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Antibacterial activity of 4 medicinal plants against 8 clinically isolated multidrug resistant bacteria

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KEYWORDS

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A B S T R A C T

To investigate the antibacterial activity, using hot extraction procedures with some solvents, ethanol, methanol and water to validate medicinal uses of aloe vera, *neem*, oregano, rosemary controlling infections; and to qualitatively estimate phytochemical constituents of leaf-extracts of the plant.The antibacterial activity of leaf-extracts was evaluated by the agar-well diffusion method against 8clinically isolate from both Gram-positive and -negative multidrug resistant (MDR) pathogenic bacteria *in vitro*. The presence of reducing sugar, saponins all the leaf extracts was established. Pathogenic bacteria used were, *Escherichia coli*3, *Klebsiellapneumonia*2, *Staphylococcus aureus* (*S. aureus*) 3, along with standard bacterial strains. These MDR bacteria had been recorded to have significant inhibitions by leaf extracts, obtained by hot extraction procedures with two solvents. Imipenem 10µg/discwas the positive/reference control and the diluting solvent, 10% dimethyl sulphoxide was the negative control. Leaf-extracts ethanoland methanolhad shown significant antibacterial activity against all bacteria. methanol extract of oregano and aloe vera indicated glycoside and tannins as major active compounds against methicillin-resistant *Staphylococcus aureus*this leaf-extract could be used in treating infectious diseases, caused by the range of tested bacteria, as complementary and alternate medicine.

Introduction

Antibiotics have saved the lives of Millions of people and have contributed to the major gains in life expectancy over the last century. However, the clinical efficacy of many existing antibiotics is being threatened by the emergency of Multi-drug resistant MDR pathogens.The World Health Organization estimated that about an 80% population of developing countries relies on traditional medicines, mostly plant drugs, for their primary health care needs (WHO 2008, and Loeraa JA, *et al*., 2007). Particularly in rural India, uses of raw plant products as well as some concoction of plant products in Ayurvedic medicines are sought after to a great proportion, because of cheap

availability, and in urban areas too those are increasingly popular for cultural nuances that exist(De Silva T, *et al*., 2007). Further, a large number of phyto-drugs are popular and are preferred to over synthetic ones—*a priory*, for healthier or rather harmless effects (Sindhia VR, *et al*.,2010); almost all the viral infections are always addressed with plant products, as it is known. In ethno-botanical literature of India, several hundreds of plants are known to have the potential to treat many diseases (Kirtikar KR, *et al*., 1935).

Infections with both Gram-positive (GP) and Gram-negative (GN) bacteria have clinically become intractable, slowly, due to the emergence of multidrug resistant (MDR) strains. Among GP pathogens, strains of *Staphylococcus aureus* (*S. aureus*), methicillin resistant *S. aureus* (MRSA) and vancomycin resistant *S. aureus* (VRSA), strains of *Enterococcus* sp. are noteworthy (Dubey D,*et al*., 2012). Moreover, GN bacteria, *Acinetobacter* sp., *Klebsiellapneumoniae* (*K. pneumoniae*), *Citrobacterfreundii* (*C. freundii*), *Escherichia coli* (*E. coli*) and *Pseudomonas aeruginosa* (*P. aeruginosa*) are commonly found as pathogens of urinary tract; while *E. coli*, *K. pneumoniae*, are pathogens of gastrointestinal tract. Presently, these pathogens are too MDR, recorded in several reports (Dubey D,*et al*., 2012).

