



Screening of methanol & acetone extract for antimicrobial activity of some medicinal plants species of Indian folklore

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ABSTRACT

In search for new and safe antibacterial agent medicinal plants have been continuously investigated. The methanol and acetone extract of *Alstonia scholaris* (stem bark), *Achyranthus aspera* (whole plant) *Moringa oleifera* (leaves), *Tinospora cordifolia* (stem), and *Enicostema hyssopifolium* (stem) were screened for their antibacterial activity using the agar diffusion method. The susceptibility of the microorganisms to the extracts of these plants was compared with each other and with selected standard antibiotics. The antimicrobial activities of these plants were discussed according to their phytochemical components. Finding of current research was suggest *A. scholaris* and *M. oleifera* can be explored as a new source of antibacterial compound, for *Enterobacter aerogens* (ATCC13048), *Staphylococcus aureus* (ATCC9144), and *Micrococcus luteus* (ATCC4698).

Keywords: Antibacterial activity, *Alstonia scholaris*, Antibiotic, *Staphylococcus aureus*.

INTRODUCTION

There is an urgent need to discover new antimicrobial agents for human and veterinary therapeutic uses, as resistance to current drugs increases in severity and extent (Robert & Meunier, 1998; Shah, 2005; Pauli *et al.*, 2005; Rios & Recio, 2005). Plants are invaluable sources of pharmaceutical products (Olalde Rangel, 2005). Plants are recognized for their ability to produce a wealth of secondary metabolites and mankind has used many species for centuries to treat a variety of diseases (Cragg *et al.*, 1999). Despite the wide availability of clinically useful antibiotics and semisynthetic analogues, a continuing search for new anti-infective agents remains indispensable because some of the major antibacterial agents have considerable drawbacks in terms of limited antimicrobial spectrum or serious side effects (Olila *et al.*, 2001).

The negative health trends call for a renewed interest in infectious disease in the medical and public health communities and renewed strategies on treatment and prevention. Proposed solutions are outlined as a multi-pronged approach that includes: prevention, (such as vaccination); improved monitoring; and the development of new treatments. It is last solution that would

encompass the development of new antimicrobials (Fauci, 1998). To avoid the futuristic disaster due to side effect of chemically synthesized antimicrobial in form of drug, food additives and preservatives mankind has to shift to natural antimicrobial compound. Ample source of potential anti microbial are given as gift to mankind by nature in form of medicinal plants. The identification of new natural products with antimicrobial activity, extraction methods, and hopefully new modes of action, is one of the ways of tackling this problem. Lack of scientific knowledge has often constituted a major constraint to consideration of the use of traditional herbal remedies in conjunction with or as an affordable alternative to orthodox medical treatment. In present study methanol and acetone extract of five medicinal plants are studied for their antimicrobial activity in crude form and after treatment. Traditionally these plants are used in many Ayurvedic preparations for treating various diseases and used increasingly as dietary supplement to fight or prevent common disease. Centre focus of present work was to evaluate antibacterial activity and find ways to increase their effectiveness.

MATERIALS AND METHODS

Plant material

Authentic powder samples (By Prof. M.H.Parabia, Head of bioscience department, Bapalal Botanical Vaidya Research Center) of *Alstonia scholaris* Linn. R.Br. (Stem bark, Apocynaceae), *Achyranthus aspera* Linn. (Whole plant, acantheceae), *Moringa oleifera* Lam. (Leaves-Morinaceae), *Tinospora cordifolia* (Stem-

