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Screening of Phytochemicals against Multiple Drug Resistant Bacteria from Clinical Isolates

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A B S T R A C T

Plants, a rich source of therapeutic compounds because its tremendous applications in the pharmaceutical industry. To find a new and effective antimicrobial compound from the selected plant, the solvents like methanol, Ethanol, Chloroform, Hexane, Xylene, and water were used to evaluate systematically. In vitro antimicrobial activity was performed using Multi-Drug Resistant (MDR) clinical isolates by Kirby Bauer Method. Solvent extracts showed more antimicrobial activity compared to aqueous extracts of both plants. Phytochemical analysis of *Piper betel* leaves showed the presence of Glycoside, Tannin, Terpenoid, Coumarin, Flavonoid, and Phenol whereas *Achyranthes aspera roots were lacking* Phenol and Tannin. Thus, present studies suggest the medicinal use of bioactive components from traditional plants *Piper betel* and *Achyranthes aspera* in the treatment of various infectious diseases.

Introduction

India is a well-known country for use of herbal drugs. Now a days the leading countries also prefer the use of herbal drugs for curing old diseases. There are so many believers in India and in the UK, and Japan like countries are using herbs and many herbal drugs for prophylaxis of aged disease takes a bit longer time but it cures the diseases. According to the WHO, world's population, more than 75% is using herbs and herbal drugs as a healthcare needs. (Pan *et al.,* 2014). There is renewed and latest interest in deriving an antimicrobial product from the natural compound, especially from the plant material (James *et al.,*). (Redfern *et al.,* 2014) The results from the herbs prove us correct that the herbs have antimicrobial activity as well as some intellectual properties which are still to be researched. It's very vast to understand its side effects like in term of pharmacokinetics and pharmacodynamic. As we know the change is must the microbial world shows the same change in the observation while studying their properties characters and genetic properties after modern drug discovery the study of the microbial world became too easy and quicker but the problem of multi drug

resistant bacteria against majority of antibiotics has created a alarming situation and due to the emergence of antibiotic resistant microorganisms exhibits simultaneous resistance to two or more classes of antibiotics. Plants produce secondary metabolites, which constitute the main source of biologically active substances. (Akhtar *et al.*, 2013)

Application of plant derived products as photochemical either as in pure form or as standardized extracts, could be addition of great significance in the treatment of many bacterial infections. In general, studies on the antimicrobial activity of plant extracts have been restricted to standard bacterial strains, but not on multi-drug resistant clinical isolates of regional Etiologic. (Debnath *et al.*, 2013)

Microorganisms exhibit intrinsic resistant to certain antibiotics but most of microorganisms become resistant through genetic mutation either by horizontal gene transfer or by plasmid mediated. (Aarestrup, 2005; Kausar *et al.*, 2022) Over use of antibiotics leads to majority part of the community burden of antibiotic use which contributes dramatically to the rise prevalence of resistance amongst many human pathogens. (Dahiya and Purkayastha, 2012) Drastically Increased resistance among micro organisms leads to increase nosocomial and community-acquired infections is known to be related to the widespread utilization of antibiotics. (Jamalifar *et al.*, 2011)

To stop such burdens the only way is to use medicine as prescribed by the doctor. The *Piper betel* and *Achyranthes aspera* both have biochemical properties as well as phytochemical activity, the details will be discussed in the next part. Plant which contains phytochemical property that plant can be used for medicinal use.

Study aim to evaluate the properties of plant's secondary metabolites in vitro. The screening and antimicrobial activity of plant materials against Multi-Drug Resistant isolates will confirm us to use the medicinal plant.

Introduction of *Piper Betel*

As we know herbs are full of surprises when we use them for curing diseases, the *Piper betel* also playing an important role in curing diseases. The *piper betel* belongs to the genus *Piper* and species *Piper betel*. The classification is as described below:

The *Piper betel* belongs to the *Piperaceae* family. It has two main types white and black. This is the reference of Rajanighantu. As the great rishi said it has 7 main types shrivati, amlavati, satasa, guhagare, amlarasaa, patuulika and vehasaneya.

