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PRELIMINARY PHYTOCHEMICAL ANALYSIS OF THE INDIAN MEDICINAL PLANTS FOR ANTIBACTERIAL ACTIVITY AGAINST BOVINE MASTITIS PATHOGENS

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The Antibacterial activity of four different extract viz. hexane, ethyl acetate, methanol and aqueous of six medicinal plants of Gujarat, India each belonging to different families were evaluated against mastitis pathogens viz. *Staphylococcus aureus, Streptococcus agalactiae, E.coli, Pseudomonas mendocina, Micrococcus pyogens*. The *in vitro* antibacterial activity was performed by agar well diffusion method. The aqueous and hexane extracts were less active but methanol and ethyl acetate extracts showed high degree of antibacterial activity against the tested bacterial strains. The most active antimicrobial plants were *Rauvolfia serpentina, Cinnamomum zeylanicum* and *Azadirachta indica. The Staphylococcus aureus* was the most resistant bacteria while *Pseudomonas mendocina* was the most susceptible bacteria. Amongst the plant species screened, methanol extract of *Rauvolfia serpentina* root showed best antibacterial activity. Primary qualitative analysis of phytochemicals showed presence of different phytochemicals in the extracts used in this study.

Key words: Mastitis, Antibacterial activity, medicinal plants, phytochemicals

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Mastitis is an inflammation of mammary glands and is characterized by physical, chemical and usually bacteriological changes in milk and pathological changes in glandular tissues (Radostits, *et al.*, 2000; Sharma et al., 2012). Mastitis can be infectious or non infectious (Bradley, 2002). In India, the average incidence of clinical mastitis has been found to be 1-10%, whereas incidence of subclinical mastitis have been reported to vary from 10-15% in cattle and 5-20% in buffaloes (Kumar, 1988 and Singh, 1991). Mastitis is the most economically devastating disease in the dairy industry worldwide. The factors causing the high cost involvement include treatment cost, milk disposable due to high bacterial load, reduction in milk production, culling of high producing affected animals and extra labour cost while managing affected animals (Miller et al., 1993). Estimates suggest mastitis costs the dairy industry upwards of two billion dollars each year. Today the increase in mastitis cases causes great concern for animal breeders, particularly with the improvement in productivity. The incidence of antibiotic resistance and lack of availability of proper vaccine have made the conventional mastitis control measures largely unsuccessful. Mastitis is considered to be a multifactorial disease. Over 200 microbial species, sub species and serotype have been isolated from bovine mammary gland (Mallikarjunaswamy and Krishnamurthy, 1997) and identified as causative agents of mastitis. However, other group of micro-organisms, virus, fungi etc can also cause mastitis in bovine.

One important reason for treatment failure is assumed to indiscriminate use of antibiotics without testing in vitro sensitivity of causal

organisms and insensitivity of some antibiotics (Saxena et al., 1993). This practice at one hand increases economic losses and on other side results in development of resistance to commonly used antimicrobials (Owens et al., 1997). The emergence of multiple drug resistant bacteria has become a major cause of failure of the treatment of infectious diseases (Gibbons, 2005; Kepil, 2005; Alam et al, 2009). Antimicrobial drug resistance is a global concern today as the resistant microorganisms have emerged and spread throughout the world because of their genetic plasticity (Kunin, 1993; Blondeau, 1999). The fast growing health consciousness among the consumers regarding the use of antibiotics for treatment of mastitis has triggered a search for a genetic defense mechanism and an alternate mode of treatment.