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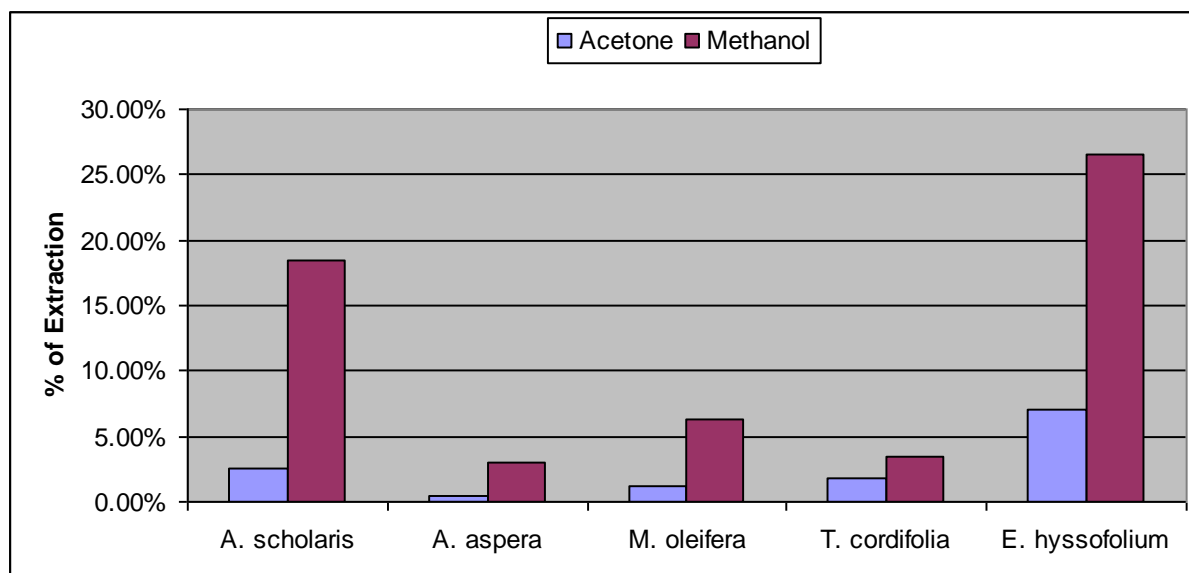


Figure 1: Extractive value in methanol and acetone

Table 1: Phytochemical screening of methanol extract

Phyto constituents	<i>A.scholaris</i>		<i>A.aspera</i>		<i>M. oleifera</i>		<i>T.cordifolia</i>		<i>E. hyssofolium</i>	
	M ₁	M ₂	M ₁	M ₂	M ₁	M ₂	M ₁	M ₂	M ₁	M ₂
1. Alkaloids	+	+	+	+	+	+	+	+	+	+
2. Carbohydrates	+	-	+	-	+	-	+	-	+	-
3. Protein & A.A	+	-	+	-	+	-	+	-	+	-
4. Steroids	-	-	-	-	-	-	-	-	-	-
5. Glycosides	+	+	-	-	+	+	-	-	+	+
6. Saponin	+	+	+	+	+	+	+	+	+	+
7. Flavanoids	+	-	+	-	+	+	+	-	+	+
8. Tannins	+	+	-	-	+	+	+	+	+	+
9. Triterpenoids	+	+	-	-	+	+	+	+	+	+
10. Fixed oils	-	-	-	-	-	-	-	-	-	-

+ = present, - = absent, M₁ = Crude Methanol Extract, M₂ = Methanol extract treated with lead acetate

Menispermaceae), and *Enicostema hyssopifolium* (Willd) (Stem-Gentianaceae), collected from Bapalal Botanical Vaidya Research Center Surat (Gujarat).

Extraction

Coarsely powdered air-dried material 4 g was placed in a glass stoppered conical flask and macerated with 100 ml of analytical grade solvents (methanol, and acetone) shaking frequently, and then allowing it to stand for 18 hours. Filter it rapidly through Whatman No. 1 filter paper, taking care not to lose any solvent. Transfer 100 ml filtrate to flat-bottom dish and evaporate solvent on a water bath. Dry at 50 °C for 6 hours, cool in a desiccator for 30 minutes and weigh without delay, calculate the content of extractable matter in mg/g of air-dried material (WHO, 2002).

Treatment

Lead acetate is known for precipitation of carbohydrates, protein and pigments from liquid extract and using in many isolation procedures (Neelakantam et

al., 1935; Hassid, 1938; George & Crocker, 1932). With aiming to remove such compounds from curd extracts (10 ml) of acetone and methanol solvent were treated with 200µl of 50 % lead acetate (Hi-media, Mumbai), mix it properly and centrifuge at 10000 rpm (REMI, India). Carefully remove the supernatant in watch glass, evaporate at room temperature, dry at 50°C for 6 hours, cool in a desiccator for 30 minutes.

Phytochemical screening

All four types of extracts; crude methanol extract (M₁), crude acetone extract (A₁), treated methanol extract (M₂), treated acetone extract (A₂) of all five plants were subjected to qualitative phytochemical screening for protein, carbohydrate, saponin, tannin, glycoside, alkaloids, flavanoids, terpenoids, steroids and fixed oil according to the method of Harbone (Harbone, 1984).