It has chemical components like 3.1% protein, 6.1% carbohydrate, khanija 2.3%, and tannin 2%. It has calcium, phosphorus, iron, iodine, potassium, and vitamin A, B, and C. Betel oil contains 0.7-2.6 % of gaseous oil which contains phenol and terpene. Due to the phenol, the leaf contains fragrance. The percentage of the essential oil decides the taste of the leaf. The higher content of the terpene higher the bitterness in the taste. The leaf contains live chemical components like Eugenol, chavibetol, and hydroxychaviol.

It is specially used for killing the microorganism in the stomach as well as it keeps the mouth fresh when we chew the leaf. The use of the betel leaf enhances hunger. It is also used as a medicine for cardiac arrest. The use of leaves enhances the power of the heart. It is also used in curing fever.

The gaseous substance present in betel oil which is also known as chavicol, chavicol has a powerful antiseptic property. It is 5 times stronger than carbolic acid and twice as strong as eugenol. The juice is also antiseptic and used in catarrhal and inflammation of the throat and bronchi in diphtheria (R. N. Khory, Part ii., P 156).

The essential oil is widely use in catarrhal affection, in inflammation of the throat, larynx, and bronchi; it has an antiseptic action. It's used in diphtheria as a gargle and as a inhaler too. The dose is one drop in 100 gm of water. In India, the juice of four leaves

may be used similarly diluted (Dymock, P186).

Betel leaves are of great holistic significance and are also preferred to chew. Being always at hand, pan leaves are used as a domestic remedy in many ways, application of the stalk of pan leaf with oil is introduced into the rectum in constipation and tympanites of children, with the object of inducing the bowels to act. The leaves are smeared to the temples headaches to relieve pain, too painful and swollen glands for promoting the secretion, and to the mammary gland with the object of checking the secretion of milk. Pan leaves are used as a ready dressing for foul ulcers, which seem to improve under them. (Hind. Mat. Med., P.245)

Introduction of *Achyranthes aspera*

The *Achyranthes aspera* belongs to the family *Amaranthaceae*, Genus *Achyranthes*, and species *aspera*. It has two types white and red *Aspera*. The red aspera stem and flowers are red-colored. Some leaf also has some patches of red color. It grows in dry places.

Chemical contents in the *Aspera*: The ash of plant contains the potassium and the rice contains a large percentage of alkaline. (Materia Medica of India – R. N. Khory, II., P.504)

Aspera is used curing for intestinal diseases, cardiac, blood, nasal infection**,** and skin diseases. The Aspera root is used for snack bites with the combination of black paper.

Astringent, diuretic, and alternative, given in menorrhagia, Diarrhoea, and Dysentery. Khar is largely used in *Anasarca ascites* and Dropsy. It is given in cutaneous affections and enlargements of glands, and to loosen expectoration on a cough.

It has a great reputation in dog bites, and bites of snakes and other venomous reptiles, for which purpose it is given internally and also applied externally. The juice is sometimes applied to toothache and the paste as eye salve (Anjan) in the opacity of the cornea. Medicated oil is dropped into the ear in deafness and noise in the ears (Do. II. 504-5).

The property of diuretics of the plant are well-known to the natives of India, and European physicians agree as to its value in dropsical affections; if one ounce of the plant allow to boiled in ten ounces of water for 15 minutes, and from 1 to 2 ounces of the decoction be given 3 times a day (Dymock, III, P. 136).

The useable plant parts are: root leaf stem and flower i.e., rice

Materials and Methods

Chloroform, Ethanol, Xylene, DMSO (dimethyl sulfoxide), Muller Hinton agar, Whatman paper no 14, Filter paper, Nutrient agar, Distilled water, Conc. H2SO4, Hexane, NaOH, FeCl3, etc.

Sample collection

Piper betel leaves were collected from the Mandai Shaniwar Peth, Pune, and the *Achyranthes aspera* plants were collected from Sandeepani Vedagurukulam Ramayampet, Telangana.