Herbalism isa traditional medicine practice based on the use of plants and plant extracts. Recently there has been a shift in universal trend from synthetic to herbal medicine (*Return to Nature).* Traditional use of medicines is recognized as a way to learn about potential future medicines. At present, nearly 30% or more of the pharmacological drugs are derived directly or indirectly from plants and their extracts dominating in traditional medicine systems and a common element in Ayurvedic, Homeopathic, Naturopathic etc. (Murugesan et al., 2011; Jabeen et al, 2007; Banso, 2009; Ahamunthunisa and Hopper, 2010). The effect of plant extract on bacteria has been studied by a large number of researchers worldwide (Reddy et al., 2001). Much work has been done on ethanomedical plants in India (Erdogrul et al., 2002). Among the 120 active compounds currently isolated from the higher plants and widely used in modern medicine today, 80 percent show a positive correlation between their modern therapeutic use and the traditional use of the plants from which they are derived. (Fabricant et. al., 2001). Herbalists tend to use extracts from parts of plants, such as the roots or leaves but not isolate particular phytochemicals (Vickers and Zollman, 1999). The utilization of herbal drugs is on the flow and the market is growing step by step (Kamboj, 2000).

The present studies were undertaken to determine antibacterial activity of six Indian medicinal plants of different families (*Azadirachta indica, Adhatoda vasica, Allium sativum, Asparagus racemosus, Rauwolfia serpentina and Cinnamomum zylanicum*) against Mastitis causing bacteria especially *Pseudomonas mendocina, Staphylococcus aureus, Streptococcus agalactiae, Micrococcus pyogenes and Escherichia coli* and qualitative analysis of phytochemicals of selected medicinal plants.

MATERIALS AND METHODS

Collection of the plant samples

Fresh plant parts were collected from the semi-arid region of Anand, Gujarat, India. The details of plant/ plant parts screened, their families, vernacular names and their therapeutic uses are given in Table 1. The taxonomic identities of these plants were confirmed by the taxonomist and the voucher specimen numbers of the plants were preserved. Fresh plant material were washed under running tap water, air dried and then homogenized to fine powder and stored in airtight bottles at 4°C.

Preparation of plant extracts

As described earlier (Dash et al., 2011), the plant extracts were prepared by proceeding with sequential method (cold maceration method) using Hexane, Ethyl acetate, Methanol and Distilled Water. Approximately 50 gm of plant material was soaked in 250 ml hexane for 24 hours at room temperature. This solution was filtered with the help of Whatman No. 1 filter paper. The filtrate was collected in 15 cm petridishes and evaporated the solvent at room temperature. The solid dried extract was stored in 2 ml appendroff tube and powder was used for antimicrobial assays. The filter cake was dried at room temperature and stored separately. The dried powder of filter cake was sequentially resuspended in 250 ml ethyl acetate, methanol and distilled water to prepare dried extract in each solvent. After extraction in

each solvent remained filter cake was dried and further used with next solvent for extraction. These dried extracts were stored at 4°C.

Table 1: Medicinal plants used in this study

Plant Name	Common Name	Family	Parts used	Medicinal properties
Azadirachta indica	Neem	Meliaceae	Leaves	Antiulcer effect, Antimalarial activity, Anti-microbial activity , Anticancer activity, Antioxidant activity, Anti-inflammatory, Antiarthritic, Diuretic, antidiabetic
Adhatoda vasica	Ardusi	Acanthaceae	Leaves	Anti-allergic activity, Anti-asthmatic activity, Anti-inflammatory activity, Anti-microbial activity, Anti-tubercular activity, Bronchodilatory activity, antioxidant activity
Allium sativum	Garlic	Alliaceae	Bud	Anti-microbial activity , blood purifier, anti-hypertensive, swelling, antioxidant, anti cancer, diuretic
Asparagus racemosus	Satavari	Asparagaceae	Leaves	antioxidant, antibacterial, immunomodulator, digestive, antidiarrhoeal
Rauwolfia serpentina	Sarpagandha	Apocynaceae	Root	Anticoagulant, antibacterial, antioxidant, anti allergic, tonic, anti-hypertensive,
Cinnamomum zeylanicum	Cinnamon	Lauraceae	Bark	Anti-allergic activity, antioxidant activity, Anti-microbial activity, Anti-tubercular activity, Bronchodilatory activity

Preparation of sample

Samples for antimicrobial activity were prepared by dissolving 100 mg of each extracts in 1 ml of dimethyl sulphoxide (DMSO).