Procuring of bacterial strain

Pure cultures of test organisms (eight no.) were collected from the Microbiology Department of Sardar

Table 2: Phytochemical screening of acetone extract

Phyto constituents	<i>A.scholaris</i>		<i>A.aspera</i>		<i>M. oleifera</i>		<i>T.cordifolia</i>		<i>E. hyssofolium</i>	
	A ₁	A ₂	A ₁	A ₂	A ₁	A ₂	A ₁	A ₂	A ₁	A ₂
1. Alkaloids	-	-	+	+	+	+	-	-	-	-
2. Carbohydrates	+	-	+	-	+	-	+	-	+	-
3. Protein & A.A	+	-	+	-	+	-	+	-	+	-
4. Steroids	-	-	-	-	-	-	-	-	-	-
5. Glycosides	+	+	+	+	+	+	+	+	+	+
6. Saponin	+	+	+	+	+	+	+	+	+	+
7. Flavanoids	+	-	+	-	+	+	+	-	+	+
8. Tannins	+	+	-	-	+	+	-	-	-	-
9. Triterpenoids	+	+	+	+	+	+	+	+	+	+
10.Fixed oils	-	-	-	-	-	-	-	-	-	-

+ = Present, - = absent , A1 = Crude Acetone Extract, A2 = Acetone extract treated with lead acetate

Table 3: Antimicrobial activity of methanol extract

Plants	Inhibition zone (mm)								
		BS	EA	KP	ML	PA	SA	ST	SPA
<i>Alstonia scholaris</i>	M ₁	-	-	2	6	2	-	-	-
	M ₂	-	-	4	10	5	-	-	-
<i>Achyranthus aspera</i>	M ₁	-	-	-	2	-	4	2	-
	M ₂	-	-	-	5	-	8	6	-
<i>M. oleifera</i>	M ₁	-	4	-	2	4	2	2	2
	M ₂	-	8	-	5	9	7	6	6
<i>T. cordifolia</i>	M ₁	-	2	-	-	-	-	-	-
	M ₂	-	7	-	-	-	-	-	-
<i>E. hyssofolium</i>	M ₁	-	-	-	-	-	-	2	-
	M ₂	-	-	-	-	-	-	6	-

- = inhibition was not observed, M1 = Crude Methanol Extract, M2 = Methanol extract treated with lead acetate

Table 4: Antimicrobial activity of acetone extract

Plants	Inhibition zone (mm)								
		BS	EA	KP	ML	PA	SA	ST	SPA
<i>A. scholaris</i>	A ₁	6	20	-	10	-	4	-	-
	A ₂	8	22	-	14	-	7	-	-
<i>A. aspera</i>	A ₁	4	4	-	2	-	3	-	-
	A ₂	7	8	-	4	-	4	-	-
<i>M. oleifera</i>	A ₁	7	8	-	10	6	10	6	2
	A ₂	9	12	-	13	9	13	10	5
<i>T. cordifolia</i>	A ₁	2	5	-	-	-	5	-	8
	A ₂	6	9	-	-	-	10	-	12
<i>E. hyssofolium</i>	A ₁	4	6	-	-	-	7	-	-
	A ₂	8	9	-	-	-	10	-	-

- = inhibition was not observed, A1 = Crude Acetone Extract, A2 = Acetone extract treated with lead acetate

Patel University, V.V.Nagar, Anand - Gujarat. Among eight collected bacterial strain the Gram-positive bacteria were *Staphylococcus aureus* (ATCC9144) (SA), *Micrococcus luteus* (ATCC4698) (ML), *Klebsiella pneumoniae* (ATCC15380) (KP), *Bacillus subtilis* (ATCC 6051) (BS), and *Pseudomonas aeruginosa* (ATCC25668) (PA), *Enterobacter aerogens* (ATCC13048) (EA), *Salmonella typhi* (NCTC 8394) (ST) and *Salmonella paratyphi A* (SPA) were Gram-negative bacteria. Purity of culture

was maintained on Nutrient Agar by periodical transfers.

Agar diffusion assay

Antimicrobial screening was done using agar well diffusion methods (Perez, 1990). For this 25 ml of sterile Mueller –Hinton Agar No.2 (Hi-media), was poured in sterile autoclaved petri plates, before pouring 100µl of activated culture of bacteria was added, and then al-

