tools for control of microbial growth. The extracts were prepared using solvents of different polarities and the antimicrobial activity was tested against multi-drug resistant strains of *Escherichia coli, Candida albicans, Enterococcus faecalis, Staphylococcus aureus, Streptococcus mutans, Klebsiella pneumonia* and *Mycobacterium smegmatis* using MIC method. The

direct TLC bioautography method is used as a tool to detect and isolate the antimicrobial compounds from plant extracts directly even in small amounts and is considered to be a good method compared to agar dilution methods.

Aloe vera is a succulent plant well known for its medicinal properties belongs to the family of "Aloaceae". It is a stemless plant grows up to a height of 60-100 cm and the leaves are thick and fleshy [8]. Polysaccharides, monosaccharides, chromones, anthraquinones, organic compounds, inorganic compounds, vitamins and enzymes are some of the active compounds present in *Aloe vera* were responsible for antibacterial, antifungal, antioxidant, wound healing and anti-diabetic properties [9,10]. The thick, colorless mucilaginous gel material present within the leaves of *Aloe vera* is considered to be effective in curing gastrointestinal diseases [11], burns and wounds [12]. It is also used to boost up the immune system in humans [13] and the products obtained from *Aloe vera* are used widely in cosmetic, food and pharmaceutical industries [14].

Coriandrum sativum also known as dhania is an annual herb belongs to the family of "Apiaceae". It grows up to a height of 50 cm. The leaves are variable in shape, broadly lobed at the base of the plant. It is used to treat diarrhea, vomiting, rheumatism, cough, dysentery, joint pains and indigestion [15]. It possesses antidiabetic, anti-inflammatory, lipolytic, antihypertensive, nerve relaxing and antiseptic properties. The coriander extracts possess free radical scavenging, antioxidant, antimutagenic, anticancerous and antibacterial properties [16].

Allium sativum commonly called as garlic is a bulbous plant belongs to the family of "Amaryllidaceae". It has good anti-bacterial and anti-fungal properties. Garlic is used to prevent high blood pressure, high cholesterol and heart diseases, provides protection against cancer [17].

Spinacia oleracea commonly known as Palak/spinach used widely in India is an edible flowering plant belongs to the family of "Chenopodiaceae". It is used to treat leprosy, asthma, urinary diseases, lung inflammation, joint pains, thirst, sore throat, scabies, vomiting, ringworm, sore eye, cold, sneezing, fever and the diseases related to brain and heart [18].

Zingiber officinale commonly called as ginger is an herbaceous perennial flowering plant belongs to the family of "Zingiberaceae". Ginger is widely used as a spice possesses medicinal value. It is used to treat asthma, nausea, colic, cough, cold, loss of appetite, swelling, heart diseases, dyspepsia and rheumatism [19].

Materials and Methods

Collection of Plant Material

The plant materials considered for the present are *Aloe vera, Spinacia oleracea, Zingiber officinale, Coriandrum sativum* and *Allium sativum* which were collected from the local market of Visakhapatnam.

Microorganisms Used

The microbial strains considered for the present study are *Escherichia coli, Staphylococcus aureus, Enterococcus faecalis, Streptococcus mutans, Candida albicans, Mycobacterium smegmatis* and *Klebsiella pneumonia.* All the strains were obtained from the National Collection of Industrial Microorganisms (NCIM), Pune, Maharashtra, India.

Preparation of Extracts

Aloe vera leaves*, Spinacia oleracea* leaf*, Zingiber officinale* rhizome*, Coriandrum sativum* leaves and *Allium sativum* cloves were washed thoroughly under running tap water and then with distilled water to remove dirt. They were then air-dried under shade, away from sunlight for 4-5 days and made into a fine powder using mortar and pestle. Extracts were prepared using the solvent methanol, ethyl acetate, petroleum ether and chloroform at a concentration of 1:10 ratio. The extraction is carried out with vigorous shaking for 48-72 h followed by filtration. The extract is then concentrated using Rota evaporator and is further diluted to required concentration using DMSO just before use.