Microorganisms

In vitro antimicrobial activity was examined for hexane, ethyl acetate, methanol and aqueous extracts from six medicinal plants used by traditional healers. Microorganisms were obtained from Veterinary College. Microorganisms were maintained at 4°C on nutrient agar slants. Amongst five microorganisms investigated two Gram negative bacteria were *Escherichia coli* and *Pseudomonas mendocina* while three Gram positive bacteria were *Staphylococcus aureus, Streptococcus agalactiae* and *Micrococcus pyogenes.*

Antibacterial bioassay

Preparation of bacterial suspension

Colonies of different strains of bacteria *(E.coli, S.aureus, S.agalactiae, Micrococcus pyogenes* and *P. mendocina)* were transferred to the different fresh nutrient broth in sterile conditions and were incubated at 37°C for 24 hrs. These suspensions were preserved in 250 ml sterile flasks for use.

Standardization of inoculums

Exactly 0.2 ml of 24 hours old culture of each organism was dispensed into 20 ml of sterile nutrient broth and was incubated for 3-5/hours to standardize the culture to 10^6 cfu/ml (Collins and Lyne, 1970). This suspension was used for inoculums.

Agar well diffusion method

The antimicrobial activities were of the test samples were carried out by disc diffusion method (Bauer et al., 1966). A volume of 0.5 ml of overnight broth culture of each clinical isolates containing 10^6 cfu/ml was aseptically transferred to the solidified nutrient agar and spread evenly on the agar

surface using a sterile glass spreader. Four 6 mm wells were bored unto the agar and each well was filled with 100 µl different extracts of plant. For each bacterial strain controls were maintained where pure solvents were used instead of the extract. The results were compared with the standard antimicrobics Gentamycin (10 µg /disc), Tetracycline (30 µg /disc), Streptomycin (10 µg /disc) and Erythromycin (15 µg /disc). The Petri dishes were incubated at 37°C for 18-24 hours and the inhibition zones were measured. The experiment was repeated three times to get mean values. The results were compared with the standard antimicrobics Gentamycin (10 µg /disc), Tetracycline (30 µg /disc), Streptomycin (10 µg /disc) and Erythromycin (15 µg /disc).

Minimum Inhibition Concentration (MIC) of the extract

The MIC was defined as the lowest concentration that completely incubated the growth of microorganism for 24 hrs (Thongson et al., 2004). The MIC of the extracts was also carried out by two fold serial broth dilution method. Plant extract showing inhibition zone of more than 8 mm were selected for MIC.

Qualitative analysis of Phytochemicals

The extracts of different plants were analysed for the presence of alkaloids, cardiac glycosides, steroids, phenols, saponins and tannins according to standard methods (Harborne, 1998).

