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Comparative evaluation of antimicrobial activity of newer herbal disinfectant formulations on hospital microbial flora

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***Abstract*---**Background: The world is now focusing on use of herbals because they are eco-friendly and less toxic on human health and environment Objectives:To evaluate antimicrobial activity of newer herbal disinfectant formulations on hospital microbial flora Methods: Herbal disinfectants were prepared with medicinal plant leaf extracts of Tridax procumbens, Withania somnifera, Tinospora cordifolia, Costus igneus and Nepata cataria. Hospital microbial flora was isolated by rubbing and rotating sterile swabs from targeted spots. Extract formulations were tested for the antimicrobial activity against five isolatedbacterial strains i.e., Escherichia coli, Pseudomonas aeruginosa, Enterobacter sp., Enterococcus faecalis and Staphylococcus aureus using Kirby-bauer disc diffusion method. Results: Extracts exhibited antimicrobial activity against all tested microorganisms. Among the five different herbal disinfectants, Tinospora cordifolia showed good antimicrobial activity against

Escherichia coli, Enterobacter and Enterococcus faecalis with 27mm, 20mm and 14mm of zone of inhibition respectively. Costus igneus showed high antimicrobial activity to Pseudomonas aeruginosa with 22mm of zone of inhibition. The herbal disinfectant formulations in the present study were reported effective results against hospital microbial flora which cause nosocomial infections Conclusions: Herbal disinfectants Tinospora cordifolia, Costus igneus, Withania somnifera exhibits antimicrobial activities against MDR bacterial pathogens isolated from hospital wards.

***Keywords*---**antimicrobial activity, plant extracts, disinfectants, microorganisms, hospital wards.

Introduction

According to a review of the literature, there are only a few well-organized chemical entities that are efficient in managing and controlling the normal growth of microorganisms on non-living items. Such a small number of chemical agents will potentially reduce microbial load significantly [1].Alcohols, organic acid compounds, phenols, surface active agents, halogens, aldehydes, quaternary ammonium salts, and other disinfectant variants are widely used due to their individual merits and outstanding characteristic characteristics. Through coagulating proteins, lysis of cell membranes, or inhibition of enzyme activity, these agents inhibit microbial growth or destroy microbes etc. Apart from being effective antimicrobials, they can have drawbacks such as skin irritation, unpleasant odour, and bacterial endospores have been found to be much more resistant than their vegetative forms. There are two types of disinfectants available commercially: chemical disinfectants and herbal disinfectants. Herbal disinfectants are now widely used instead of chemical disinfectants because chemical disinfectants have shown to have negative side effects, and herbal disinfectants are plant-based, environmentally friendly solutions[2]. In the fields of pharmacology and microbiology, extensive research is underway to produce novel antimicrobial agents with strong disinfectant properties. Saponin-containing plants have good antimicrobial properties, according to the literature [3, 4]. The primary mode of transmission of multidrug-resistant pathogens and infection to patients is through the hands of health care staff. Hand hygiene is an easy and low-cost method of preventing health-care-associated infections, especially those originating from environmental surfaces. Plants are known to possess various metabolites with profound antimicrobial properties hence they have been extensively used in traditional medicines since years[5, 6]. HAIs (Hospital Acquired Infections) are a major health-care provider and patient safety concern. Proper disinfection and sterilisation steps are critical for controlling hospital-acquired infection, as failure will result in increased morbidity and mortality in a variety of hospital-acquired infections. Surface disinfection (e.g., disinfecting tables, instruments, walls and floors, etc.) or dipping infected substances in disinfectant solution are the most common methods of infection control in hospitals. Disinfectant formulations are used for a variety of purposes [7].The present study was carried out to evaluate the antimicrobial activity of herbal disinfectant formulations prepared by using some indigenous medically