Secondly, the resistance of pathogenic bacteria to antibiotics is of high clinical concern. Rather the concept of the control of drug resistance is a matter of clairvoyance for dovetailed antimicrobials today. A suitable epitome is the superbug, multidrug resistant (MDR) *S. aureus* in the human health domain worldwide, as its different strains or rather incarnations have generated β-lactamase activities in degrading all sorts of penicillin derived antibiotics, in addition to resistance to other groups/generations of antibiotics (Clarke CR. 2006). Multidrug resistance of *Staphylococcus, Pseudomonas, Escherichia* and a few more pathogenic bacteria to a wide range of antibiotics has been reported to have been due to non-prudent uses of same antibiotics against infections of food- and pet-animals worldwide(Middleton J,*et al*., 2005). (Maple PAC, *etal*., 1989), including man. MDR-MRSA strains carry resistance markers for other antibiotics and instances of resistance up to 23 antibiotics in some strains have been reported(Maple PAC, *etal*., 1989). The emergence of VRSA is of further concern. Today, the management of the consortium of MDR strains of both GN and GP pathogens has become increasingly difficult because of the β-lactamase production by *Staphylococcus, Bacillus, Pseudomonas, Proteus, Klebsiella, Neisseria, Salmonella, Haemophilus* and a few more pathogens (Rath S,*et al*., 2012), and pandrug resistance (PDR, resistance of bacteria to all antibiotics in present use) to different classes of antibiotics in GN ones (Falagas ME, *et al*., 1989). Meek appreciation of failures in the control of MDR strains would be inhuman, which generates the impetus on a systematic global search for new drugs from natural resources like plants, worldwide (Davidovich C, *et al*., 2008 andDubey D, *et al*., 2012); chemicals from plants could be chosen for the control in a future crusade against MDR pathogens. Moreover, accumulated ethnomedicinal reports of different countries lend themselves well to the basic information needed for further work on drug-targeting against MDR pathogens (Dubey D, *et al*., 2012).

The research was designed to study the antimicrobial potentiality of four medicinal plants viz.*aloe barbadensis* (aloe vera), *Azadirachta indica* (neem), *Origanum vulgare* (oregano) and*Rosmarinusofficinalis*

(rosemary) against a series of MDR bacteria of clinical relevance. Phytochemical screening was carried out of identify major biological active phytoconstituents.

Materials and Methods

Preparation of plant extract

The air-dried powdered leaf material (in 40 g lots) of *aloe barbadensis* (aloe vera), *Azadirachta indica* (neem), *Origanumv ulgare* (oregano) and *Rosmarinus officinalis* (rosemary) was extracted with 400 mL volumes of solvents, methanol, ethanol and distilled water. For extraction, in a soxhlet apparatus, a lot of 40 g of powder-mass was placed in the extractor and a volume of 400 mL of a solvent was used during 24 h of soxhletion, till colourless extracts precipitated in the extractor. After filtration, each extract was concentrated by the rotary evaporator. The resultant sticky-mass was dried in a desiccator; the solid mass was stored in a suitable volume of 10% dimethyl sulphoxide (DMSO) with a drop of Tween-80. The stock concentration of each extract was maintained at 30 mg/mL, for further use.

Qualitative test for phytochemicals

Phytochemical screening was carried out to assess the qualitative chemical composition of crude extracts using commonly employed precipitation and colouration procedure to identify the major natural chemical groups, as described earlier (Dubey D, *et al*., 2012). Reducing sugar, Alkaloids, Glycosides, Anthraquinone, Flavonoids, Tannins, Sterols, saponins, triterpenoids and phlobatanins were assessed.

Bacterial cultures and growth condition

In Kanchipuram district in and around hospitalized patients of wards and cabins of Hospital, 8 bacterial strains Three GP species, *S. aureus*, (2 isolates) MRSA, and GN bacteria, *E. coli* (3 isolates), *Klebsiella pneumonia* (2 isolates),were isolated. All these 8 strains were identified by standard biochemical tests and were maintained as axenic cultures in suitable media, as described previously (Dubey D, *et al*., 2012 and Rath S, *et al*., 2012). Different clinical samples were collected from patients of wards, cabins, intensive care unit, neonatal care unit in the hospital, and were used for the growth of bacteria in nutrient agar, MacConkey agar, blood agar, eosin methylene blue (EMB) agar. Microbial type culture collection (MTCC) strain of each bacterium was used as the reference control during identification (see Table 1).

Antibiotic sensitivity pattern

All bacterial strains were subjected to antibiotic sensitivity test by Kirby-Bauer's method, using a 6 mm thick Mueller-Hinton agar medium, as described previously (Sahu MC*et al*., 2012) and results were determined basing upon the standard guidelines (Clinical Laboratory Standard Institute Performance standard for antimicrobial susceptibility testing 2011). For the control, Imepenem 10 µg/disc, Vancomycin 30 µg/disc was used and it was sensitive to all test bacteria and its inhibition zone range was 18-19 mm.

