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M.A.M. MONDOL

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M.A.M. MONDOL

School of Science and Technology, Bangladesh Open University, Board Bazar, Gazipur-1705, Bangladesh.

Corresponding author & address: M.A. Mojid Mondol, E-mail: drmojidmondol@gmail.com
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ABSTRACT

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Antimicrobial resistance (AR) is recognized as one of the greatest threats to public health and in global concern. Consequently, the increased morbidity and mortality, which are associated with multidrug resistant bacteria, urgently require the development of novel and more efficient drugs. The *in vitro* antibacterial activity of methanol and ethyl acetate extracts obtained from 10 medicinal plants (*Abroma augusta, Desmodium motorium, Blumea balsamifera, Eclipta prostrate, Plumbago indica, Leonurus sibiricus, Cassia alata, Plumbago capensis, Costus speciosus* and *Aerva sanguinolenta*) and 13 standard antibiotics (cefradine, amoxycilline, doxycycline, cefixime, erythromycin, ceftriaxone, ciprofloxacin, nitrofurantoin, clindamycin, imipenem, cloxacillin, azithromycin and vancomycin) was assessed against three Gram-negative multidrug resistant clinical isolates (*Pseudomonas aeruginosa, Escherichia coli* and *Klebsiella* sp.). The zone of inhibition as determined by disk diffusion assay method varied with the plant extract, the solvent used for extraction, and the organism tested. Methanol extracts obtained from medicinal plants, *Leonurus sibiricus* and *Costus speciosus*, were found to be more potent being capable of exerting significant inhibitory activities against the multidrug resistant tested pathogens. These two extracts may be used as source for the development of new, natural antimicrobial drugs. Other plant extracts showed either less activity or now activity at all. Almost all the standard antibiotics except imipenem used in the concentration failed to suppress the growth of the multidrug resistant tested pathogens.

Key words: *medicinal plants, multidrug resistant, pathogens, antibiotics, activity*

INTRODUCTION

Approximately, 500,000 species of both identified and unidentified plants have been estimated on Earth. Among them, only 1-10% is being used as foods by animals and humans (Borris 1996; Cowan 1999). Plants are the key source for alternative medicines and pesticides for fighting against diseases since ancient times. The self-medication with plant substances is common due to easy availability. The use of plant-derived natural products in medical treatments is attracting more attention due to its potential efficacy and no side effects (Cowan 1999).

After discovery of first antibiotic, penicillin, by Alexander Fleming from a fungus, *Penicillium notatum* in 1940s was once called a "miracle drug". Nowadays, drug resistant pathogens are widespread especially in developing countries like Bangladesh due to overuse and misuse in human and animal, self-medication and often given without proper justification. More than 70% clinically isolates methicillin (oxacillin)-resistant *S. aureus* (MRSA) strains are resistant to almost all commonly used antibiotics causing infections in hospital and community with higher morbidity and mortality than methicillin (oxacillin)-sensitive *S. aureus* (MSSA) (Moellering 2012; Kluytmans and Diederen, 2008; Rehm 2008). An outbreak of vancomycin resistant enterococcal strains (VRE) with a 73% mortality rate has highlighted the seriousness of the situation (Edmond *et al.* 1995). Infections caused by the drug-resistant pathogens increase cost of treatment; prolong sufferings and greater risk of morbidity and mortality. Due to rise of antimicrobial resistance, millions of people die every year worldwide (WHO 2014).

In ancient era, plants were the only available source of medication to treat human illness. WHO estimates that around 80% of the world's population relied on medicinal plants as their primary healthcare source (Farnsworth *et al.* 1985). The dependence on remedies derived from medicinal plants is particularly important in developing countries where modern medicine is often absent or simply too expensive. It has been shown that, there is a correlation between the ethnomedical usage of medicinal plants and modern medicines discovered from those plants (Fabricant and Farnsworth, 2001). To establish the correlation through an experiment, 88 single chemical entities isolated from 72 medicinal plants have been introduced into modern therapy, many of which have the same or a similar therapeutic purpose as their original ethnomedical use. It is estimated that 60% of anti-tumour and anti-infective drugs already on the market or under clinical trial are of natural origin (Shu 1998).

Bangladesh is a tropical country. More than 4 thousand different plant species have been found in Bangladesh. Among them 550 plants have medicinal properties (Green 2015). Traditional healers in Bangladesh have been using these medicinal plants for thousands of years to treat numerous infection diseases such as dysentery, skin infection, diarrhea, pain, heart disease, cough, indigestion, weakness, etc. Bangladeshi medicinal plants remain totally unexplored for the discovery/development of new drugs.