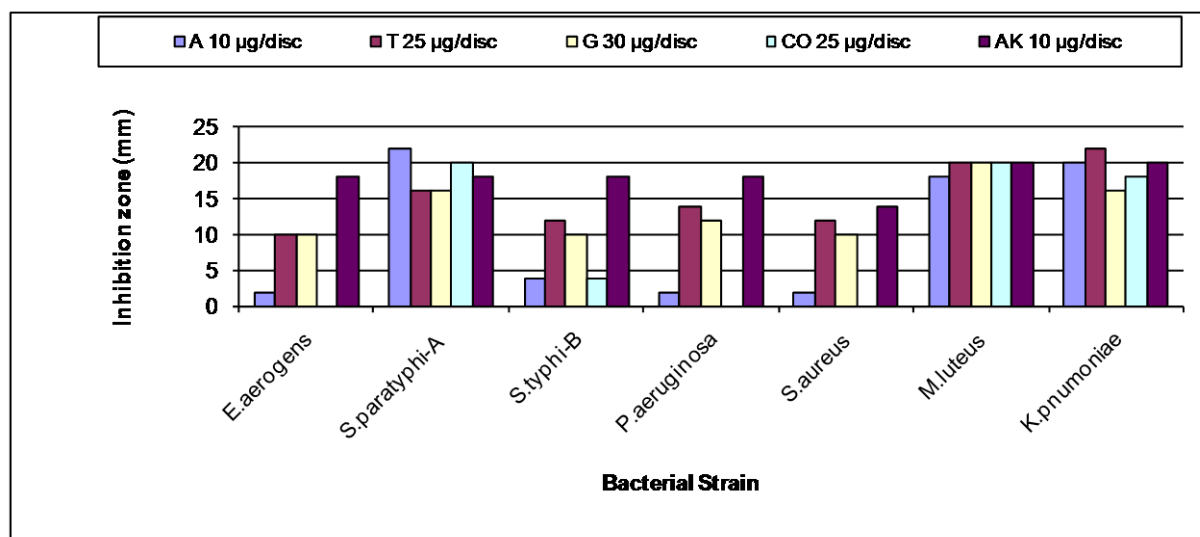


Figure 2: Antibiotic susceptibility of bacterial strain to antibiotics

A- Ampicillin, T- Tetracycline, G- Gentamicin, Co- Co-Trimoxazole, AK- Amikacin

lowed to solidify completely. The wells were prepared with the help of sterile 10 mm diameter cork-borer. Then 100 µl of plant extract from stock (25mg dry powder extract/ml) solution were poured into the wells. The plates were sealed with plasticine and transferred to the refrigerator to diffuse out for 30 mins. The plates were then incubated in the incubator at 37 °C for 24 hrs. Triplicate plates were prepared for each treatment and the average zone of inhibition excluding well, were recorded, 9.0% DMSO was used as negative control. Inoculum turbidity was maintained constant throughout the experiment to 0.8 OD at 660 nm. Level of turbidity is equivalent to approximately 1×10^8 CFU/ml.

Antibiotic susceptibility of selected bacterial strains

Susceptibility of selected bacterial strains were done against the standard antibiotics (Ampicillin(10µg), Tetracycline(25µg), Gentamicin(30µg), Co-Trimoxazole(25µg), Amikacin(10µg)) by redeemed paper disc (Hi-media). Twenty ml of sterilized nutrient agar seeded with activated bacterial culture was poured in petri dish, allowed to solidify and paper disc containing antibiotic was placed gently on surface by pointed forceps. Seal the plates with plasticine and incubate in the incubators at 37 °C for 48 hrs.

RESULTS

Extractive value

The results of extractive value in acetone and methanol are shown in graph 1. Extractive value in methanol as extractive solvent was found higher compared to acetone. The maximum yield among five plants of acetone extract was found in *E. hyssofolia* (7.11 %), same plant has highest extractive value (26.5 %) methanol used as extractive solvent.

Phytochemical screening

Results of phytochemical screening for methanol extract (M_1 & M_2) and acetone extract of all five plants are shown in Table 1 & Table 2 respectively. Alkaloids were present in M_1 extract of *E.hyssofolia*, *A.scholaris*, *T.cordifolia*, *M.oleifera*, *A.aspera* (Table 1), while absent in A_1 extracts of *E.hyssofolia*, *A.scholaris*, *T.cordifolia* (Table 2). Primary metabolites like carbohydrate and protein were detected in both M_1 and A_1 extract of all selected plants, while absent in M_2 and A_2 extract. Steroids were absent in both crude extract (M_1 and A_1) of *E.hyssofolia*, *A.scholaris*, *T.cordifolia*, *M.oleifera*, *A.aspera* (Table 1). M_1 extract of *A.scholaris*, *M.oleifera* and *E.hyssofolia* were showed positive result for glycoside (Table 1), while glycoside was present in A_1 extract of all plants (Table 2). Both M_1 and A_1 extract of all five plants showed presence of flavanoids (Table 1 & 2). But M_2 and A_2 extracts of *M.oleifera* and *E.hyssopifolium* were only showing presence of flavanoids (Table 1 & 2). Saponin was detected in M_1 , M_2 , A_1 , A_2 extracts of all plants (Table 1 & 2). Terpenoids was absent in M_1 extract of *A.aspera* (Table 1), while positive for the A_1 extract (Table 2). M_1 , A_1 and M_2 , A_2 extract of *E.hyssofolia*, *A.scholaris*, *T.cordifolia*, *M.oleifera* were positive for the presence of terpenoids (Table 1 & 2). Crude extract (A_1) of *A.scholaris* and *M.oleifera* were only positive for the tannin (Table 2). Fixed oil was not detected in any types of extracts in all selected plants.