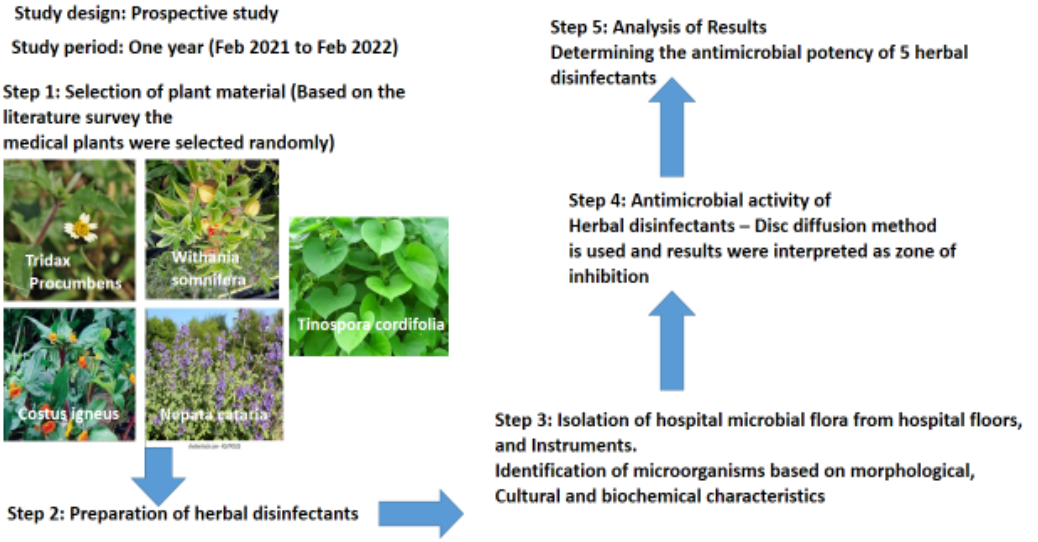
important plant extracts (Tridax procumbens, Withania somnifera, Tinospora cordifolia, Costus igneus, and Nepata cataria) which are effective on killing or inhibiting bacterial flora like Staphylococcus aureus, Coagulase negative Staphylococci (CONS), Escherichia coli, Pseudomonas aeruginosa, Enterococcus faecalis, Klebsiella species, Proteus species, Enterobacter species isolated from different hospital sites.

Materials and Methods

Selection of plant material

The plant specimens were Tridax procumbens, Withania somnifera, Tinospora cordifolia, Costus igneus, and Nepata cataria are identified in the department of Botany, Andhra University, Visakhapatnam. The research plan was described in the Figure 1.

Figure 1: Planning of research



Material and Methods

Collection of plant material

All the five different plants (Tridax procumbens, Withania somnifera, Tinospora cordifolia, Costus igneus, and Nepata cataria) were identified and the leaves were collected. Then the leaves were cleaned in water to eliminate dirt, and were sun dried to remove moisture. Then each plant leaves were made into powder and stored in dry place. The chemical composition of the selected plants was described in the Table 1 [8, 9, 10, 11, 12].

Table 1: Chemical composition of plants

Plant Name	Parameter	Dry weight composition
Tridax procumbens	Phytochemicals (mg/100g)	
	Carotenoids	94.57
	Saponins	103.52
	Tannins	4.72
	Phytochemicals (%)	
Withania somnifera	Total Phenolics	180.8±0.01
	Total flavonoids	136.97±0.01
	Total carbohydrates	19.01±0.03
	Total alkaloids	119.7±0.58
	Total tannins	0.6±0.01
Tinospora cordifolia	Phytochemicals (%)	
	Saponin	1.92±0.22
	Tannins	0.56±0.001
	Alkaloids	0.25±0.001
	Total phenols	5.7±0.07
	Flavonoids	0.81+0.02
	Carotenoids	0.98±0.01
Costus igneus	Phytochemicals (mg/100g)	
	Ascorbic acid	81±2.08
	Beta-carotene (µg)	667±5.12
	Alpha-Tocopherol	149±2.10
	Glutathione (m mols)	75±0.50
	Total phenols (g/100g)	4.5±0.10
	Total flavonoids	0.84±0.01
Nepata cataria	Total flavonoids	0.46±0.0045
	Total phenols	1.40±0.028
	Rosmarinic acid	0.15±0.04