Plant material

Piper betel leaves weighing around 3-3.5 gm were used and *Achyranthes aspera* roots were about 2.-2.5 gm in weight for the extraction purpose.

Extraction

Extraction is the process that in which the separation of active compound of plant or animal tissues from inactive or inert compound by using selective solvents in the standard extraction process. The obtained product from plants is relatively impure in form of liquid, semisolids or powders which intended only for oral or external use. The extraction was done by process of maceration.

Antimicrobial activity assay

Antimicrobial activity can be defined as a collective term for all active components (agents) that inhibit the growth of bacteria, prevent the biofilm formation, and may destroy microorganisms.

Clinical isolates used

Antimicrobial activity was performed against the following strains: *Klebsiella pneumoniae*, *Escherichia coli*, *Staphylococcus haemolyticus* and *Pseudomonas aeruginosa*.

Kirby Bauer Method

The fresh bacterial culture suspension was compared to McFarland standard i.e. 0.5.and was spread on sterile Muller Hinton Agar (MHA) plates using sterile swabs. Bacterial culture suspension of 100 ul volume was used for spreading. The Whatman paper 4, 6 mm disc was kept on the agar by dipping them into the solvent extract. The plates were incubated for 24 hrs at 37^0C. Finally, the zone of inhibition was measured using the ruler. The solvent used for extraction was used as a control. (Kausar *et al.*, 2022)

Antibiotic susceptibility testing for screening of Multi-Drug Resistant clinical isolates

Susceptibility means microorganisms such as bacteria and fungi are not able to grow in presence of one or more antimicrobial drugs. Susceptibility testing is performed on bacteria or fungi causing an individual's infection after they have been recovered in a culture of the specimen. Testing is used to determine the potential effectiveness of specific antibiotics on the bacteria and/or to determine if the bacteria have developed resistance to certain antibiotics. The results of this test can be used to help for selection of drugs that will likely be most effective in treating an infection. When someone is treated with an antimicrobial drug, the most susceptible microorganisms will kill first. If treatment is stopped before all of the pathogens are killed, the survivors may develop a resistance to that particular antimicrobial drug. If next time same microorganism are exposed to the same drug, it may be ineffective as the bacteria and their progeny are likely to retain resistance to that antimicrobial drug. Resistance can spread when resistant microbes share their genetic material with susceptible ones. (https://www.testing.com/tests/antibiotic-susceptibility-testing/)

By knowing antibiotic susceptibility testing, to check the susceptibility of the microorganism, we have chosen some of the Multi-Drug Resistant organisms, whether they are susceptible or not we found some of them as susceptible and resistant.

The resistant organism showed the zone of clearance against the *Piper betel* and *Achyranthesaspera*. These results would give a new insight into studies regarding role of bioactive compound of herbs against to fight infection.

Source of organisms

Different organisms were isolated from Kamala Nehru Hospital, Pune from different types of samples i.e., Pus, Urine, and Blood.

Microorganism identification

The classification of chosen Multi-Drug Resistance (MDR) bacteria is as follows:

Most of the bacteria cause urinary tract infection, nervous system damage, skin diseases, hematologic diseases, heart attack, etc. to kill these disease-causing bacteria there are plenty of antibiotics available on the market and humans consume those medicine. With the use of so many drugs without a prescription from the doctor and incomplete consumption of the drug, the bacteria became susceptible as well as resistant. As they resist the drug the illness becomes serious and, in the end, the result may be death.

