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RESEARCH ARTICLE

A STUDY OF ANTIBACTERIAL POTENTIALITY OF SOME PLANTS EXTRACTS AGAINST MULTI-DRUG RESISTANT HUMAN PATHOGENS

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ABSTRACT

The present study has been designed to determine the role of leaf and seed ethanol and aqueous extracts of Tribulus terrestris, Convolvus arvensis, Malva parviflora, Melilotus indicus, Rumex chalepensis and Anchusa arvensis for potential antibacterial activity, if any, against two Gram-positive include Staphylococcus aureus NCTC7428 Bacillus subtilis MTCC 441 and four Gram-negative include Pseudomonas aeruginosa MTCC 2453, Escherichia coli MTCC 739, Enterobacter aerogenes ATCC 13048 and Klebsiella pneumoniae MGH 78578 pathogenic multi-drug resistant bacteria. The MIC values of both aqueous as well as alcoholic leaf and seed extracts of the plants have been determined for each microorganism and compared with standard antibiotics of β-lactams, flouroquinolones, tetracyclines and aminoglycosides.

KEY WORDS: Plant extracts, ATCC, MTCC, NCTC, Zone of inhibition, MIC

INTRODUCTION:

In recent years, further concerns about the possible spread of bacterial resistance in bacteria isolated from food and the environment has been proposed (Palaniappan and Holley *et al.*, 2010).[1] Antibiotics are used widely in animal products during the past centuries. Excessive and uncontrolled use of antibiotics as routine supplements could lead to an increase in the number of antibiotic-resistant bacteria (Huyghebaert *et al.*, 2010).[2] Although awareness of antibacterial resistance in humans with the use of antibiotics in animal feed many have suggested that ultimately limits their use in European.

So today these ideas are being used by growing much attention worldwide to evaluate natural alternatives to antibiotics (Regulation 1831/2003/EC).[3] Medicinal plants and other herbaceous plants over the years have been used in the treatment of several human and animal diseases. Today many as supplements in animal popularize herbal extracts have found it often to antimicrobial growth enhancers in animal feed due to the residual effects that leave for restricted use. These cases as instances of anti-bacterial, anti-oxidant, anti-cancer, anti-fungal, relaxing, pesticides and insecticides, as well as growth enhancers are introduced (Tipu *et al.*, 2006).[4] Recently, the effects of pathogenic microorganisms and resistance against antibiotics that have acquired the properties of the extracts and compounds of biological species are the center of attention. Antimicrobial of herbal compounds are one of the most valuable resources in medicine. As a results the spread of infectious diseases, to identify more of these extracts and compounds useful in the treatment of patients. Antimicrobial compounds in plants are numerous therapeutic capabilities (Kokoska *et al.*, 2002).[5] Since the very wide range of infectious diseases and antibiotic resistance due to the limitations caused by consumption, it is necessary to find new antibiotics (Hammerum and Heuer *et al.*, 2009).[6] The utilize of plant extracts and phytochemicals, with known antibacterial characteristic, may be of immense significance in therapeutic treatments.

In the many studies have been conducted in different countries to substantiate such efficiency (Almagboul *et al*., 1985; Sousa *et al*., 1991; Kubo *et al*., 1993; Shapoval *et al*., 1994; Artizzu *et al*., 1995; Izzo *et al*., 1995; Shanab *et al*., 2004; Nair *et al*., 2005; Ngemenya *et al*, 2006; Abeysinghe *et al.*, 2010; Gull *et al*., 2012; Moghadam *et al*., 2012; Rakholiya and Chanda *et al.*, 2012).[7-19]

This study has been designed to determination potential of antibacterial activity leaf and seed ethanol and

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aqueous extracts of six plants (*Tribulus terrestris, Convolvus arvensis, Malva parviflora, Melilotus indicus, Rumex chalepensis and Anchusa arvensis*) against two Gram-positive (*Staphylococcus aureus* NCTC7428, *Bacillus subtilis* MTCC 441) and four Gram-negative (*Pseudomonas aeruginosa* MTCC 2453, *Escherichia coli* MTCC 739, *Enterobacter aerogenes* ATCC 13048 and *Klebsiella pneumoniae* MGH 78578) pathogenic multi-drug resistant bacteria.