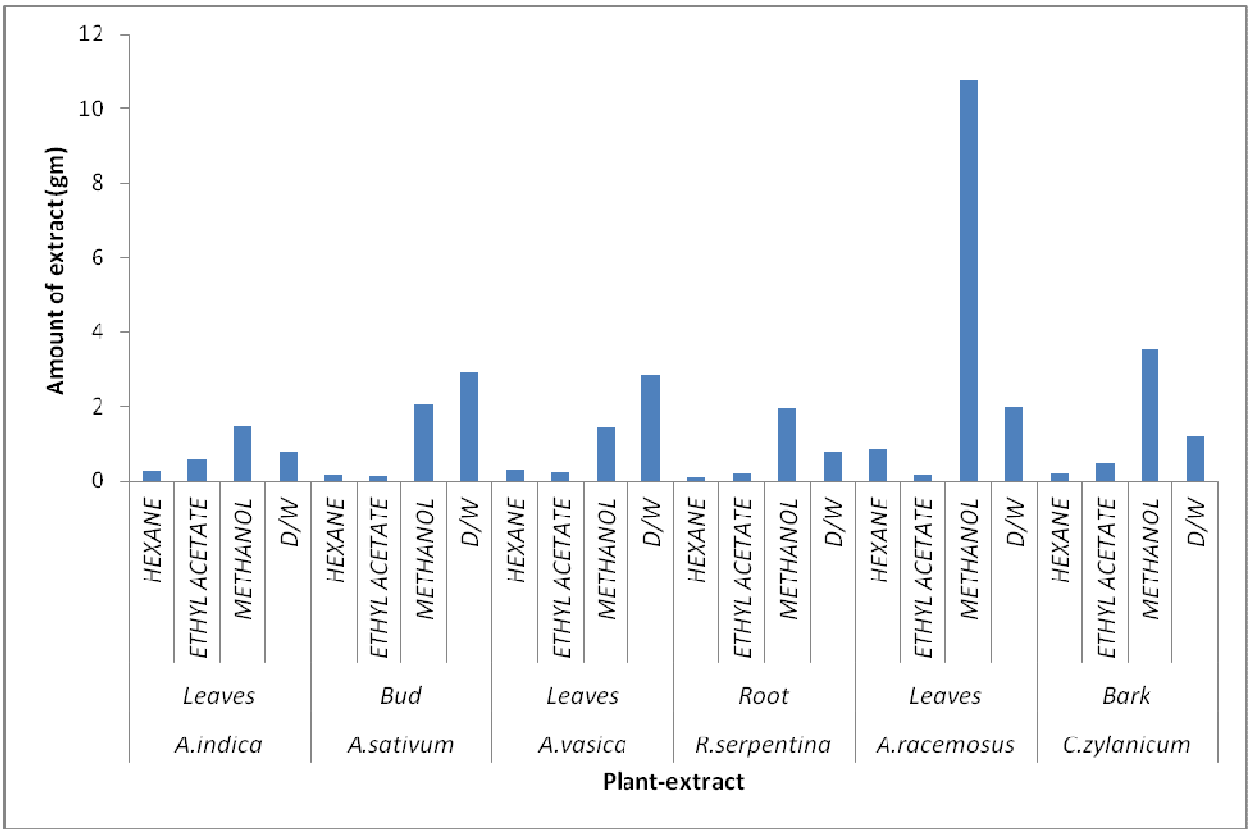
1) *Test for Alkaloid (Hager's test)*
   1 ml of plant extract was taken and then and add 1 ml of saturated solution of picric acid was added. Appearance of yellow colour indicates the presence of alkaloids.
2) *Test for tannins*
   About 0.5 g of the extract was boiled in 10 ml of water in a test tube and then filtered. A few drops of 0.1% ferric chloride was added and observed for brownish green or a blue-black coloration.
3) *Test for saponins*
   0.5 g of extract was added in 5 ml of distilled water in a test tube. The solution was shaken vigorously and observed for a stable persistent froth. The frothing was mixed with 3 drops of olive oil and shaken vigorously after which it was observed for the formation of an emulsion.
4) *Test for cardiac glycosides (Keller-Killiani test)*
   0.5 g of extract was diluted to 5 ml in water was added 2 ml of glacial acetic acid containing one drop of ferric chloride solution. This was under laid with 1 ml of concentrated sulphuric acid. A brown ring at the interface indicated the presence of a deoxysugar characteristic of cardenolides. A violet ring was appeared below the brown ring, while in the acetic acid layer a greenish ring may form just above the brown ring and gradually spread throughout this layer.
5) *Steroids (Salkowski's test)*
   About 100 mg of dried extract was dissolved in 2ml of chloroform. Sulphuric acid was carefully added to form a lower layer. A reddish brown colour appeared. This colour confirmed the presence of steroidal ring.
6) *Phenol (Folin's test)*
   2 ml of extract was taken and add 2 ml of Folin's reagent. Appearance of violet/brown colour indicates presence of phenol.
7) *Flavonoids*
   1ml of aqueous extract was added in 1ml of 10% lead acetate solution. The formation of a yellow precipitate was taken as a positive test for flavonoids.