Inoculum Preparation

UTI agar medium is used to culture the strains of *Escherichia coli* and *Enterococcus faecalis.* Mannitol Salt agar medium is used for the growth of *Staphylococcus aureus*. The Streptococcus Selection medium used for *Streptococcus mutans* and Rose Bengal medium used for culturing *Candida albicans* strains respectively. The agar slants with respective cultures were prepared and incubated at 37°C for 24 h. The colonies of test organisms grown overnight were inoculated into 0.85% normal saline and the turbidity adjusted to 0.5 Mc Farland using the standard which is equal to 1.5×10^8 CFU/ml. It was further diluted to obtain the final inoculum of 5×10^5 CFU/ml.

Antimicrobial assay using MIC method

MIC was performed as per Clinical and Laboratory Standards Institute guidelines using extracts against bacterial and fungal pathogens in a 96 well u-bottomed microtitre plates. The plant extracts were serially diluted from the concentration of 500mg/ml to 0.02 mg/ml and then added with the final inoculum of 5×10^5 CFU/ml. The anti-microbial compound and the final inoculum were in the ratio of 1:1 (v/v). Each test performed in triplicate with positive and negative controls. After the addition of inoculum, plates were sealed with aluminum foil and incubated at 37°C for 24 h in the case of bacterial cultures and for 48 h at 28°C for fungal cultures respectively in an incubator. At the end of incubation period, the wells were added with 20 µl of 0.1 mg/ml

resazurin dye and incubated for 30 min for the color development. Presence of bacterial or fungal growth is indicated by a change in the color of the medium to pink, whereas no color change indicates the absence of growth of the organism and the least concentration where there is no growth is considered as an MIC value of that particular compound against bacterial and fungal strains used. The experiment is carried out in triplicate using the standards streptomycin, fluconazole, cefixime and rifampicin.

Thin Layer Chromatography

Thin Layer Chromatography (TLC) was performed using 0.2 mm silica coated aluminum sheet purchased from Merck. The mobile phase of chloroform: methanol in the ratio of 9:1 is used for the separation of bioactive compounds present in the plant extracts. 10 µl of each extract was spotted separately on the TLC sheet, allowed for the migration of compounds. After complete elution; the spots were identified and calculated R_f values for each band.

$R_{f=}$ Distance travelled by solute

Distance travelled by solvent

Direct TLC Bioautography Method

Bioautography is a rapid analytical technique used in the identification of bioactive lead/ scaffolds in complex matrices of plant extracts. In this method of direct TLC Bioautography, the developed TLC plate was sprayed with fungal and bacterial suspensions incubated at 25°C for 48 h under humid conditions. After the incubation period, resazurin dye was sprayed and again incubated at 37°C for 3 to 4 h. The antimicrobial activity is indicated by the appearance of clear white zones against a purple background on the TLC plate.

Results and Discussion

Antimicrobial Assay

The antibacterial and antifungal study is carried out against the strains of *Escherichia coli, Staphylococcus aureus, Enterococcus faecalis, Streptococcus mutans, Candida albicans, Mycobacterium smegmatis* and *Klebsiella pneumonia* using the extracts of *Aloe vera*, *Spinacia oleracea, Zingiber officinale*, *Coriandrum sativum* and *Allium sativum*. All the extracts showed antimicrobial activity against the tested pathogenic strains and the results were shown in Table 1. The petroleum ether and methanolic extracts of *Aloe vera* and *Coriandrum sativum* is effective against *M. smegmatis* compared with other strains. The extracts prepared with solvents of chloroform, methanol, petroleum ether and ethyl acetate showed antimicrobial activity at a concentration ranging from 250-31.25 mg/ml respectively against all the tested strains. *Spinacia oleracea* and *Zingiber officinale* petroleum ether, chloroform and ethyl acetate extracts showed effective inhibitory activity against the strains of *M. smegmatis* and *K. pneumoniae* at a concentration of 31.25 mg/ml, whereas, the MIC value ranged from 250-31.25 mg/ml against the other strains used in the study. *Candida albicans* and *Klebsiella pneumoniae* were inhibited at a concentration of 31.25 mg/ml with *Allium sativum* methanol and chloroform extracts.