MATERIALS AND METHODS:

Plant materials:

The plant material used in this study consisted leafs of *Tribulus terrestris, Convolvulus arvensis, Malva parviflora, Melilotus indicus, Rumex chalepensis and Anchusa arvensis*, collected from area Jammu-Kashmir. Taxonomic determination of the plant was confirmed by the Department of Biotechnology, Mehsana Urban Institute of Science, Mehsana. The seeds of all plants were purchased from a seed company, Ahmedabad. List of used in this study summarized in Table 1.

Preparation of Ethanolic Extract:

The leaf and seed separately were powdered and their dissolved in 200 ml ethanol 85% using a shaker water bath for 24 h at room temperature. After filtration with Whatman No. 1 filter paper, a rotary evaporator at 40°C for 40 min to remove solvent from the extract concentrated the resulting solution. The semisolid extract produced was kept in a freezer at -80°C overnight and then subjected to freeze dried for 24 h, at -70°C in 200 ml vacuum. For further use, the extract was stored in airtight container at 4°C in refrigerator.

Preparation of Aqueous Extract:

20 gram of leafs and seeds of *S.arvensis* was finely ground utilizing a homogenizer and was extracted with distilled water at room temperature for 24 hours. This mixture was then filtered using Whatman No.1 filter paper to remove debris and a volatile extract was then evaporated at 40ºC using a rotary evaporator. For further use, the extract was stored in airtight container at 4°C in refrigerator.

Microorganisms:

The two Gram-positive include *Staphylococcus aureus* NCTC7428, *Bacillus subtilis* MTCC 441 and four Gram-negative include *Pseudomonas aeruginosa* MTCC 2453, *Escherichia coli* MTCC 739, *Enterobacter aerogenes* ATCC 13048 and *Klebsiella pneumoniae* MGH 78578 were obtained from the Department of Biotechnology, Mehsana Urban Institute of Science, Mehsana. Susceptibility of six reference bacterial strains to antibiotics in nutrient agar summarized in Table 2. The microorganisms were inoculated on to nutrient agar slants at 37°C and maintained at -80°C.

ANTIMICROBIAL ACTIVITY:

All the dried extracts were exposed to UV rays (200-400 nm) for 24 h and checked frequently for sterility by streaking on nutrient agar plates (Chessbrough *et al*., 2000).[20] Antimicrobial activity was based on the disc diffusion method (Thitilertdecha *et al*., 2008) using a cell suspension of microorganisms.[21] The concentration of the cell suspension was equilibrated to a 0.5 McFarland standard and 50 µl of each microorganism's suspension was spread on a Mueller-Hinton agar plate. In addition, 50 µl of diluted leaf and seed extract was pipetted onto sterile paper discs (6 mm in diameter), which were allowed to dry in an open sterile Petri dish in a biological laminar flow bench. Discs were placed on the surface of inoculated plates and incubated at 37°C for 24 h. Diameters (mm) of the zones of bacterial inhibition minus the discs diameter were recorded (Ilçim *et al*., 1998).[22] The surfaces of the media were inoculated with bacteria from a broth culture. High potency bio-discs (Himedia) were placed on the agar. After 18 h of incubation at a 37°C, the plates were examined and the diameters of the inhibition zones were measured to the nearest millimeter.