RESULTS AND DISCUSSION

On the basis of extraction capacity of solvents, compounds from powdered form of plant were extracted in the proper solvent. Highest extraction of *A. racemosus* (10.76 gm) was obtained by using methanol. Higher extraction of *A. indica*, *R. serpentina* and *C. zeylanicum* was also observed by using methanol whereas, *A. sativum* and *A. vasica* showed higher extraction in D/W. In general, methanol was proved to be good

solvent for extraction. Hexane and ethyl acetate showed poor extraction. The amount of extracts by using different solvents, and the characteristics of extracts mentioned in graph- 1 and table-2 respectively.

Antibacterial activity was tested by agar diffusion method. Clear zone around well was indicator of inhibition of bacterial growth. The zone is referred as zone of inhibition (ZOI). The finding of antibacterial activity of extracts obtained from six medicinal plants has been indicated in table 3.



Graph-1: The amount of extracts

All the solvent extract of various plants showed good activity against the test bacteria ranging from 1-15 mm. Some of extracts have not shown any activity against bacteria. Ethyl acetate and methanol extract of *Azadirachta indica* showed activity (2-11 mm) against all five bacteria whereas, methanol extract of *Rauwolfia serpentine* showed maximum activity (2-15 mm) against all bacteria. Methanol extract of *Cinnamomum zeylanicum* also showed good activity (2-15 mm) against all five bacteria. Many of the workers (Parekh and Chanda, 2007; Alam et al., 2009) have reported that methanol is highly potent solvent for extraction the phytochemicals from the plant material. However, in present studies antibiotics like Erythromycin, Streptomycin, Gentamycin and Tetracycline showed maximum zone of inhibition against all five bacteria as compared to plant extracts. All the studies indicate that plants have also potential antibacterial activity. Because of the differences in plants and the plant parts that are extracted it is natural that there is differences in antibacterial activity. The results of the present investigation highlight the fact that the some organic solvent extracts exhibit good antibacterial activity because the active principles were either polar or non-polar and were extracted only through successive organic solvents (Britto, 2001; Muhanasundari et al., 2007).

Table 2: Characteristics of plant extracts

PLANT	PART	SOLVENT	WEIGHT (gm)	COLOUR	CHARACTERISTICS
Azadirachta indica	**Leaves**	Hexane	0.287	Hay (Green straw)	Hard, Opaque
		Ethyl Acetate	0.572	Bottle green	Shiny, Hard, Opaque, Powder
		Methanol	1.45	Black	Gummy, Oily, Shiny, Opaque, Hard
		D/W	0.76	Brown	Shiny, Hard, Opaque
Allium sativum	**Bud**	Hexane	0.18	colourless	Gummy, Oily, Transleuscent, Soft
		Ethyl Acetate	0.15	colourless	gummy, hard, Transleuscent
		Methanol	2.062	Rust brown	Gummy, Oily, Opaque, Soft
		D/W	2.908	Dark brown	Gummy, Oily, Hard, Transluscent, Shiny
Adhatoda vasica	**Leaves**	Hexane	0.312	Lemon yellow	Gummy, Hard, Opaque
		Ethyl Acetate	0.226	Orangish brown	Gummy, Transluscent, Soft
		Methanol	1.438	Dark brown	Gummy, Oily, Soft, Transluscent
		D/W	2.86	Black	Gummy, Oily, Shiny, Opaque, Soft
Rauvolfia serpentina	**Root**	Hexane	0.102	Greenish	Oily, Gummy, Soft, Opaque
		Ethyl Acetate	0.205	Yellowish orange	Soft, Opaque
		Methanol	1.969	Brown	Hard, Opaque, Shiny
		D/W	0.78	Light Brown	Shiny, Hard, Transluscent
Asparagus racemosus	**Leaves**	HEXANE	0.88	Greenish	Soft, Opaque, Gummy
		ETHYL ACETATE	0.16	Orangish brown	Hard, Opaque
		METHANOL	10.76	Orange	Oily, Gummy, Soft, Transluscent
		D/W	2.001	Sand color	Shiny, Hard, Transluscent
Cinnamomum zeylanicum	**Bark**	HEXANE	0.198	Yellow	Oily, Gummy, Soft
		ETHYL ACETATE	0.46	Brown	Oily, Gummy, Soft, Opaque
		METHANOL	3.538	Rust brown	Shiny, Opaque, Hard
		D/W	1.192	Brown	Oily, Gummy, Soft, Opaque