Plant material extraction

10g of powdered plant extract was taken and heated in water bath at 60^0C for 60mintues with 100ml of ethanol solution; the ratio at which solution was prepared was 9 parts of ethanol: 1 part of water (9:1) (Table 2). The mixture was filtered and stored.Carbopol was added primarily to deionized water with constant stirring. Later, after uniform mixing, triethanol amine (TEA) was added gradually by stirring to avoid lump formation and the above mixture was kept aside for 24 hours. The plant extract was added to denatured alcohol along with glycerin, polysorbate 20 and were mixed with aqueous phase. Finally, 0.25% of methyl (or) propyl parabens were added as preservatives and 0.5% of perfume was added and mixed with gentle stirring to attain a uniform product; prepared product was stored in airtight high density polythene (HDPE) containers.

Table 2: Preparation of herbal disinfectant

S.No	Item	Quantity (%)
1	Deionized Water	31
2	Alcohol Denatured	62
3	Plant Extract	2
4	Carbopol 940	0.5
5	Triethanol Amine (TEA)	0.7
6	Glycerin	2.3
7	Polysorbate 20	0.5
8	Perfume	0.5
9	Preservative	0.5

Process of collecting swabs

Sterile swabs were collected from floors and instruments/equipments of different departments (Surgery, Paediatrics, Obstetrics and Gynaecology, Orthopedics,etc.) in hospital. The floor is wet mopped twice a day in general wards and with phenol disinfectant solution. Examination desk and worktops are cleaned twice a day with 70% Isopropyl alcohol wipes. Tools are disinfected with Cidex as per user manual. Fumigation of OT, ICUs is achieved by fumigator chock-full with quaternary ammonium compound solution (SOT 125 TM) on weekly basis.Total 30 samples were collected from frequently used areas in hospital by visitors, doctors and patients. Samples were collected from the targeted spots by rubbing and rotating sterile swabs.

Isolation and identification of bacterial isolates

Swabs were inoculated instantly after collection by streaking on the surface of 5% Sheep Blood agar and MacConkey agar plates, Nutrient agar. Inoculated plates were directly incubated at 37 °C. Identification of the isolates was made by standard microbiological techniques such as colony morphology, biochemical reactions, microscopic features were identified based on Gram staining.

Identification of Bacterial isolates

The pathogens were identified and classified by using microscopic findings, culture methods and biochemical methods. Microorganisms were examined for their motility by hanging drop method. The log phase of broth culture is taken and placed it on a cover slip. The concave slide is inverted over the cover slip which is closed around the ends with gel. The slide is inverted around rapidly and hanging drop was observed. Organisms were focused and motility was detected. The morphological examination of the microorganisms was performed through Gram's staining. Based on Gram's staining, shape, arrangement of organisms were noted. The bacteria isolated were in pure growth for identification of antimicrobial susceptibility. The isolates were streaked on the Mueller Hinton agar plates and incubated at 37^0C for 16 hours. Culture methods need to be followed by biochemical tests comprises carbohydrate fermentation tests, IMViC (Indole production, Methyl red test, Voges-proskauer test, Citrate utilization),

Urease test, Oxidase test, Catalase test, Nitrate reduction test, Triple sugar iron agar test etc.

Antibiotic susceptibility test

Disc diffusion method

Well diffusion method was performed with herbal disinfectants and with standard antibiotics. Well diffusion refers to the dispersion of an antimicrobial agent of a quantified concentration in well, into the solid culture medium that has been inoculated with the respective bacterium isolated in a pure culture in the form of a lawn culture. Well diffusion is centred on the purpose of an inhibition zone related to the bacterial susceptibility to the antimicrobial present in the well. The diffusion of the antimicrobial agent into the inoculated culture media results in an incline of the antimicrobial. AST was accomplished according to CLSI guidelines using Mueller-Hinton agar (MHA) plates using the concentration of antibiotics per well, mentioned by the WHO experts committee on biological standardization. The plates were incubated at 37°C for 16-18h hrs.