Table.1

Clasification of Bacteria				
Bacteria Name:	*K. Pneumoniae*	*P. aeruginosa*	*E. coli*	*S. haemolyticus*
Kingdom	Bacteria	Bacteria	Bacteria	Bacteria
Phylum	Pseudomonadota	Pseudomonadota	Pseudomonadota	Bacillota
Class	Gammaproteobacteria	Gammaproteobacteria	Gammaproteobacteria	Bacilli
Order	Enterobacterales	Pseudomonadales	Enterobacterales	Bacillales
Family	Enterobacteriaceae	Pseudomonadaceae	Enterobacteriaceae	Staphylococcaceae
Genus	*Klebsiella*	*Pseudomonas*	*Escherichia*	*Staphylococcus*
Species	*K. Pneumoniae*	*P. aeruginosa*	*E. coli*	*S. Haemolyticus*

Table.2

			AST Results	
Sr.No.	Source	Isolate *	Resistant	Susceptible
1	Urine	*Klebsiella pneumoniae*	Amikacin	-
			Gentamicin	-
			Ertapenum	-
			Meropenum	-
			Cefepime	-
			Ceftazidime	-
			Ceftriaxone	-
			Ampicillin	-
			Amoxyclav	-
			Piperacillin - tazobactam	-
			Norfloxacin	-

			AST Results	
Sr. No.	Source	Isolate *	Resistant	Susceptible
2	Blood	*Staphylococcus haemolyticus*	Penicillin	-
			Gentamycin	-
			Erythromycin	-
			Clindamycin	-
3	Urine	*Esherichia coli*	Ceftazidime	Amikacin
			Ceftriaxone	Gentamycin
			Amoxyclav	Ertapenum
			Norfloxacin	Imipenum
			Ciprofloxacin	Cefoxitin
4	Pus	*Pseudomonas aeruginosa*	Ciprofloxacin	Amikacin
			Colistin	Gentamycin
				Tobramycin

*Method: Automated by BD Phoneix M50

Table.3 Disc Diffusion Result against 391

Disc Diffusion Result			
Isolate no. 391			
Sr. No.	Solvent Extract	Abrevation	Diameter in mm
1	Piper betal	HBE1	17
2	Banarasi Piper betal	BBE2	5
3	Achyranthes aspera	AE3	10
4	Piper betal	HBC1	7
5	Banarasi Piper betal	BBC2	9
6	Achyranthes aspera	AC3	6
7	Piper betal	HBX	12
8	Piper betal	HBH	9
9	Achyranthes aspera	XA	9
10	Achyranthes aspera	HA	9

Table.4 Disc Diffusion Result against 392

Disc Diffusion Result			
Isolate no. 392			
Sr. No.	Solvent / Solvent Extract	Abrevation	Diameter in mm
1	Piper betel	HBE1	10
2	Banarasi Piper betel	BBE2	10
3	Achyranthus aspera	AE3	12
4	Piper betel	HBC1	7
5	Banarasi Piper betel	BBC2	7
6	Achyranthus aspera	AC3	7
7	Piper betel	HBX	12
8	Piper betel	HBH	20
9	Achyranthus aspera	XA	7
10	Achyranthus aspera	HA	10

Table.5 Disc Diffusion Result against 411

Disc Diffusion Result			
Isolate no. 411			
Sr.No.	Solvent / Solvent Extract	Abrevation	Diameter in mm
1	Piper betal	HBE1	17
2	Banarasi Piper betal	BBE2	5
3	Achyranthes aspera	AE3	10
4	Piper betal	HBC1	7
5	Banarasi Piper betal	BBC2	9
6	Achyranthes aspera	AC3	6
7	Piper betal	HBX	6
8	Piper betal	HBH	6
9	Achyranthes aspera	XA	7
10	Achyranthes aspera	HA	7

Table.6 Disc Diffusion Result against 78

Disc Diffusion Result			
Isolate no. 78			
Sr.No.	Solvent / Solvent Extract	Abrevation	Diameter in mm
1	Piper betal	HBE1	17
2	Banarasi Piper betal	BBE2	5
3	Achyranthes aspera	AE3	10
4	Piper betal	HBC1	7
5	Banarasi Piper betal	BBC2	9
6	Achyranthes aspera	AC3	6
7	Piper betal	HBX	6
8	Piper betal	HBH	7
9	Achyranthes aspera	XA	7
10	Achyranthes aspera	HA	11