Result and Discussion

Isolation and biochemical identification of bacteria

Specific colony morphology of each pathogen was noted, for which a corresponding MTCC strain was used, parallely (Table 1). For example, LF, flat dry pink, irregular colonies were of *E. coli*. After growth, a single colony was subjected

to Gram-staining and basing upon it, other biochemical tests were performed for identification (Table 2).

For example, *E. coli* was negative for oxidase, Voges-Proskauer, citrate and urease tests, while bile-esculin was not done; it was positive for catalase, indole, and methyl red, triple sugar iron and nitrate reduction tests. Similarly, the rest bacteria were typified. Three GPs, *S. aureus*(3 isolates), MRSA, and Five GNs, *E. coli*(3 isolates), *K. pneumonia* (2 isolates).

Phytochemical analyses

Ethanol, Methanol and water extracts were subjected to phytochemical analyses, to ascertain the presence of metabolites such as reducing sugar, Alkaloids, Glycosides, Anthraquinone, Flavonoids,Tannins, Sterols, saponins, triterpenoids and phlobatanins.(Kuklinski C *et al*., 2000).

It was recorded that antibiotics (µg/disc), amikacin 30 was resistant to two and sensitive to six bacteria; amoxyclav 30 was resistant to six and sensitive to two bacteria; cefixime was resistant to all the bacteria; Cefoparazone+Sulbactum was resistant to four and sensitive to four bacteria; cefotaxime was resistant to seven and sensitive to one bacteria; cefotriaxone was resistant to seven and sensitive to one bacteria; gentamicin 30 was resistant to four bacteria and sensitive to four bacteria; gatifloxacin 30 was resistant to three and sensitive to five bacteria; imipenem 10 was sensitive to all the bacteria; levofloxacin 5 was resistant to four bacteria and sensitive to four bacteria; Meropenemwas resistant to two and sensitive to sixbacreria; ofloxacin 5 was resistant to seven and sensitive to one strain;piperacillin/tazobactam 100/10 was resistant to three and sensitive to six bacterium.

Antibacterial activities

Antibacterial activity of water and solvent extracts was determined by agar well diffusion method anti-bacterial activity against cited GP bacteria and five GN bacteria. Imipenem 10µg/disc used as standard. The case of detailed information of antibacterial activities of extracts and inhibition zone sizes were recorded (Table 5). National Type Culture Collection bacterial strains (drug sensitive strains of *S. aureus*, *P. aeruginosa* and *E. coli*), with the highest sizes of zones of inhibition against the used bacteria, at around 100 mg/mL with the aqueous extract of the plant (Lohitha P, et al., 2011).

MRSA strains reported from Nepal were at 40.1% of the total bacterial isolates, and those strains were multiple resistant to trimethoprim/ sulfamethoxazole, cephalexin, amikacin, ciprofloxacin and norfloxacin, in addition to the usual penicillin derivatives, but all those were vancomycin sensitive (Tiwari HK, et al., 2009). But the most effective way to prevent clinical crisis due to MRSA has been with daptomycin, nowadays (Holloway K. 2000 and Sorlozano A,et al.,2009). In Brazil, about 40% to 60% nosocomial infections in urinary and respiratory tracts, boils and surgical wound infections were by MRSA alone, and the presence of *mec*A gene with those was proved, probably because of suchgreater infection prevalence (Perez LRR, et al., 2008). In a study from Malaysia, it was reported that among 287 pathogens, 52% were GNs with *Proteus* sp. 25%, *P. aeruginosa* 25%, *K. pneumoniae* 15%, *E. coli* 9%, and the rest 45% were GP bacteria with *S. aureus* 40%, Group B *Streptococci* 25% and *Enterococcus* sp. 9%; antibiograms indicated the susceptibility to imipenem and amikacin in GN and vancomycin in GP bacteria (Raja NS. 2007). Among

intracellular pathogens isolated, both *S. aureus* and *Staphylococcus epidermidis* were frequently present, the latter species being coagulase-negative *Staphylococcus*; and *S. aureus* strains were mostly MRSA. Indeed, *S. aureus* was not invasive intrinsically, but MRSA was reported as invasive through eye (Kato T, et al., 1998). Further, in a classical study from New York, it was reported that the colonization rate of MRSA was more in intravenous drug abusers (Berman DS, etal., 1987).