Results

Most of the pathogenic bacteria are harmful to humans and other living organisms. These required treatment otherwise it might cause epidemic, endemic, pandemic etc.Table 3depicted the isolation of various microorganisms from different hospital wards include Emergency, NICU (Neonatal intensive care unit), OBG and Paediatric wards. The samples were also including Minor OT, Gynaeic OT and Casualty. Out of 48 swab samples collected from floors, walls, examination tables, bed and stethoscopes from different departments; 36 samples showed positive for microbial growth. Both Gram-positive cocci and Gram negative bacilli were isolated and identified based on standard microbiological techniques. The microorganism isolated were Staphylococcus aureus, Coagulase negative Staphylococci, Escherichia coli, Pseudomonas aeruginosa, Enterococcus faecalis, Klebsiella species, Proteus species, Enterobacter species. The isolated microorganisms were tested for the antimicrobial susceptibility using various standard antibiotics through Kirby-Bauer Method. The most frequently isolated microorganisms was Pseudomans aeruginosa. In the current study a total of 16 strains of P. aeruginosa were isolated. Out of 16 strains, 13 strains showed maximum sensitivity to Imipenem (IMP) and 9 strains showed sensitivity to Amikacin. Most of the strains showed highest resistance to Ampicillin (AMP), AMC, and Ciprofloxacin (CIP). Out of 11 isolated E.coli strains, 9 strains showed maximum sensitivity to carbapenem drug Imipenem. Majority of E.coli strains showed highest resistance to antibiotics and were considered as MDR isolates being resistant to Ampicillin, Amoxycillin clavulanic acid (AMC), Ceftriaxone (CTR), ciprofloxacin, Gentamycin (GEN), Cotrimaxazole (COT) and Amikacin (AK). Among 5 Klebsiella species strains, all the strains showed resistance to AMP, CIP, 2 strains showed resistance to other tested antibiotics. Majority of klebsiella strains 4 showed sensitivity to Imipenem and 3 strains to Amikacin.Out of 4 Enterobacter species isolated 3 strains showed highest susceptibility to IMP and AK and highest resistance to AMP, CTR, CPP, and CIP. A total of 8 Enterococcus faecalis strains isolated, among them 7 strains showed maximum susceptibility to

IMP, AK, and 6 strains showed sensitivity to COT and maximum resistance to AMP, AMC, CP, GEN.The second frequently isolated organism was CONS. out of 15 strains isolated, 12 strains showed maximum sensitivity to IMP, 10 strains to AK and 9 strains to COT. Most of the CoNS strains showed maximum resistance to AMP, AMC, CTR, CFP, and CIP.The third frequently isolated microorganisms Staphylococcus aureus of nearly 14 strains, 12 strains showed maximum sensitivity to IMP, 9 strains to AK and 8 strains to COT. The remaining strains showed multidrug resistance to AMP, AMC, CTR, CFP, CIP, GEN. Among 5 Proteus species isolated 4 strains showed maximum susceptibility to IMP and AK and 3 strains to COT and all the other isolated strains showed resistance to other class of antibiotics.From the Table 4 it was found that most of strains isolated were multidrug resistant isolated to commonly tested antibiotics. Out of total 78 isolated microorganisms, 14 strains showed Carbapenem resistance through modified Hodge test which include 2 strains of E.coli, 3 strains of P. aeruginosa, single strain of Klebsiella species, Enterobacter species, Proteus species and Enterococcus faecalis, 2 strains of S. aureus and 3 strains of CoNS (Table 5).All these 14 isolated carbapenem resistance strains isolated from various swabs collected from the hospital wards were tested for their antimicrobial susceptibility with 5 different herbal disinfectant formulations (Figure 2 and Figure 3A, 3B, 3C) (Table 6).Among the two E.coli strains, one E.coli strain showed sensitivity to formulation-4 containing T. cordifolia leaf extract and complete resistance to other formulations. However, the two carbapanem resistant S. aureus strains showed resistance to all 5 different formulations. Among the 3 carbapenem resistant P. aeruginosa strains only one strain showed sensitivity to formulation-1 containing C.igneus leaf extract. Out of 3 CoNS strains, one strain showed sensitivity to formulation -3 made up of Nepata cataria leaf extract and resistant to other tested formulations. Klebsiella species showed complete resistance to all 5 different formulations. Whereas Enterobacter species showed sensitivity to formulation-4 with T. cordifolia. The isolated E. faecalis strain is resistant to all tested formulations-1 to 4. Proteus species showed good sensitivity to two different formulations, Formulation-1 containing C.igneus and formulation-4 containing W. somnifera(Figure 3A, 3B, 3C).