Due to favorable and poor hygienic conditions, microbial diseases are commonly found especially in rural people of Bangladesh. Irrational use of antibiotics, multidrug resistant human pathogens are also widespread in Bangladesh. Nowadays, infectious diseases are not cured by the common antibiotics as earlier. So, the poor people who suffer from drug resistant infection diseases and do not have ability to buy expensive antibiotics go to the traditional medicinal plant healers to treat their infectious diseases. Preliminary screening of extracts obtained from Bangladesh medicinal plants used by the traditional healers indicates the huge potential for the

development of new/novel lead antibiotics against multidrug resistant human pathogens (Chowdhury 2013). The components of medicinal plants are already compatible with human body; so there is a high chance of new drugs obtained from medicinal plants to be less toxic to the human body compare to other sources.

This research was an attempt to estimate the antibacterial activity of methanol and ethyl acetate extracts of ten medicinal plants against antibiotic-resistant clinical Gram-negative bacterial isolates with a view to find out potential sources of new drug development.

MATERIALS AND METHODS

General experimental procedures

Ethyl acetate (Scharlau, Spain), methanol ((Scharlau, Spain), *n*-hexane (Daejung Chemicals and Metals Company Ltd, Korea), peptone (Qualikans, India), yeast (Qualikans, India), agar (Merck, Germany), H_2SO_4 (Merck, Germany), $BaCl_2.H_2O$ (Merck, Germany), sterile filter paper disk (BioMaxima S.A., Poland), filter paper (Whatman Int. Ltd, Maid Stone, England), heavy duty blender (Havells, India) and 13 standard antibiotics (HiMedia, India) were bought from local suppliers. Sterilization, aseptic works and solvent evaporation were done using vertical autoclave machine (Model: LVA-202, Labocon, UK), horizontal laminar airflow cabinet (Model: LLFH-204, Labocon, UK) and rotary evaporator (Model: HS-2005S-N, Hahnshin S&T Co., Ltd, Korea). All used solvents were either analytical grade or distilled prior to use.

Medicinal plants

50 fresh medicinal plants were collected through an expedition from Mirzapur under Tangali, Chattogram and Rajshahi districts. These plants (about 1 kg each) were collected in plastic bags. The plant parts (leaf and stem) were washed with running tap water, cut into small species and dried under shade for 2 weeks in the biological science lab, BOU. After drying, the plant materials were grinded into fine powdered form by using a blender, kept in plastic bags and subjected later to extraction. 10 out of 50 medicinal plants were subjected to activity screening in this research (Table 1) against multidrug resistant pathogenic bacteria.

Extraction of medicinal plants

The powdered form of each medicinal plant (100 g) was taken in 2 L conical flasks and 1 L ethyl acetate solvent was added in it and was left for overnight. Then the plant material was removed by filtration using Whatman filter paper and the filtrate was concentrated to dryness using rotary evaporator at reduced temperature (40°C). After drying, each plant material was extracted again with methanol, filtered and concentrated to dryness as ethyl acetate extract. Both ethyl acetate and methanol extracts were partitioned between methanol and *n*-hexane; the *n*-hexane phase mainly contains fats was discarded and methanol phase was concentrated to dryness using rotary evaporator at reduced temperature (40°C). These methanol extracts were subjected to activity screening against drug resistant clinical isolates.

Table 1. List of medicinal plants used for activity screening against drug resistant pathogens

SL	Vernacular Name	Scientific Name	Family
1	Ulotkombol	*Abroma augusta*	Malvaceae
2	Turichondal	*Desmodium motorium*	Papilionaceae
3	Lackdan	*Blumea balsamifera*	Compositae
4	Vootraj	*Eclipta Prostrata*	Asteraceae
5	Roktochita	*Plumbago Indica*	Plumbaginaceae
6	Roktodron/Hemanggi	*Leonurus sibiricus*	Lamiaceae
7	Dudmordon	*Cassia alata*	Caesalpiniaceae
8	Nil-chita	*Plumbago capensis*	Plumbaginaceae
9	Kau	*Costus speciosus*	Costaceae
10	Nuriya	*Aerva sanguinolenta*	Amaranthaceae

Collection of test pathogens

Three multidrug-resistant test pathogens (namely *Pseudomonas aeruginosa*, *Escherichia coli* and *Klebsiella* sp.) were collected from the microbiology laboratory, Rajshahi Medical College, Rajshahi, Bangladesh. These test pathogens were isolated from the clinical samples of the patients.