Plant species	Strain name	MIC Values mg/ml			
		Chloroform	Methanol	Petroleum ether	Ethyl acetate
Aloe vera leaf	*E. coli*	250	250	250	250
	S. aureus	250	250	125	250
	E. faecalis	250	250	125	250
	S. mutans	250	250	250	250
	C. albicans	62.5	62.5	125	125
	M. smegmatis	62.5	31.25	62.5	125
	K. pneumonia	250	125	250	125
Aloe vera gel	*E. coli*	125	125	250	250
	S. aureus	125	250	250	250
	E. faecalis	250	125	125	125
	S. mutans	125	250	250	125
	C. albicans	62.5	125	125	62.5
	M. smegmatis	62.5	31.25	31.25	62.5
	K. pneumonia	31.25	62.5	125	62.5

Spinacia oleracea leaf	*E. coli*	125	250	250	250
	S. aureus	250	250	250	125
	E. faecalis	125	250	250	125
	S. mutans	250	250	250	250
	C. albicans	125	125	62.5	62.5
	M. smegmatis	62.5	31.25	31.25	62.5
	K. pneumonia	125	62.5	125	62.5
Zingiber officinale rhizome	*E. coli*	62.5	125	125	62.5
	S. aureus	250	125	250	125
	E. faecalis	125	125	125	250
	S. mutans	125	250	125	125
	C. albicans	125	62.5	250	250
	M. smegmatis	125	62.5	31.25	62.5
	K. pneumonia	62.5	31.25	250	31.25
Coriandrum sativum leaf	*E. coli*	125	250	125	125
	S. aureus	250	250	250	125
	E. faecalis	125	250	250	125
	S. mutans	250	250	250	250
	C. albicans	125	125	62.5	62.5
	M. smegmatis	62.5	31.25	31.25	62.5
	K. pneumonia	125	62.5	125	62.5
Allium sativum clove	*E. coli*	250	250	250	250
	S.aureus	125	250	250	250
	E. faecalis	125	125	125	250
	S. mutans	250	125	125	250
	C. albicans	62.5	31.25	125	62.5
	M. smegmatis	125	125	62.5	125
	K. pneumonia	31.25	62.5	250	62.5

Table 1: Antimicrobial activity of herbal extracts.

From the above study, it was observed that all the extracts showed potent antimicrobial activity against all the screened bacterial and fungal strains. The plant extracts effectively inhibited the growth of tested pathogenic strains in the order of *Mycobacterium smegmatis*> *Klebsiella pneumoniae*> *Candida albicans*> *Enterococcus faecalis*> *Staphylococcus aureus*> *Escherichia coli*> *Streptococcus mutans* and the inhibitory effect of plant extracts was in the order of *Spinacia oleracea* leaf> *Aloe vera* leaf> *Aloe vera* gel> *Coriandrum sativum* leaf> *Allium sativum* cloves> *Zingiber officinale* rhizome. The methanol extracts showed higher antimicrobial activity followed by chloroform, petroleum ether and ethyl acetate. The polarities of solvents play a vital role in the extraction of plant secondary metabolites that influence the antimicrobial potential of extracts [20]. The toxicity of solvent, handling of extracts, total yield and the duration of extraction are some of the important factors need to be considered to increase the extraction efficiency in order to obtain maximum product out of the plant material used [21].