Table 3: Isolation of microbial flora from different hospital clinical settings

Ward	No. of objects sampled	No. of culture positive samples	Microorganisms isolated
Emergency ward	Floor (2)	2	S. aureus, CoNS, E. coli, Pseudomonas, Enterococci (sp.)
			S. aureus, CoNS, Klebsiella sp.
	Wall (2)	2	S. aureus, CoNS, E. coli, Pseudomonas sp.
			S. aureus, CoNS, Enterococci, Proteus sp.
	Examination table (2)	1	CoNS, Pseudomonas sp.
	Stethoscope (2)	1	S. aureus
NICU ward	Floor (2)	2	CoNS, E. coli
			Pseudomonas sp.
	Wall (2)	2	S. aureus

			CoNS
	Examination table (2)	0	No growth
	Bed (2)	1	E. coli
Minor OT	Floor (2)	2	Pseudomonas, Staphylococcus aureus, Enterobacter sp.
			Pseudomonas, Staphylococcus aureus, Klebsiella sp.
	Wall (2)	2	Enterococci sp., CoNS, Proteus sp., Pseudomonas sp.
			Klebsiella sp.
	Examination table (2)	1	CoNS
OBG	Stethoscope (2)	1	Pseudomonas sp., CoNS
Gynic OT	Floor (2)	2	Proteus sp., Pseudomonas sp., E. coli, Staphylococcus sp.
			CoNS, Enterococci sp.
	Wall (2)	2	Pseudomonas sp.
			CoNS
	Examination table (2)	0	Nil
	Stethoscope (2)	0	Nil
Paediatric ward	Floor (2)	2	Pseudomonas sp.
			E. coli
	Wall (2)	2	Staphylococcus aureus, E. coli
			CoNS, Enterobacter sp.
	Stethoscope (2)	1	Pseudomonas sp.
	Bed (2)	2	E. coli, Enterococci sp.
			CoNS
Casualty	Floor (2)	2	Proteus, E. coli, Pseudomonas sp., CoNS, Enterococci sp.
			Staphylococcus sp., Klebsiella sp.
	Wall (2)	2	Pseudomonas sp., Enterococci sp.
			E. coli, Staphylococcus sp.,, Enterobacter sp
	Examination table (2)	2	Klebsiella, proteus, Staphylococcus sp., Pseudomonas
			CoNS, Enterobacter sp.
	Bed (2)	2	Staphylococcus sp., E. coli
			Enterococci sp., Pseudomonas sp.

Table 4: Antimicrobials and their effect on bacterial isolates

Bacterial isolates	AMP		AMC	CTR	CFP				CIP		GEN		IMP		COT		AK	
	S	R	S	R	S	R	S	R	S	R	S	R	S	R	S	R	S	R
Escherichia coli (11)	1	10	3	8	2	9	2	9	0	11	3	8	9	2	3	8	4	7
Pseudomonas aeruginosa (16)	3	13	5	11	6	10	7	11	2	14	6	10	13	3	8	8	9	7
Klebsiella sp. (5)	0	5	1	5	1	5	2	3	0	5	1	5	4	1	2	3	3	2
Enterobacter sp. (4)	1	3	2	2	1	3	1	3	1	3	2	2	3	1	3	1	3	1
Enterococcus faecalis (8)	2	6	3	5	4	4	4	4	2	6	3	5	7	1	6	2	7	1
Staphylococcus aureus (14)	4	10	6	8	5	9	5	9	3	11	7	7	12	2	8	6	9	5
CoNS (15)	2	13	5	10	6	9	6	9	5	10	8	7	12	3	9	6	10	5
Proteus sp. (5)	1	4	1	4	2	3	3	2	1	4	2	3	4	1	3	2	4	1