Preparation of the suspension (inoculums) of the test pathogens

To prepare the suspension of the test pathogens, first the test pathogens were streaked on the sterile nutrient agar medium (5 g peptone, 3 g yeast extract, 15 g agar and 5 g NaCl and distilled water 1L) from stock culture and then incubated at 30°C for 24 hours. Then 50 ml nutrient broth medium (5 g peptone, 3 g yeast extract, and 5 g NaCl and distilled water 1L) was prepared, sterilized and inoculated from 24-hour test pathogen cultures and then incubated at 30°C for 24 hours. This culture suspension was used for activity screening of medicinal plant extracts. All the microbial culture works were done under aseptic condition.

Antibacterial activity assay

Antibacterial activity of the solvent extracts was determined by agar disk diffusion assay method (NCCLS 1993). At first, the suspension of the 24-hour test pathogen culture was diluted with sterilized distilled water in such a way that its turbidity matched with the turbidity of the 0.5 McFarland reagent [A 0.5 McFarland standard was prepared by mixing 0.05 ml of 1.175% barium chloride dihydrate ($BaCl_2.2H_2O$), with 9.95 ml of 1% sulfuric acid (H_2SO_4)]. 0.5 McFarland reagent represents 1.5×10^8 (generally, range is 1.0×10^8 to 2.0×10^8) bacteria/ml.

To prepare the activity assay plate, nutrient agar medium was prepared (see above) and sterilized at 121°C for 20 min by autoclave machine. The medium was poured on the sterilized Petri dish (90 mm) and left to solidification in laminar airflow cabinet. Inoculum containing of each bacterial culture to be tested was spread on nutrient agar plates with a sterile swab moistened with the bacterial suspension. Each medicinal plant extract was dissolved and diluted with appropriate solvent combination. From the diluted each test extract, specific amount of sample was taken out by the micropipette so that it contained 1 mg extract and impregnated in the sterile microbial susceptibility testing paper disk (6 mm). After drying, all the disks containing test samples were transferred about 2 cm apart by sterile forceps on the surface of the previously test pathogen spread agar plate. These plates were kept in deep fridge overnight for diffusing extracts in the surrounding media. Then all the test plates were incubated at 30°C for 24 hours. After incubation, zone of growth inhibition for each extract was measured in mm. 13 different standard antibiotic disks and one sterile microbial susceptibility testing paper disk (6 mm) were used as positive (standard) and negative controls in this experiment, respectively.

RESULTS AND DISCUSSION

Table 2. Amount of extracts obtained from tested medicinal plants

SL	Vernacular name	Methanol extract (ME)	Ethyl acetate extract (EAE)
1	Ulotkombol	710 mg	780 mg
2	Turichondal	760 mg	240 mg
3	Lackdan	2.56 g	2.15 g
4	Vootraj	1.46 g	2.40 g
5	Roktochita	730 mg	350 mg
6	Roktodron/Hemanggi	3.71 g	3.16 g
7	Dudmordon	730 mg	420 mg
8	Nil-chita	1.52 g	360 mg
9	Kau	1.44 g	520 mg
10	Nuriya	1.44 g	-

Table 3. Activity screening samples and zone of inhibition against tested pathogens

SL	Vernacular name	Amount/disk	Zone of inhibition (in mm)		
			P. aeruginosa	***E. coli***	***Klebsiella* sp.**
1	Ulotkombol	1 mg (ME)	-	-	-
		1 mg (EAE)	16	-	-
2	Turichondal	1 mg (ME)	-		-
		1 mg (EAE)	-	-	-
3	Lackdan	1 mg (ME)	20	18	-
		1 mg (EAE)	-	10	-
4	Vootraj	1 mg (ME)	-	-	-
		1 mg (EAE)	16	16	-
5	Roktochita	1 mg (ME)	-	-	-
		1 mg (EAE)	-	-	-
6	Roktodron/Hemanggi	1 mg (ME)	32	30	14
		1 mg (EAE)	-	-	-
7	Dudmordon	1 mg (ME)	-	-	-
		1 mg (EAE)	-	-	-
8	Nil-chita	1 mg (ME)	-	20	-
		1 mg (EAE)	14	18	-
9	Kau	1 mg (ME)	44	40	-
		1 mg (EAE)	-	-	-
10	Nuriya	1 mg (ME)	-	-	-