Table.7 Phytochemical test of *Piper betel* and *Achyranthes aspera*

Phytochemical tests	**Observation**	**Betal**	**Aghada**
Glycoside test	yellow colouration	+	+
Tannin test	yellow ppt	+	-
Terpanoid test	redish/ brown	+	+
Cumarin test	yellow colouration	+	+
Ferric Chloride test	bluish black colouration	+	-
Flavonide test	yellow ppt	+	+

Table.8 Abbreviations

391	Klebsiella pneumonia
392	Pseudomonas aeruginosa
78	Eschericia coli
411	Staphylococcus haemolyticus
Con 1	Ethanol
Con 2	Chloroform
Con 3	Xylene
Con 4	Hexane
HBE	*Piper betel* Ethanol
HBC	*Piper betel* Chloroform
HBX	*Piper betel* Xylene
HBH	*Piper betel* Hexane
BBE	Banarasi *Piper betel* Ethanol
BBC	Banarasi *Piper betel* Chloroform
AE	*Achyranths aspera* Ethanol
AC	*Achyranths aspera* Chloroform
AX	*Achyranthes aspera* Xylene
AH	*Achyranthes aspera* Hexane
Ppt	Precipitation
$FeCl_3$	Ferric Chloride
H_2SO_4	Sulfuric Acid
NaOH	Sodium Hydroxide
DMSO	Dimethyl Sulfoxide