DETERMINATION OF MINIMUM INHIBITORY CONCENTRATION (MIC):

The MIC is defined as the lowest concentration at which the microorganism does not demonstrate visible growth. The minimal inhibitory concentration (MIC) values were also studied for the microorganisms, which were determined as sensitive to the extracts in the disk diffusion assay. The inoculum (100 µl), initially adjusted to the density cited above, was spread onto 20 ml Mueller–Hinton agar supplemented with the seed at concentrations ranging from 2–10 µl/ml in Petri dishes, with each one of its equivalent in 10% dimethylsulfoxide (DMSO). These serially diluted cultures were then incubated at 37±1°C for 24 h. The MIC is defined as the lowest concentration at which the microorganism does not demonstrate visible growth. As control, 10% dimethylsulfoxide was used (Khadidja *et al*., 2010).[23]

RESULT:

In this study, Antibiogram of some usual antibiotics against test microorganism - Antibiogram of the Gram negative and Gram positive bacteria revealed that all the

bacterial strains were resistant to some greatly utilized broad-spectrum antibiotics. Nevertheless, all the bacteria were sensitive to the new generation antibiotics except *B.subtilis* because due to complex growth requirements, definitive NCCLS (1993) cut off values for antibiotics susceptibility and resistance has not been established (Table 2).[24] Result showed that, all the extracts of plants (ethanolic leafs extract, aqueous leafs extract, ethanolic seeds extract, aqueous seeds extract) recorded different degrees of antibacterial activity against multi-drug resistant bacteria as evidenced by the zone of inhibition (Table 3-6). *Tribulus terrestris* L. in both ethanolic and aqueous leaf extracts, affected on all the bacteria. Both ethanolic and aqueous of seeds extract not affected on all bacteria. In both seed extraction (ethanolic and aqueous) not showed effect on all bacteria. Maximum inhibition among ethanolic and aqueous leafs extract was related on *Malva parviflora* L. that it have inhibition zone 30, 23 mm on *P.aeruginosa* respectively. Seed extracts (ethanolic and aqueous) than leaf extracts have lower effects on all bacteria. The result showed that ethanolic leaf extract compared with aqueous leaf extracts having greater antibacterial activity. Bhattacharjee *et al*., (2006) also reported that methanol extracts of the leaves and seeds of the *A.mexicana* showed greater antibacterial activity than the corresponding water extracts.[25]

Minimum Inhibitory Concentration (MIC) of the six different plants extracts varied against different test pathogens. Some plants extract did not show any activity. The MIC of the plant extract required for the test pathogens in presented in Fig 1-4. Lowest MIC of ethanolic and aqueous leaf extracts related to *K.pneumoniae* (*Anchusa arvensis* L. 116.4 mg/ml), *E.aerogenes* (*Rumex chalepensis* L. 214.1mg/ml) respectively. Highest MIC of ethanolic and aqueous leaf extracts related to *K.pneumoniae* (*Tribulus terrestris* L. 498.4 mg/ml), *P.aeruginosa* (*Melilotus indicus* L. 497.4 mg/ml) respectively. Lowest MIC of ethanolic and aqueous seed extracts related to *E.aerogenes* (*Tribulus terrestris* L. 209.2 mg/ml), *S.aureus* (*Rumex chalepensis* L. 274.9 mg/ml) respectively. Highest MIC of ethanolic and aqueous seed extracts related to *K.pneumoniae* (*Tribulus terrestris* L. 498.4 mg/ml), *P.aeruginosa* (*Melilotus indicus* L.497.4mg/ml) respectively.

Table 1: List of the plants studied

Studied plants	Family	Floristic unit
Anchusa arvensis L.	Boraginaceae	Leaf, seed
Convolvulus arvensis L.	Convolvulace	Leaf, seed
Tribulus terrestris L.	Zygophyllaceae	Leaf, seed
Rumex chalepensis L.	Polygonaceae	Leaf, seed
Malva parviflora L.	Malvaceae	Leaf, seed
Melilotus indicus L.	Fabaceae	Leaf, seed

Table 2: Susceptibility of six reference bacterial strains to antibiotics in nutrient agar Diameter of the inhibitory zones (mm)