Standard antibiotics					
11	Cefradine	25 µg	-	-	-
12	Amoxycilline	30 µg	-	-	-
13	Doxycycline	30 µg	-	-	8
14	Cefixime	5 µg	-	-	-
15	Erythromycin	15 µg	-	-	-
16	Ceftriaxone	30 µg	-	-	-
17	Ciprofloxacin	5 µg	-	-	-
18	Nitrofurantoin	300 µg	-	-	8
19	Clindamycin	2 µg	-	-	-
20	Imipenem	10 µg	20	17	
21	Cloxacillin	5 µg	-	-	-
22	Azithromycin	15µg	-	-	-
23	Vancomycin	30 µg	-	-	-
24	Negative control	Sterile disc	-	-	-

″-″ Not active in tested concentration

The antibacterial activity of the extracts (Table 2) was quantitatively assessed by the presence or absence of inhibition zone and by measuring the diameter of the inhibition zone around the discs. The results of antimicrobial activity of the plant extracts are presented in Table 3.

Antibacterial activity of ten plants belonging to nine botanical families was evaluated *in vitro* against three drug-resistant clinical isolates, *P. aeruginosa* (known to cause pneumonia, septic shock, urinary tract infection, gastrointestinal infection, skin and soft tissue infections), *E. coli* (cause gastroenteritis, urinary tract infections, neonatal meningitis, hemorrhagic colitis and Crohn's disease. Common signs and symptoms include severe abdominal cramps, diarrhea, hemorrhagic colitis, vomiting, and sometimes fever) and *Klebsiella* spp. (cause urinary tract infections, pneumonia, septicemias, and soft tissue infections).

19 extracts (10 methanol and 9 EtOAc) obtained from ten medicinal plants, screened for potential antibacterial activity against multi-drug resistant (MDR) bacteria, the most potent extract was methanol obtained from hemanggi. It exhibited the promising broad spectrum antibacterial properties (14-32 mm zone of inhibition) against all the tested MDR bacteria in the concentration used (Table 3). The most remarkable activity was shown by the methanol extract obtained from Kau against *P. aeruginosa* and *E. coli* with zone of inhibition 44 mm and 40 mm (Fig. 1), respectively. Besides these, the extracts obtained Lackdan (methanol), Vootraj (EtOAc), Nil-chita (EtOAc) indicted pronounced antibacterial activity against *P. aeruginosa* and *E. coli* with a range of zone of inhibition 14-20 mm. Unfortunately, extracts obtained turichondal, roktochita, dudmordon and nuriya failed to suppress the growth of any tested pathogens (Table 3). The negative control did not exhibit inhibition against the tested pathogen.

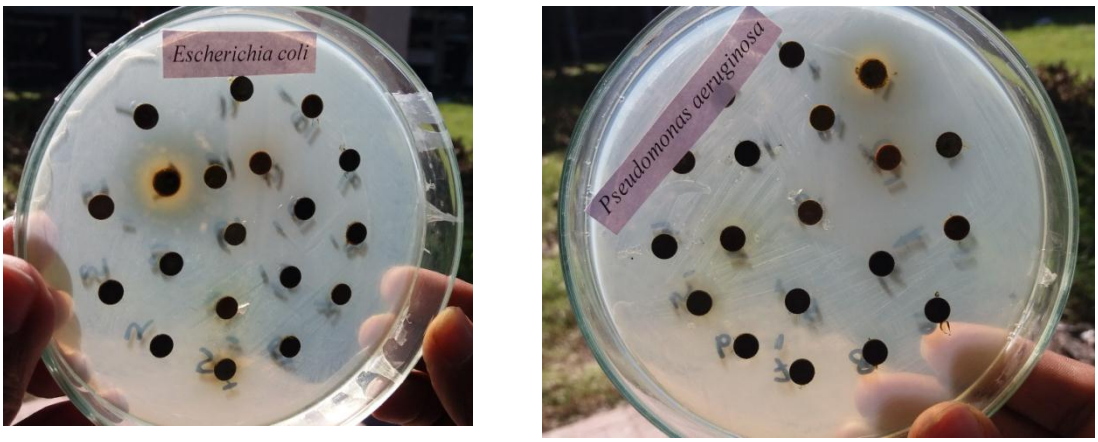

Fig. 1. Activity test plates against *P. aeruginosa* and *E. coli*

P. aeruginosa and *E. coli* were sensitive to imipenem whereas *Klebsiella* sp. was sensitive to doxycycline and nitrofurantoin. *P. aeruginosa*, *E. coli* and *Klebsiella* sp. isolates were surprisingly found resistant to cefradine, amoxycilline, cefixime, erythromycin, ceftriaxone, ciprofloxacin, clindamycin, cloxacillin, azithromycin and vancomycin. *P. aeruginosa* and *E. coli* were exhibited almost similar sensitivity towards all the tested samples whereas the most resistant species was *Klebsiella* sp. (Table 3).