Antimicrobial activity

Results of comparative antimicrobial activity of methanol and acetone extracts of *E.hyssofolia*, *A.scholaris*, *T.cordifolia*, *M.oleifera*, *A.aspera* are recorded in Table 3 and Table 4 respectively. M_1 and M_2 extracts of all five plants were completely inactive against BS (Table 3). M_1 , M_2 extracts of *M.oleifera* was found most active

against EA, ML, PA, SA, ST and SPA bacterial strains (Table 3). Methanol extract (M₂) of *A.scholaris* showed 10mm of zone of inhibition against ML, while inhibition zone of acetone extract (A₂) of same plant for same bacterial was 14 mm. Maximum zone of inhibition (22mm) in acetone extract was also observed in *A.scholaris* against *Enterobacter aerogens*. *Klebsiella pneumoniae* was completely resistant for acetone extracts (A₁, A₂) of *E.hyssofolia*, *A.scholaris*, *T.cordifolia*, *M. oleifera*, *A.aspera* (Table 4). *Bacillus subtilis*, *Enterobacter aerogens*, and *Staphylococcus aureus* were found sensitive to A₁ and A₂ extracts of all five plant (Table 4). Moderate kind of inhibition by acetone extract (A₁ & A₂) was observed in ML and SPA, and least antibacterial activity was observed for PA and ST, against A₁ and A₂ extract (Table 4). One distinct observation noticed in present study was that, crude extract of all five plants in methanol or acetone showed higher antibacterial activity after treatment with lead acetate at same concentration. Growth of all selected bacteria was not inhibited by 9.0 % DMSO.

Antibiotic susceptibility of bacterial strains

Results of antibiotic susceptibility of the selected bacterial strains are shown in (Graph.2). ML was more susceptible to ampicillin, tetracycline, gentamicin, cotrimoxazole and amikacin. EA, PA, and SA were completely resistant to co-trimoxazole. STB had shown negligible inhibition (4mm) against co-trimoxazole. Highest susceptibility (22mm) was observed by tetracycline against *Klebsiella pneumoniae*. Ampicillin at 10µg/disc was showed negligible activity against *Enterobacter aerogens* (2mm), *Salmonella paratyphi A* (4mm), *Pseudomonas aeruginosa* (2mm), and *Staphylococcus aureus* (2mm).

DISCUSSION

The yield of extractive value for *E.hyssofolia*, *A.scholaris*, *T.cordifolia*, *M.oleifera*, *A.aspera* were higher in methanol as compared to acetone as extractive solvent. Polarity of methanol and high solubility of plant secondary metabolite in methanol could be probable reason for high extractive value. In present study cold maceration was used to extract secondary metabolite is also reason for low yield of extracts, as maceration (Ibrahim *et al.*, 1997) and cold extraction (Okeke *et al.*, 2001) generally been reported to give lower yield of plant extracts compared to hot and soxhlet extraction in solvent having low boiling point (< 65 °C).