Antibiotics (µg/ml)	*K.pneumoniae*	*B.subtilis*	*E.coli*	*S.aureus*	*P.aeruginosa*	*E.aerogenes*
Ampicillin (20)	00	28	00	00	19	00
Amikacin (20)	12	13	11	19	24	10
Cotrimoxazole (20)	00	28	12	16	00	00
Ciprofloxacin (10)	25	20	00	00	06	00
Cloxacillin (25)	00	00	00	00	00	00
Cefadroxil (20)	00	00	00	00	00	00
Cefuroxime (20)	13	00	00	00	00	11
Doxycycline (20)	11	12	11	10	23	06
Erythromycin (10)	25	26	00	00	00	15
Gentamycin (10)	00	14	15	00	21	00
Kanamycin (20)	00	26	12	00	17	00
Nalidixic acid (20)	12	00	00	18	00	00
Norfloxacin (10)	12	00	11	07	16	14
Penicillin-G (10)	10	00	00	00	00	00
Sparfloxacin (10)	16	14	00	00	22	13
Tobramycin (10)	14	28	14	15	18	10
Tetracyclin (25)	20	27	20	12	00	00

Table 3: Antibacterial activity of the ethanolic leaves extracts of plants against multi-drug resistant bacteria

Diameter of the inhibitory zones (mm)						
Plants	*K.pneumoniae*	*B.subtilis*	*E.coli*	*S.aureus*	*P.aeruginosa*	*E.aerogenes*
Anchusa arvensis L.	09	04	09	11	16	00
Convolvulus arvensis L.	02	08	08	00	24	07
Tribulus terrestris L.	09	05	10	18	20	06
Rumex chalepensis L.	00	02	12	16	18	11
Malva parviflora L.	08	19	00	14	30	06
Melilotus indicus L.	06	00	10	08	00	13
Negative control (DMSO, 5 µl)	00	00	00	00	00	00

Table 4: Antibacterial activity of the aqueous leaves extracts of plants against multi-drug resistant bacteria

Diameter of the inhibitory zones (mm)						
Plants	*K.pneumoniae*	*B.subtilis*	*E.coli*	*S.aureus*	*P.aeruginosa*	*E.aerogenes*
Anchusa arvensis L.	06	02	08	08	19	00
Convolvulus arvensis L.	00	04	03	00	11	00
Tribulus terrestris L.	06	01	07	13	16	02
Rumex chalepensis L.	00	01	09	11	10	01
Malva parviflora L.	02	14	00	09	23	00
Melilotus indicus L.	04	00	03	06	00	11
Negative control (DMSO, 5 µl)	00	00	00	00	00	00

Table 5: Antibacterial activity of the ethanolic seed extracts of plants against multi-drug resistant bacteria Diameter of the inhibitory zones (mm)

Plants	*K.pneumoniae*	*B.subtilis*	*E.coli*	*S.aureus*	*P.aeruginosa*	*E.aerogenes*
Anchusa arvensis L.	00	04	11	00	02	00
Convolvulus arvensis L.	00	07	02	02	03	00
Tribulus terrestris L.	00	05	10	09	06	06
Rumex chalepensis L.	00	02	14	11	10	00
Malva parviflora L.	01	11	00	06	18	01
Melilotus indicus L.	00	00	11	00	00	00
Negative control (DMSO, 5 µl)	00	00	00	00	00	00

Table 6: Antibacterial activity of the aqueous seed extracts of plants against multi-drug resistant bacteria

Diameter of the inhibitory zones (mm)						
Plants	*K.pneumoniae*	*B.subtilis*	*E.coli*	*S.aureus*	*P.aeruginosa*	*E.aerogenes*
Anchusa arvensis L.	00	00	03	00	00	00
Convolvulus arvensis L.	00	01	01	02	06	00
Tribulus terrestris L.	00	00	01	06	03	00
Rumex chalepensis L.	00	01	00	04	02	00
Malva parviflora L.	02	00	00	01	16	03
Melilotus indicus L.	04	01	03	06	04	01
Negative control (DMSO, 5 µl)	00	00	00	00	00	00