The antimicrobial activity of extracts could be due to the presence of several plant bioactive compounds. The method of preparation and the type of solvent influence the antimicrobial potential of plants and the variations in the activity of tested plant species is due to the difference in the composition of phytochemicals. Antibiotics were most commonly used to kill the microbes and protect patients from infectious diseases, but there is an increased emergence of multi-drug resistant strains that leads to treatment failure. Microbes acquire resistance to antibiotics preventing destruction by means of intrinsic mechanism [22]. To control human health problems associated with increasing

population day by day, there is a need to discover new antimicrobial drugs for combating the growth of harmful microbes. Drugs with therapeutic properties obtained naturally from the source of medicinal plants are of great importance as these natural plant products are likely to be effective against multi-drug resistant strains. Secondary metabolites produced by plants are useful in treating diseases like diabetes, heart diseases, infectious diseases and cancer [23]. It was reported that the garlic has an anti-infective property and antimicrobial activity against a large number of microbes including viruses. Allicin primarily inhibits the synthesis of DNA, RNA and proteins, which is an active ingredient of garlic [24]. The antimicrobial activity of garlic is due to the presence of phenolic compounds and organosulfur compounds [25]. Similar work supporting our results of antimicrobial activity of *Aloe vera* gel against the strains of *E. coli* and *S. aureus* was also reported [26]. The antimicrobial activity of *Aloe vera* juice against the strains of *Mycobacterium smegmatis, S. aureus, Enterococcus faecalis, M. luteu*s, *B. sphericus, P. aeruginosa, K. pneumoniae, E. coli, S. typhimurium* and *candida albicans* was reported [27]. The inhibitory effect of *Aloe vera* juice against the strains of *M. smegmatis*, *K. pneumoniae*, *E. faecalis*, *M. luteus*, *C. albicans* and *B. sphericus* were also reported [28]. Caffeic acid, chlorogenic acid, ferulic acid, flavanols like quercetin, protocatchunic acid and other polyphenols are present in the leaf extracts of fresh coriander [29]. Due to the presence of polyphenols along with quercitin, coriander is used in food and pharmaceutical industries to avoid the bacterial contamination. The antimicrobial and antioxidant potential of polyphenols present in *coriander* is effective against the strains of *E. coli*, *P. aeruginosa* responsible for urinary tract infections and gastroenteritis and against *S. aureus*, causative organism of pneumonia, toxic shock syndrome and food poisoning. Ginger rhizome contains gingerol and shagelol active compounds along with several bioactive compounds exhibiting a broad range of antimicrobial activity. The inhibitory effect of the ginger ethanol extract against candida albicans was reported [30]. Recent studies revealed that zingerone showed protective action against *E. coli* responsible for diarrhea. The study shows that the methanol extracts of ginger effectively inhibited the growth of *E. coli* and *S. aureus* similar to our study [31]. The inhibition of bacterial strains *S. typhimurium, E. coli, P. multocida, M. luteus, L. bulgaricus, S. aureus, K. pneumoniae, P. vulgaris* and *S. epidermis* by *S. oleracea* was reported [32]. Alkaloids, tannins, steroids, glycosides and terpenoids are some of the phenolic compounds present in the extracts of *S. oleracea* responsible for the antimicrobial activities.

TLC Separation of Bioactive Compounds

Thin layer chromatography is performed to identify the number of bioactive compounds present within each extract. The methanol extracts of *Coriandrum sativum* leaf, Spinach leaf, *Aloe vera* leaf and *Zingiber officinale* rhizome showed 6, 8, 5 and 6 distinct bands respectively (Figure 1). All the extracts used in the study gave impressive results which directly represent the presence of phytochemicals. It was observed that the *Spinacia oleracea* leaf extract possesses a large number of Phyto-constituents compared to others which could be the reason of higher antimicrobial activity.