Table 3: Antibacterial activity

Plant species	**Solvent**	Zone of inhibition (mm)				
		E. coli	***P. mendocina***	***M. pyogenes***	***S. aureus***	***S. agalactiae***
Azadirachta indica	Hexane	0	5	0	0	3
	Ethyl Acetate	2	7	11	5	5
	Methanol	2	6	4	3	4
	D/W	3	0	0	0	1
Adhatoda vasica	Hexane	0	0	0	0	0
	Ethyl Acetate	0	0	5	2	0
	Methanol	0	0	0	0	0
	D/W	0	3	0	0	0
Allium sativum	Hexane	0	5	4	0	3
	Ethyl Acetate	0	5	0	2	4
	Methanol	0	0	8	0	0
	D/W	0	0	0	0	0
Asparagus racemosus	Hexane	2	2.5	0	0	1
	Ethyl Acetate	2	4	4	0	2
	Methanol	2	6	0	0	7
	D/W	0	5	0	0	0
Rauwolfia serpentina	Hexane	2	2	0	0	3
	Ethyl Acetate	0	3	0	0	3
	Methanol	2	11	14	15	13
	D/W	2	0	0	0	4
Cinnamomum zeylanicum	Hexane	3	7	0	0	7
	Ethyl Acetate	0	3.5	2	0	5
	Methanol	2	11	4	2	15
	D/W	0	2	6	2	0
Erythromycin		8	14	35	30	13
Streptomycin		12	17	8	10	18
Gentamycin		11	14	28	26	14
Tetracycline		5	11	22	22	15

The results of phytochemical analysis of the test plants are given in table-4. All the secondary metabolites; alkaloids, cardiac glycosides, steroids, phenols, saponins and tannins were commonly present in the test plants. The presence of one or more of these secondary metabolites indicated that the antibacterial activity is due to those active compounds present in different parts of the test plants. The antibacterial activity of extracts from many plants has been widely reported (Jigna and Sumitra, 2006; Chanda and Baravalia, 2010; Chattopadhyay et al., 2009; Ahmed and Beg 2001) but the test bacteria and zone of inhibition varied.

Table 4: Phytochemical analysis of metabolites against bacteria

Name of test	*A.sativum*				*A.indica*				*A.vasica*				*C.zeylanicum*				*A.racemosus*				*R.serpentina*			
	H	EA	M	D/W	H	EA	M	D/W	H	EA	M	D/W	H	EA	M	D/W	H	EA	M	D/W	H	EA	M	D/W
Alkaloids	-	+	+	-	+	-	-	+	+	+	+	-	+	+	-	+	+	-	+	-	-	-	+	+
Saponins	-	+	-	+	-	-	+	-	-	-	-	+	-	-	+	+	-	+	+	+	-	-	+	-
Tannins	-	-	-	-	-	+	+	-	-	-	+	+	-	-	+	+	-	-	-	-	-	-	-	-
Steroids	-	-	+	+	-	+	+	+	-	-	+	+	-	-	+	+	-	-	-	+	-	+	+	+
Cardiac Glycoside	-	-	-	-	+	+	-	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-
Flavanoids	-	+	-	-	-	-	-	-	-	-	+	-	-	-	+	-	-	-	-	+	-	-	-	-
Phenol	-	-	-	-	-	-	+	-	-	-	-	+	-	-	+	-	-	-	+	-	-	-	-	

Where: H = Hexane, EA = Ethyl acetate, M = Methanol and D/W = Distilled Water

Minimum Inhibitory Concentration (MIC) of Bacteria

Plant extract showing highest zone of inhibition in agar well diffusion method were selected further for MIC method. In this method plant extract with different concentration ranging from 100µg/ml – 0.18µg/ml was prepared by two fold double dilution method and depending on appearance of red colour of 2,3,5-triphenyl tetrazolium dye in ependorff tube minimum inhibitory concentration of extract was determined in table 5.