Fig.1 Degree of susceptibility to various antibiotics

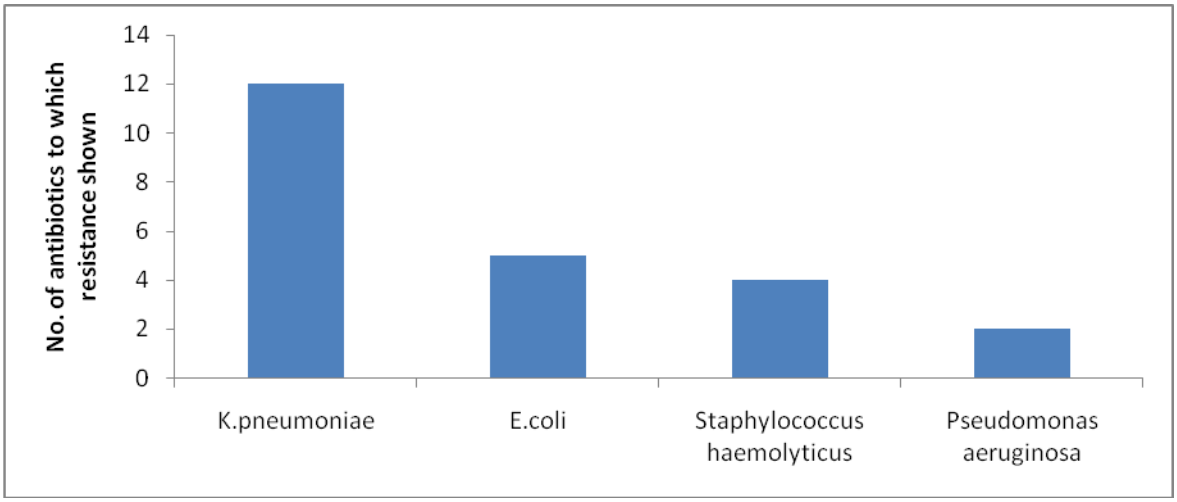
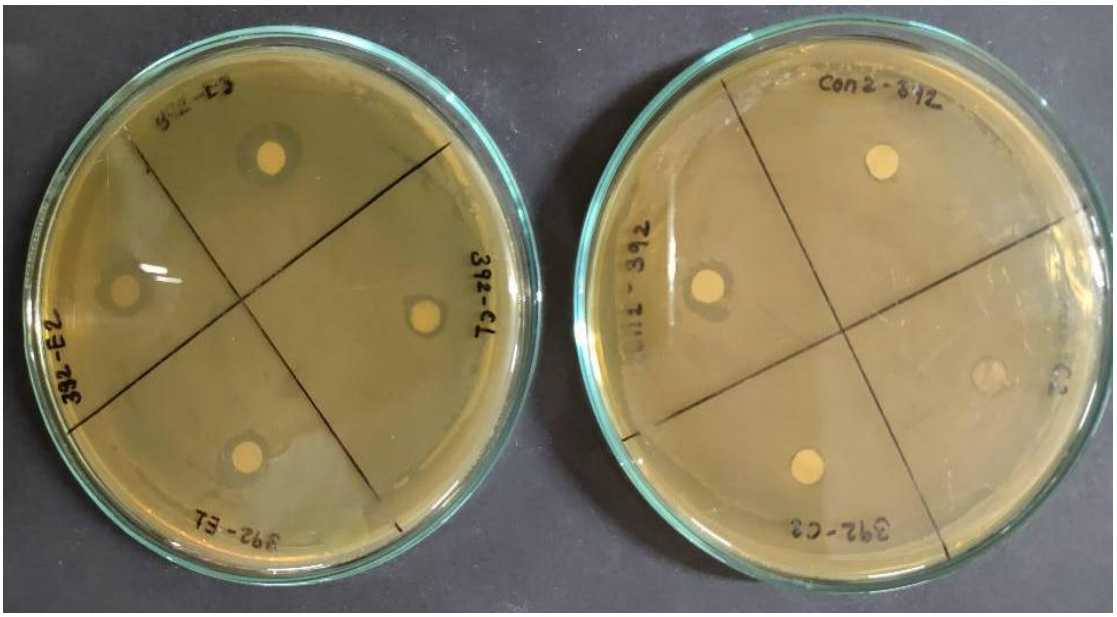
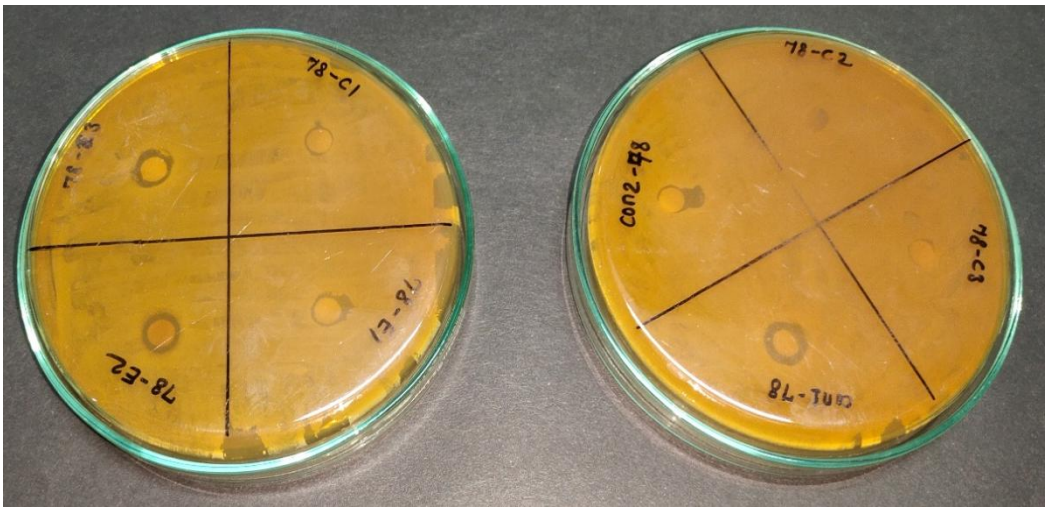


Fig.2 Zone of inhibition using ethanol and chloroform solvent against 78

Zone of inhibition of using ethanol and chloroform solvent against 392



Zone of inhibition using ethanol and chloroform solvent against 411



Screening of Multi-Drug Resistant microorganisms

Multi-drug resistant organisms were identified from different samples like pus, blood, and urine by BD Phoenix M50 automated method, where different antibiotic sensitivity patterns were observed**.**

List of Antibiotics

Data provided by Ms. Rajal Dave, In-charge Microbiology, Krsnaa Diagnostic ltd, Kamala Nehru Hospital, Pune.