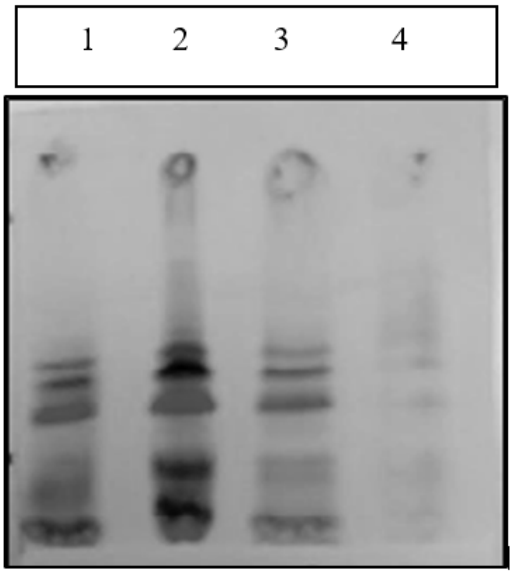

Figure 1: TLC separation of bioactive compounds using methanolic extracts of Lane **1**: *Coriandrum sativum* leaf, Lane **2**: Spinach leaf, Lane **3**: *Aloe vera* leaf and Lane **4**: *Zingiber officinale* rhizome.

The separation of the bio active compounds present within the plant extracts is achieved using the technique of thin layer chromatography, revealed the presence of several phytochemicals with different R_f values. The suitable mobile phase can be selected based on the R_f values of each compound. The polarity of the compounds can be detected using the R_f values. The technique of TLC gives an idea for the selection of appropriate solvent system for separation of compounds in pure form for further studies.

Direct TLC Bioautography Method

The method of direct TLC bioautography is used to detect the compounds with antimicrobial potential present within the plant extracts. Effective inhibition of the tested plant extracts against harmful pathogens was in the order of *Mycobacterium smegmatis* > *Klebsiella pneumoniae* > *Candida albicans* > *Enterococcus faecalis* > *Staphylococcus aureus* > *Escherichia coli* >*Streptococcus mutans.* All the herbal extracts used were found to be most effective against *Mycobacterium smegmatis* and *Klebsiella pneumoniae* showing clear white zones against a purple background on the TLC plate indicating the antimicrobial activity (Figure 2).

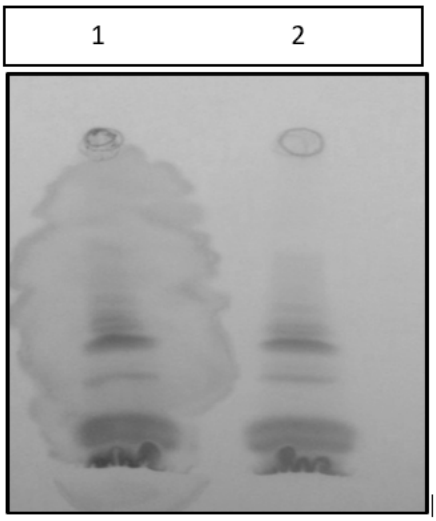

Figure 2: Direct TLC bioautography of herbal extracts against Lane **1**: *Mycobacterium smegmatis* and Lane **2**: *Klebsiella pneumoniae*.

Plant extracts contain a large number of bioactive compounds and thus the screening of compounds exhibiting the antimicrobial activity is necessary which is made easier with the use of the TLC bioautography method. The zones of inhibition with creamy spots against purple background are visualized after spraying the plates with resazurin dye. This method is considered to be convenient for obtaining the reliable information on the activity of single compounds. Study of herbal extracts suggests the use of herbal preparations for the preservation of foods, curing infectious diseases caused by pathogenic microbes and to prevent the microbial deterioration of food products. The isolation and characterization of active compounds with biological activities of these herbal plants could be used to generate a novel drug for future prospects.

Conclusion

From the above study, it can be concluded that all the plant extracts showed promising antimicrobial activity against harmful pathogenic strains. Extracts exhibiting variations in the antimicrobial activity are due to differences in the composition of bioactive compounds extracted depending upon the polarity of the solvent. Further research is going on in this area to isolate and characterize the bioactive compounds of *Aloe vera*, *Coriandrum sativum, Allium sativum* and *Zingiber officinale,* which may provide a scope of developing more effective drugs for combating infectious diseases.

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