While analysing the infection dynamics of pathogens, it was obvious that antibiotic sensitive pathogens have a limited capacity of virulence as the employed antibiotic controls them. At several levels, the host defence system also helps the control of pathogens when the later are in a smattering number. Most often than not, an infection from a MDR bacterial strain leads to a disease, particularly when an emulating control-agent/antimicrobial is absent, *i.e.*, the employed antibiotic has been won over by it. Indeed, in the presence of a stress factor—an antibiotic, the bacterial cell undergoes intrinsic or acquired genetic changes via, conjugation/transformation, involving exchanges of resistance markers, exemplified with the *mar*-locus of *E. coli*(George AM, et al., 1983), if at least, the natural selection for the emergence of mutants is slow. Spontaneous mutation in bacteria occurs at the rate, 1 in 10^7 cells usually. Eventually, some drug-resistant mutant predominates with the replacement of all sensitive strains by the resistant strain, the later serving as if a doppelgänger. Since, the emergence of resistant mutants is a self-repetitive process in conditions ideal for pathogens, serial/continual resistant events to a gamut of diverse antibiotics land at the emergence of multidrug resistance in a bacterium, at least in an aged/immune-compromised body. Indeed, the horizontal transfer of genetic materials from one organism to another appears faster than mutational changes, a phenomenon popularly called as, evolution of quantum leaps, operates naturally (Groisman EA, 1996). It is because, genes for the drug-resistance mechanism are operative in antibiotic-producing cells, and those are transferred naturally to sensitive strains (Martinez JL, et al ., 2002), as an event of natural selection. Ultimately, antibiotic resistance remains as the clinical determinant of the pathogenesis. Slowly, the use of numbers of antibiotics for the control of infectious diseases in last decades have led to multiple resistances in one cell, the MDR strain of a species, paradigmatically with any of notorious pathogens. As conjectured from retrospective follow-ups, it is clear that older antibiotics slowly became obsolete, by the resistant mechanism. The clinical concern is that antibiotic resistance was reported in several pathogenic bacteria for which, particular antibiotics were never applied. Is this the mechanism of the transformation of a harmless commensal to a perilous MDR pathogen in the present antibiotic era? Not surprisingly, drug resistant bacteria gain the capability of surviving and multiplying under stress conditions. The biological rule, any limiting condition for the majority would be an excellent opportunity for the minority. When in presence of a drug *in vivo*, all the drug sensitive strains are eliminated and the resistant strain survives, multiplies, and predominates, culminating in a disease. Drug resistant strains and their control by newer antibiotics are leitmotivs in the odyssey of the emergence of MDR and PDR strains of umpteen pathogens in the last 4-5 decades and more. MDR-MRSA is the intractable, ghoulish example rising to a great notoriety of being marked as the superbug of health domain, worldwide (Dubey D, et al., 2013).

Table.1 Isolation and characterization of pathogenic clinical isolates with individual colony characteristics

Bacteria	Standard strain	Agar media	Colony morphology
E. coli	MTCC 443	Nutrient agar	Flat dry, irregular colonies
		MacConkey agar	LF, flat dry pink, irregular colonies
		EMB agar	Flat dry, irregular colonies, with metallic green colour
K. pneumoniae	MTCC 4031	MacConkey agar	LF, pink, mucoid colonies
S. aureus, MRSA	MTCC 7443	Blood agar	Medium to large, smooth, entire, slightly raised, creamy yellow, with green/β hemolytic colonies
		Nutrient agar	As in blood agar without hemolytic activity
MRSA: methicillin resistant *S. aureus*; LF: lactose fermenting colonies; EMB: eosin methylene blue agar.			