Phytochemical Activity testing

Test for Glycoside

For determination of glycoside dissolve a small amount of aqueous extract of fresh or dried material in one ml of water. Add a few drops of 10% aqueous NaOH solution. The yellow colour indicates the presence of glycoside.

Test for Tannins

For determination of tannins test, take 3ml of aqueous extract and add a few drops of 1% lead acetate. A yellow precipitate indicates the presence of tannins in the herb.

Test for Terpenoid

For determination of terpenoid the Salkowski test was performed in which 3-4 ml of aqueous extract was mixed with the 2 ml of chloroform and concentrated H2SO4 3ml was added slowly drop by drop to form a monolayer.

After the addition of the few drops of concentrated H2SO4, the mixture starts boiling with plenty of bubbles the boiling remains continues until we stop the addition of the solvent. It takes around 30-40 seconds to stop the bubbling. The test remains hot for around 5-6 minutes. Brownish red coloration indicates the terpenoid.

Test for Coumarin

For determination of coumarin 3 ml of 10 % of NaOH was added to 2 ml of aqueous extract formation of yellow color indicates contains coumarin.

Test for Phenol (Ferric Chloride Test)

For determination of phenol ferric chloride test was performed, for testing the extract take the ethanolic extract and add a pinch of FeCl3 powder the bluish-blackcolor indicates the presence of phenol.

Test for Flavonoid

For determination of flavonoid the lead acetate solution test was performed, for testing add a few drops of 10 % of lead acetate in 2ml aqueous extract a yellow color formation indicates the presence of flavonoid and it disappears on standing but the precipitation remains the same.

Results and Discussion

Antimicrobial activity

The aqueous extract and solvent extract was used for the screening of antimicrobial activity; the aqueous extract didn't show a clear zone of inhibition as compared to solvent extraction.

Phytochemical analysis

The comparison of phytochemical analysis of *Piper betel* and *Achyranthesaspera* is mentioned in the chart.

Selection of the plant to cure any disease is very difficult but if the properties of the plant and chemicals are known then it is very easy to consume and avoid the hazardous effect of the plant. In this work, we have performed so many tests to confirm the chemical properties and antimicrobial activity against MDR bacteria.

To perform the antimicrobial activity there are two methods 1. agar well diffusion method and 2. Kirby Bauer Method i.e., disc diffusion method. We have performed the antimicrobial activity with Kirby Bauer Method. We found this method more effective than well diffusion method.

The Antimicrobial activity was also tried using aqueous extract on Nutrient agar, but it failed to give results. Muller Hinton Agar proved to be best for testing antimicrobial nature. The Muller Hinton agar is commonly used for antibiotic susceptibility testing for the Kirby- Bauer disc diffusion method or standard antibiogram. Muller-Hinton agar is therefore used for clinical diagnosis. Nowadays the clinical isolates which are the root cause of many diseases have become resistant to so many drugs. The table of susceptibility testing describes to which drug the specific bacteria is susceptible and resistant. The susceptibility screening was performed by an automated method using the BD Phoneix M 50 machine.

Around six Phytochemical tests were performed for *Piper betel* and *Achyranthes aspera*. Glycoside test, Tannin test, Terpenoid test, Coumarin test, Ferric Chloride test, and Flavonoid test were positive for both *Piper Betel* and *Achyranthes aspera* except tannin and ferric chloride test was negative.

In this study ethanolic and hexane extract of betel leaves and *Achyranthes aspera* were found to have promising antimicrobial activity as compared to Chloroform and xylene. Ethanolic extract of both plants showed a detectable zone of inhibition against all four Gram-negative and Gram-positive MDR clinical isolates. The highly resistant *Klebsiella pneumonia* showed maximum sensitivity to ethanol extract of *piper betel* and moderate sensitivity to ethanol extract of *A.aspera*. The betel oil contains 0.7-2.6 % of gaseous oil which contains phenol and terpene due to which the terpenoid test was found to be positive. *Piper betel* was found to show an outstanding antibacterial activity in comparison to *Achyranthes aspera*.

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