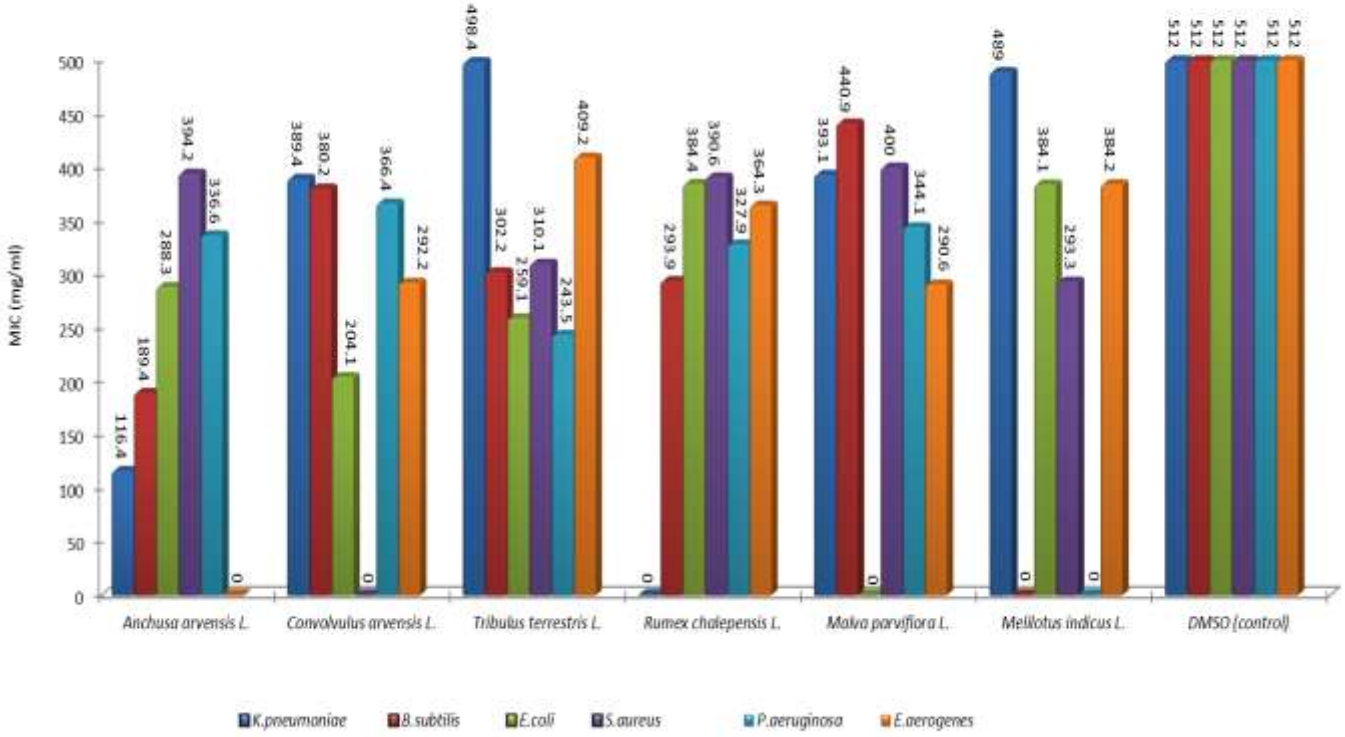

Figure 1: MIC values of ethanolic leaves extract

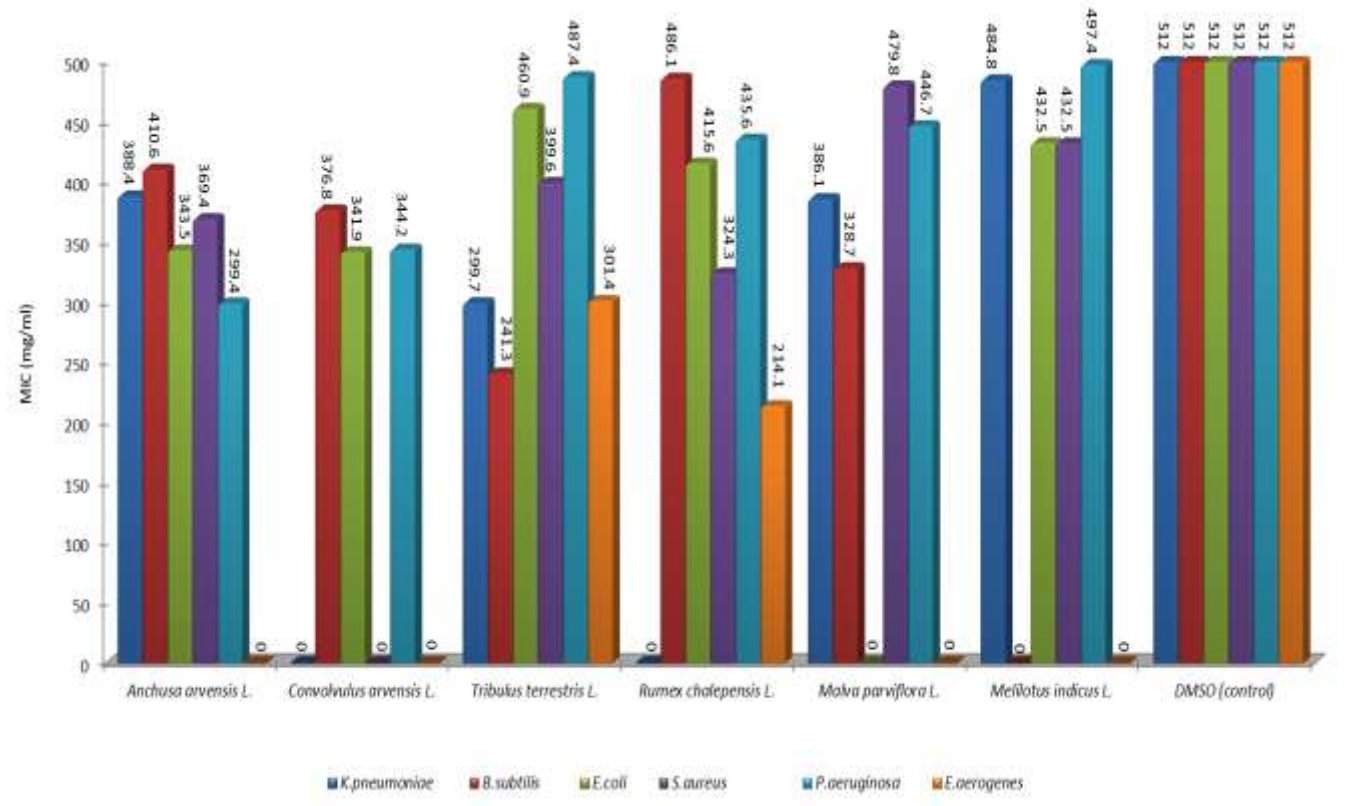

Figure 2: MIC values of aqueous leaves extract

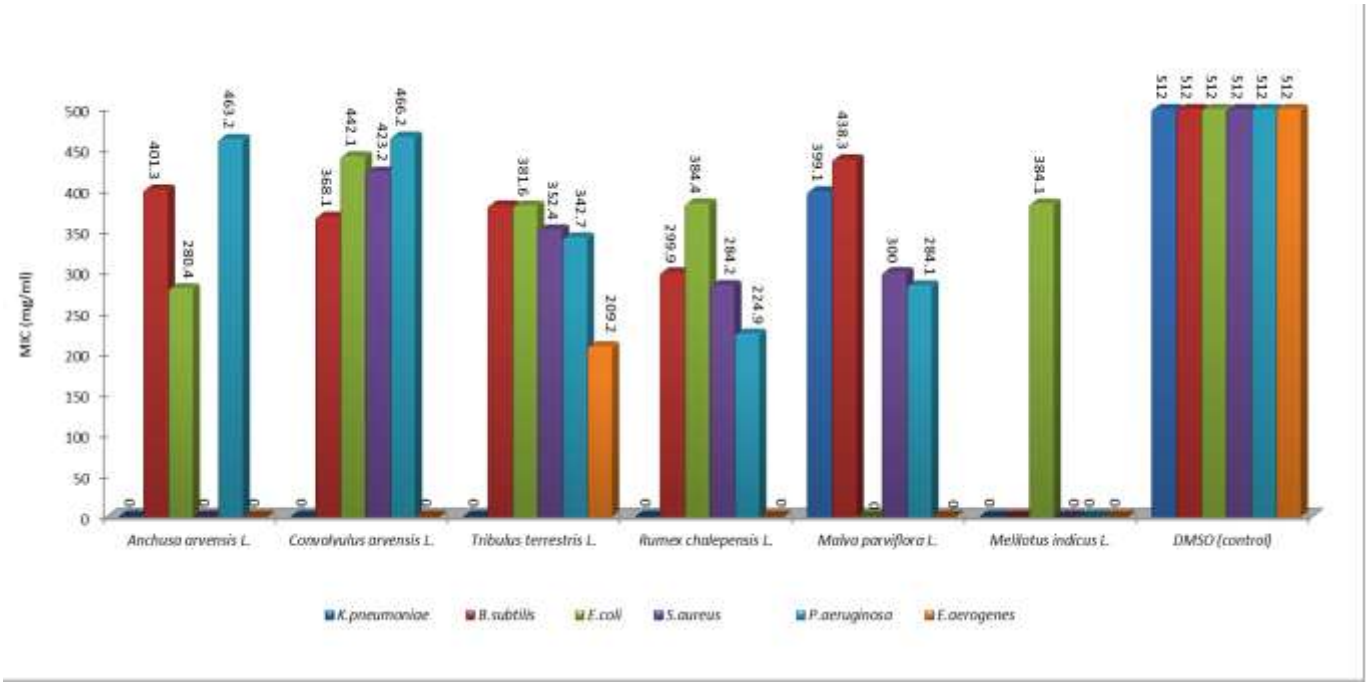

Figure 3: MIC values of ethanolic seed extracts

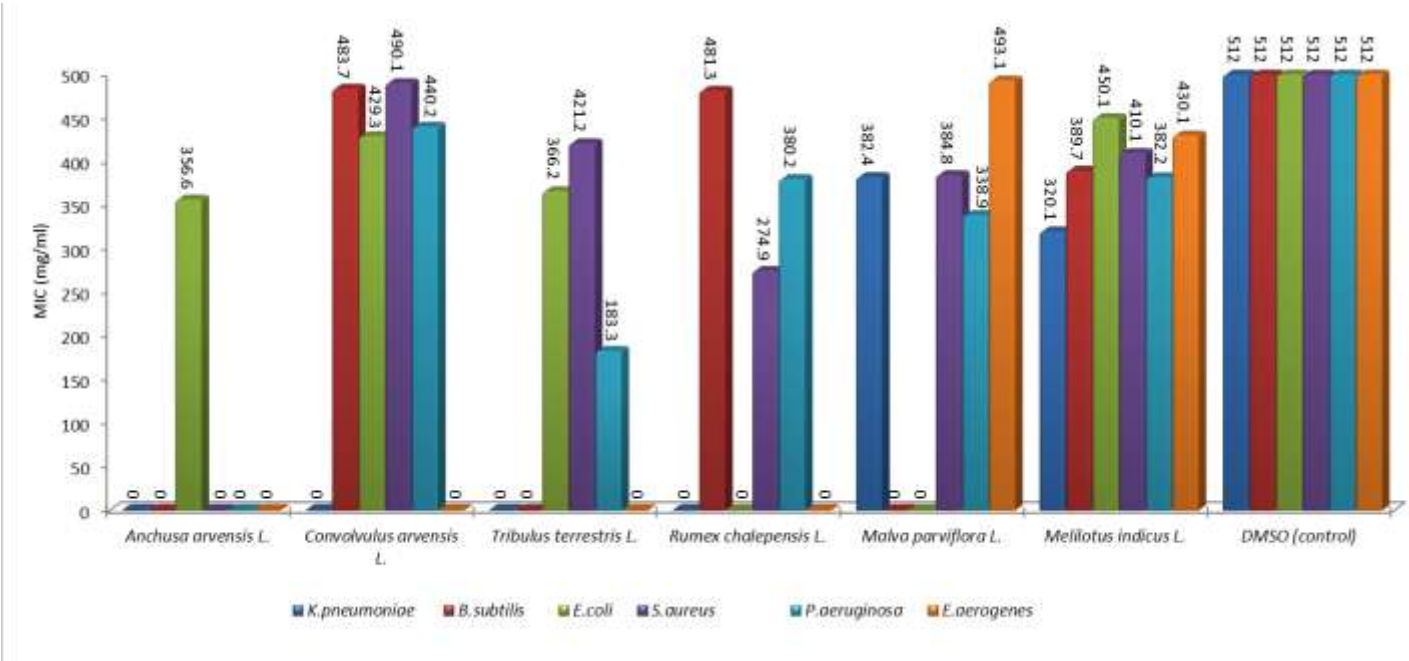

Figure 4: MIC values of aqueous seed extracts

DISCUSSION:

Use of plants as a source of medicine has been inherited and is a significant component of the health care system. Nearly 20% of the plants found in the world have been submitted to biological tests or Pharmacological (Suffredini *et al*., 2004).[26] Plants are important source of potentially functional structures for the evolution of new chemotherapeutic agents. The first step towards this aim is the *in- vitro* antibacterial activity test (Tona *et al*., 1998).