Table 5: Detection of carbapenem resistant microorganisms

Bacterial isolates	Number of strains resistant to carbapenem (imipenem)
Escherichia coli	2
Pseudomonas aeruginosa	3
Klebsiella sp.	1
Enterobacter sp.	1
Enterococcus faecalis	1
Staphylococcus aureus	2
CoNS	3
Proteus sp.	1
Total	14

Table 6: Antimicrobial susceptibility of Imipenem resistant microbial isolates to various herbal formulations used in current study

Bacterial isolates	Formulation-1 Costus igneus	Formulation-2 Tridax procumbens	Formulation-3 Nepata cataria	Formulation-4 Tinospora cordifolia	Formulatin-5 Withania somnifera
Escherichia coli strain-1	5 (R)	4 (R)	10 (R)	27 (S)	12 (R)
Escherichia coli strain- 2	14 (R)	12 (R)	12 (R)	13 (R)	14 (R)
Staphylococcus aureus strain- 1	6 (R)	6 (R)	7 (R)	9 (R)	11 (R)
Staphylococcus aureus strain- 2	11 (R)	10 (R)	12 (R)	12 (R)	14 (R)
Pseudomonas aeruginosa strain- 1	5 (R)	6 (R)	4 (R)	4 (R)	7 (R)
Pseudomonas aeruginosa strain- 2	10 (R)	11 (R)	12 (R)	10 (R)	11 (R)
Pseudomonas aeruginosa strain- 3	22 (S)	16 (R)	11 (R)	9 (R)	13 (R)
CoNS strain- 1	11(R)	9 (R)	10 (R)	6 (R)	10 (R)
CoNS strain- 2	12 (R)	12 (R)	10 (R)	14 (R)	11 (R)
CoNS strain- 3	11 (R)	8 (R)	29 (S)	13 (R)	9 (R)
Klebsiella sp.	---(R)	--(R)	-- (R)	-- (R)	5 (R)
Enterobacter sp.	14 (R)	5 (R)	11 (R)	20 (S)	7 (R)
Enterococcus faecalis	6(R)	4 (R)	7 (R)	14 (R)	9 (R)
Proteus sp.	28 (S)	11 (R)	6 (R)	10 (R)	24 (S)

Figure 2: Preparation of Disinfectants

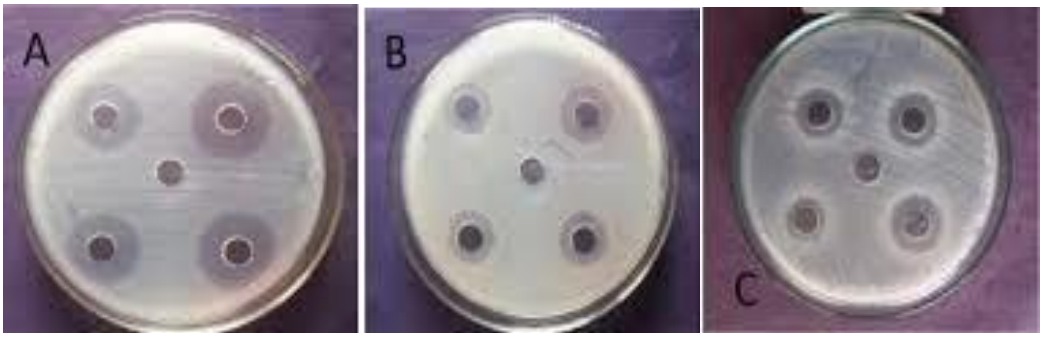


Fig. 3A; 3B; 3C: Antimicrobial activity of plant extracts. Fig. 3A showed the Zone of inhibitions to all the five formulations of E.coli Fig. 3B showed the Zone of inhibitions to all the five formulations of Pseudomonas aeruginosa Fig. 3C showed the Zone of inhibitions to all the five formulations of Staphylococcus aureus