Plant extracts have been studied against bacteria for years in the last three decades but little information is available about the activity of plants against drug-resistant hospital isolates. The result of this investigation has clearly indicated that antibiotic resistance does not interfere with the antimicrobial action of plant extracts and these extracts might have different modes of action on test organisms.

With the increase in resistance of microorganisms to the currently used antibiotics and the high cost of production of synthetic compounds, pharmaceutical companies are now looking for alternatives. Medicinal plants could be those alternatives because most of them are safe with little side effects, cost less, and affect a wide range of antibiotic resistant microorganisms (Nimri *et al.* 1999; Karaman and Kocabas, 2001).

Four ethanolic extracts (*Zanthoxylum armatum*, *Adiantum capillus-venaris*, *Artemisia absinthium*, and *Martynia annua*) inhibited (IC_{50}: 256 µg/ml) the growth of multidrug resistant strains (*E. faecium*, *S. aureus*, *K. pneumoniae*, *A. baumanii*, *P. aeruginosa*, and *Enterobacter* species) (Khan *et al.* 2018). Water extracts of 26 medicinal plant parts of Bangladesh were tested against four multidrug resistant clinical isolates (*Klebsiella* spp., *Pseudomonas* spp., *Proteus* spp. and *E. coli*). Of them, eight crude extracts obtained from *Allamanda cathartica* (leaf), *Allium sativum* (bulb), *Citrus limon* (fruit), *Tamarindus indica* (fruit), *Prunus domestica* (fruit), *Averrhoa carambola* (fruit), *Piper betle* (leaf) and *Terminalia arjuna* (leaf) exhibited potential antibacterial activity against tested pathogens (Chowdhury *et al.* 2013). The aqueous extract of *Lannea fruticosa* showed potent activity against both *P. aeruginosa* and *P. mirabilis* which was 20 mm and 19.5 mm of inhibition zone, respectively. The MIC values of aqueous extracts of *Lannea fruticosa* against *P. aeruginosa* and *P. mirabilis* was at 1.953 mg/ml and the highest MBC value was recorded at 15.86 mg/ml in the ethanol-aqueous extract of *Malva parviflora* against *P. aeruginosa* (Kidane 2019).

During the current study, almost all the bacterial isolates were resistant to the tested antibiotics. This phenomenon may be due to genetic changes since antimicrobial resistance occurs naturally over time (Oko 2016). However, the irrational usages (such as misuse, abuse, overuse etc) of antibiotics is also playing role in accelerating antimicrobial resistance process. A recent finding which strongly supports the idea is that 80%-90% of antibiotic prescriptions were found to be written by general practitioners, of that 30% are considered to be completely unnecessary (USCDC 2016). Also, inappropriate use of antibiotics, such as taking them for viral infections like flu, or for mild infections that may clear-up without treatment is known to fuel resistance. A recent study in England, reported that one in three (34%) of the samples analyzed were found to be resistant to antibiotic trimethoprim which was once the first choice treatment for urinary tract infections (ESPAUR 2017).

Antimicrobial assays on plant extracts are valuable in screening and detecting the presence of antimicrobial activities. From the above study, it can be concluded that the medicinal plants, hemanggi and kau, have great potential as antimicrobial agents against MDR clinical isolates. The biologically active components in the tested plants are not known and needs further analysis. The active plant extracts could also be considered for use as disinfectants or antiseptics. However, antimicrobial drug resistant pathogens are wide spread. Ensuring the rational use of existing antibiotics and development of new and more potent antibiotics may solve the antibiotic resistance problem and save the millions of live.

CONCLUSION

The 21st century witnesses major global health care problem which threaten the entire human life, the appearance and prevalence of multi-drug resistant pathogens. We should understand that the battle against these microorganisms is never ending, but we can beat them by changing our strategy and returning back to nature, using active ingredients from plants that survived against microbes since millions of years. The methanol extracts obtained from the medicinal plants, hemanggi and kau, exhibited promising antibacterial activities against the multidrug resistant bacteria. This result supports the use of these plants as a source for the development of new antimicrobial drugs against multidrug resistant pathogenic bacteria.

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CONFLICT OF INTEREST

There is no conflict of interest to declare.

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