[27] in this study, the ethanol extracts of the leafs and seeds of the all plants in this study showed greater antibacterial activity than the corresponding aqueous extracts. Bhattacharjee *et al*., (2006) reported that methanol extracts of the leaves and seeds of the *A.mexicana* showed immense antibacterial activity than the corresponding water extracts. Therefore, This confirms our finding in this study. These observations may be related to the nature of biologically active constituents whose activity can be increased in the presence of ethanol an also the stronger extraction capacity of ethanol could have produced more importance number of active constituents responsible for antibacterial activity (Bhattacharjee *et al*., 2006).[25] The

some ethanolic and aqueous extracts of the plants used in this study showed greater antibacterial activity and the diameter of zone of inhibition. MIC value of ethanolic leaf extract of *Malva parviflora* L. showed 440.9 mg/ml for *B.subtilis*, MIC value of aqueous leaf extract of *Melilotus indicus* L. showed 497.4 mg/ml for *P.aeruginosa*, MIC value of ethanolic seed extract of *Convolvulus arvensis* L. showed 466.4 mg/ml for *P.aeruginosa* and MIC value of aqueous seed extract of *Convolvulus arvensis* L. showed 490.1 mg/ml for *S.aureus.* Fig. 1-4.

The authors suggested that all plant used in this study, could be used to discover bioactive natural products that will lead to the development of new pharmaceutical entity such as screening of various natural organic compounds and identification of active agents must be reasonable as a productive approach in the search of new herbal drugs. In addition, leaf extracts were more impressive. However, *in-vivo* study on this medicinal plant is essential to determine toxicity of the active constituents, their side effects, serum-attainable levels, pharmacokinetic properties and diffusion in different body sites. The antimicrobial activities can be improved if the active components are purified and adequate dosage determined for proper administration. This may go a long way in preventing the administration of unsuitable concentrations, a common practice between many traditional medical practitioners. We also suggested that some of the plants in this study, which possesses strong antibacterial activity, in the treatment of diseases caused by the microorganisms tested.

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