MIC of *S. aureus* by ethyl acetate extract of *R. serpentina* was 100µg/ml. MIC of

S.agalactiae by ethyl acetate extract of *A.indica* was 25 µg/ml, 100 µg/ml by hexane extract of *A.sativum*, 100 µg/ml by methanol extract of *R.serpentina* and *C.zeylanicum*. MIC of *M.pyogenes* was 100 µg/ml by methanol extract of *R.serpentina*. MIC of *E.coli* were 50 µg/ml by EA extract of A.I., 6.25 µg/ml by M extract of R.S. and 100 µg/ml by M extract of C.Z. MIC of *P.mendocina* were 12.5 µg/ml by EA extract of A.I., 100 µg/ml by M extract of A.V., M extract of R.S., A.R. and C.Z.

Table 5: MIC of bacteria

Test organism		MINIMUM INHIBITORY CONCENTRATION(MIC) µg/ml									
		A.I. (EA)	A.S. (H)	A.V. (M)	R.S. (M)	A.R. (M)	C.Z. (M)	Control			
		leaves	Bud	Leaves	Root	Leaves	Bark	D/W	Extract	T	G
Gram +ve	*S.aureus*	-	-	-	100	-	-	+	NA	NA	NA
	S.agalactiae	25	100	-	100	-	100	+	NA	NA	NA
	M.pyogenes	-	-	-	100	-	-	+	NA	NA	NA
Gram -ve	*E.coil*	50	-	-	6.25	-	100	+	NA	NA	NA
	P.mendocina	12.5	-	100	100	100	100	+	NA	NA	NA

Where: A.I=*Azadirachta indica*, A.S=*Allium sativum*, A.V=*Adhatoda vasica*, R.S=*Rauwolfia serpentina*, A.R=*Asparagus racemosus*, C.Z=*Cinnamomum zeylanicum*, NA=Negative activity, T=Tetracycline, G=Gentamycin, D/W=Distilled water, EA=Ethyl acetate, M=Methanol, H=Hexane

Plants have formed the basis traditional medicine system and natural products make excellent leads for new drug development. Approximately 80% of the word inhabitants rely on traditional medicine for their primary health care and play an important role in the health care system of the remaining 20% of the population. (Cragg *et al*; 1999). The potential of higher plants as a source of new drugs has not been largely exploited.. Even though, pharmacological industries have produced a number of new antibiotics in the last three decades, resistance to these drugs by microorganism has increased (Gislene,

2000). In general, bacteria have the genetic ability to transmit and acquire resistance to drugs which are utilized as therapeutic agents (Cohen, 1992). Therefore actions must be taken to reduce this
problem, for example to control the use of antibiotics, research should be carried out for better understanding of the genetic mechanism and for the development of new drugs either synthetic or natural.

It has been stated that the mechanism of the antimicrobial activity of the plant extract involves the inhibition of various cellular processes, increase in plasma membrane permeability and impairment of energy or synthesis of structural components in microbial cells. The varying degree of sensitivity of the bacterial strains may be due to the intrinsic tolerance of the bacterial and the nature and combinations of phytocompounds present in the extracts as observed by Suree and Pana (2005).

Precisely the study indicated that inhibitory effects of plant extracts were higher on Gram +ve bacteria than Gram –ve bacteria. *A.indica* and *C.zeylanicum* proved their strong antibacterial activity. *A.vasica* showed less inhibition against bacteria.

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