Phytochemical profile for alkaloids, saponin, glycoside, flavanoids, tannins, and terpenoids same in methanol and acetone extraction with respective plants. Phytochemical compounds present in extract were responsible for antimicrobial activity. Several author reported these secondary metabolite as antimicrobial metabolite like tannin (Scalbert, 1991), glycosides (Aboaba & Efuwape, 2001), saponins (Hostettman & Nakanishi, 1979), terpenoids and flavanoids (Leven *et al.*, 1979),

and alkaloids (Damintoti *et al.*, 2005). Crude methanol extract (M₁) of *E.hyssofolia*, *A.scholaris*, *T.cordifolia*, *M.oleifera*, *A.aspera* was totally inactive for BS, while crude acetone (A₁) extract of same plants showed significant antibacterial activity (Table 3) against BS it is probably as to active component was extracted in much higher concentration in acetone compare to methanol. Solubility of compound such active compound is also depend on polarity of extraction solvent (Eloff, 1998; Cowan, 1999). Active extract M₁, M₂, A₁, A₂ of all five plants showed broad sensitivity against gram-positive bacteria (except KP) than the gram-negative bacteria. Resistance of gram-negative bacteria for these extract could be because of the phospholipid membrane in addition to the inner peptidoglycan layer, which makes the cell more impermeable for exogenous molecules (Nikaido & Vaara, 1985).

In present investigation methanolic extract of *A.aspera* was inactive against BS, EA, KP, PA, and SPA, while acetone extract of same plant were active against BS and KP, these findings corroborate with findings of Jigna *et al.*, (2005). Acetone extract of *A.scholaris* was showed good antimicrobial activity against BS, EA, ML and SA, while same strains were resistant for the methanolic extract. Results indicate active components in *A.scholaris* were more soluble in relatively non-polar solvent, similar findings were also reported by Khan *et al.*, (2003). Methanolic extract of *A.scholaris* had shown antimicrobial activity against KP, ML and PA, while same KP and PA were resistant for acetone extract of same plant. Phytochemical result indicate plant contain variety of constitute groups; each group has different mode of action and bioactivity (Goyal & Varshney, 1995). Comparatively *M.oleifera* was most active plant among all five plants, these activity is attributing to the presence of saponin, tannin alkaloids and phenols (Clark, 1981). But acetone extracts of *M.oleifera* was active only against ST and STA, same kind of results was also observed by Doughari *et al.*, (2006). Both bacterial strains are causative agent for typhoid fever, and recent year there has been a rapid rise in multidrug resistance by ST all over the world (Chin *et al.*, 2002; Benoit *et al.*, 2003). Present findings for A₂ extract of *M.oleifera* suggest A₂ can be explore further to develop antityphoidal agent. Methanolic extract of *E.hyssofolium* was active against only ST, while acetone extract of same plant was inhibited BS, EA and SA. Flavanoids were reported as antimicrobial agent in *E.hyssofolium* (Ghosal & Jaiswal, 1980). In present study also both extract were found positive for flavanoids. In *T.cordifolia* also acetone extract was found more active compared to methanol extract. Singh *et al.*, (2003) reported presence of terpenoids and glycoside in *T.cordifolia*, and these components have antibacterial activity (Rege *et al.*, 1989).

Methanol and acetone extracts after treatment with lead acetate solution (M₂, A₂) were more active than crude extract. Phytochemical screening revealed that,

carbohydrate and protein were absent in M₂ & A₂, while these metabolite present at higher concentration in crude extract. This metabolite may be interfered in diffusion of active component, and reason for moderate antibacterial activity. Moreover the differences of antimicrobial activity of extracts were difficult to speculate; however, many antibacterial agents may exhibit their action through inhibition of nucleic acid, protein and membrane phospholipids biosynthesis (Franklin *et al.*, 1987).

Antibacterial alternative for selected bacterial strains were always in focus because of its infectious nature and bacteria has ability to develop the genetic ability to transmit and acquire resistance to drugs, which are utilized as therapeutic agents (Cohen, 1992). Antibiotic susceptibility test depict, antibiotics had medium range of inhibition for selected bacterial strain, while selected plant extract had shown noticeable antibacterial activity. Further purification and isolation of active antibacterial compound will increase its efficiency. Several studies indicate the usefulness of plant for control of resistant strain of bacteria like SA (Anesini & Perez, 1993) and PA (Martinez *et al.*, 1996 & 1994). Among selected plants acetone extract of *A.scholaris* and *M. oleifera* can be explored as new source of antibiotics, for EA, SA and ML.

CONCLUSION

In over all study acetone extract gave relatively wide spectrum of activity (37.5% – 87.5%) against the test bacterial strains compared to methanol extract (12.5 % - 75 %). The relatively wider spectrum of activity of the acetone extracts over the methanol extract is difficult to explain since all the extracts contained same types of phytochemicals. Perhaps, the active principles were more soluble in acetone than the methanol solvents. Among five plants, *M. oleifera* and *A.scholaris* can be further investigated to developed new weapon to combat infectious diseases. Further phytochemical groups wise screening and isolation of active compound from *M. oleifera* will be the focus of future work.

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