Table.2 Summary of results of biochemical tests of eight pathogenic bacteria

Bacterium	Catalase	Oxidase	Indole	MR	VP	Citrate	Urease	TSI	NT	BE
E. coli	+ve	-ve	+ve	+ve	-ve	-ve	-ve	A/G	+ve	nd
K. pneumoniae	+ve	-ve	-ve	-ve	+ve	+ve	+ve	A/GH_2S	+ve	nd
S. aureus, MRSA	+ve	+ve	nd	nd	nd	nd	+ve	nd	nd	nd
MR: methyl red; VP: Voges-Prausker; TSI: triple sugar iron; NT: nitrate reduction; BE: bile esculin; A/G: acid and gas production; A/GH2S: acid-gas and hydrogen sulfide production; nd: not done; +ve: positive; -ve: negative.										

Table.4 Antibiogram of clinically isolated 8 bacteria by the disc-diffusion method with antibiotics

Bacteria	Antibiotics														
	Ak	Ac	Cfx	CS	Ce	Cl	G	Gf	I	Le	MR	Of	Pt	Va	Lz
K. pneumonia-1	S	R	R	R	R	R	S	S	S	S	S	R	S	-	-
K. pneumonia-2	R	R	R	R	R	R	R	R	S	R	R	R	R	-	-
E. coli-1	S	R	R	S	R	R	R	S	S	R	S	R	S	-	-
E. coli-2	S	R	R	R	R	S	S	R	S	R	S	R	S	-	-
E. coli-3	R	R	R	R	R	R	R	R	S	R	S	R	R	-	-
S.aureus-1	S	S	R	S	R	R	S	S	S	S	S	S	S	S	S
S. aureus-2	S	S	R	S	S	R	S	S	S	S	S	R	S	S	S
Sa MRSA	S	R	R	S	R	R	R	S	S	S	R	R	R	S	S
Antibiotics (µg/disc): Ak: amikacin 30; Ac: amoxyclav 30; Cfx: cefixime; Cs: Cefoparazone+Sulbactum; Ce: cefotaxime; Cl: cefotriaxone; G: gentamicin 30; Gf: gatifloxacin 30; I: imipenem 10; Le: levofloxacin 5; Mr: Meropenem; Of: ofloxacin 5; Pt: piperacillin/ tazobactam 100/10; Va: vancomycin 30. Lz: linezolid For *S. aureus*, vancomycinand linezolid was used individually and lawns had no inhibition zone. R: resistance and S: sensitivity of a bacterium; -: antibiotic was not used. Data of the second repeated experiment are presented. All values are mean of duplicate readings.															

Table.5 Size of inhibition zones of leaf-extracts with different organic solvents of Plants against MDR bacteria (mm)

Plant	**Ex**	**Test Bacteria (Zone of inhibition in mm)**								
		***K. pneumonia*-1**	***K. pneumonia*-2**	***E. coli*-1**	***E. coli*-2**	***E. coli*-3**	***S.aureus*-1**	***S.aureus*-2**	**Sa MRSA**	**Imipenem 10µg/disc**
Ne										18
	E	16	14	16	10	16	14	13	13	
	M	16	15	15	16	14	14	14	14	
	W	14	14	16	15	14	16	15	15	
Og										18
	E	6	7	14	10	12	12	15	12	
	M	7	8	12	13	11	11	13	15	
	W	5	6	11	9	8	9	11	10	
Rm										18
	E	6	9	10	11	10	15	12	14	
	M	10	11	9	10	12	14	15	14	
	W	5	7	7	8	8	10	10	10	
Av										19
	E	8	8	6	-	5	12	16	14	
	M	14	12	7	-	7	15	-	21	
	W	7	7	5	-	6	11	14	12	

Ex: extract; Ne: neem; Og: Oregano; Rm: rosemary; Av:aloevera; E: ethanol; M: methanol; W: Watersizes of inhibition zones in extracts are given. -: absence of inhibition

In conclusion from the recorded data, it could be taken that neem; Oregano; rosemary; aloeveraleaf-extracts could be used in treating infectious diseases, caused by the range of tested bacteria, as complementary and alternate medicine, since crude phyto-extracts of the plant could not be breached by MDR pathogenic bacteria. Apothecary would benefit from these findings of the plant for drugs of finesse, *i.e.*, non-microbial antimicrobials in the crusade against MDR pathogens.

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