Discussion

The usage of more chemical oriented drug as a disinfectant can pose a threat environment specifically in hospital environment by emerging the multidrug resistance bacteriacausing nosocomial infections. Besides that, chemical disinfectants cause adverse skin reactions and producing disagreeable odour to human beings.According to Joshi et al. (2008) [13]states that many chemical disinfectants could cause adverse effects such as skin irritation and resistance by pathogens and herbal disinfectant can act as and environmental friendly solution to kill pathogen. To overcome this difficulty herbal disinfectants could be one of the solutions and the present study used five different plant extracts to prepare five herbal disinfectant formulations.

All the selected five plants contain rich amount of antimicrobial compounds. The leaves of Costus igneus contain alkaloids, glycosides, polysaccharides, tannins, saponins and phenolics such as flavonoids, terpenoids, carotenoids, and steroids [14]. Withania somnifera plant contains alkaloids, steroids, saponins, phenolics, flavonoids, phytophenols, and glycoside[15].The plant Tinospora cordifolia contains diterpenoid lactones, glycosides,steroids, sesquiterpenoid,

aliphaticcompounds, phenolics, essential oils, a mixture of fatty acids, and polysaccharides [16]. The plant Nepata cataria is rich with essential oil (EO) and flavonoids [17]. Tridax procumbens leaves had high flavonoids, alkaloids, hydroxycinnamates, tannins and phytosterols, moderate benzoic acid derivatives and lignans, and low carotenoids contents [18]. The results of the present study showed all the five herbal formulations were active against Pseudomonas aeruginosa, Staphylococcus aureus, E.coli and Klebsiella species. Though very less literature was cited related to the current study,Nirmala Babu et al. 2016) [19] reported the antimicrobial activity of Costus igneus leaf extract showed high activity against Bacillus subtilis, Candida parapsilosis at low concentration (2.5µg/ml).Abalaka et al. (2012) [20] and Ashfaq et al. (2016) [21] identified the use of neem leaves responsible for the antimicrobial activities againstPseudomonas aeruginosa, Staphylococcus aureus, E.coli and Klebsiella species and in our study Costus igneus, Nepata cataria, Tinospora cordifolia showed high sensitivity towards the above species.Agarwal et al. (2019)[22]studied the extracts of T. cordifolia on S. aureus which gave zone of inhibition of 19mm whereas in our study the authors showed T. cordifolia on S. aureus strains showed less zone of inhibition 9-12mm and less antimicrobial activity.From the above results it was concluded that among the five different formulations used in the current study the Carbapenem resistant pathogens showed sensitivity to four different formulations and resistance to formulation-2 containing Tridax procumbens leaf extract, this in turn indicates that the plant extract disinfectants have opulent antimicrobial activity against most organisms.

Research limitations

Herbal disinfectants are easy to formulate and produce better results compared to chemical disinfectants. Easily available herbal plants used to prepare disinfectants with precise efficacy to decrease microbial load from hospital floors and equipment are reported in this article. The study is limited to single centre. The study is limited to disinfectant formulations only, and there should be detailed investigation is required to identify the antiseptic properties of these five herbal formulations in order to replace the existing chemical based hand rubs.

Prospects for further research

These herbs could be a potential candidate for floor disinfectants. Therefore, authors recommend the further researches on proposed herbs to validate their antiseptic properties and using nanotechnology to enhance their antimicrobial potency.

Conclusion

In the present scenario multi drug resistance is posing a severe threat for the survival of living organisms. A significant population can suffer regular intake of higher antibiotics which will be inactive on resistant bacterial isolates and recognization of existing and potential resistant bacteria from hospital wards would be the first step to short the problem of MBRS.The preparation of herbal disinfectant can reduce the risk of skin allergies and sensitivity compared to chemical disinfectant. Time studies across different plant species extracts would

indicate the sensitivity towards bacterial resistant isolates. Encouraging more herbal disinfectant could be eco-friendly and future endeavours for thick resistant bacterial isolates.

Conflict of interest
The authors declared No conflict of interest

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