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Assessment of Antibiotic-potentiating Activity of Aqueous and Methanolic Extracts of *Piper nigrum* Against Multi-Drug Resistant Phenotypes of *Pseudomonas* sp.

[1*]Charde V. N., [2]Bezalwar P. M., and [3]Patil S. P.

[1]Dept. of Microbiology, Taywade College, Mahadula-Koradi, Dist- Nagpur *(MS), India, 441 111.*
[2]Dept. of Microbiology, Chintamani College of Arts and Science, Gondpipri, Chandrapur (MS), India
[3]Department of Botany, Taywade College, Mahadula-Koradi, Dist- Nagpur *(MS), India, 441 111.*
*Corresponding author: vijaycharde@rediffmail.com

Abstract:
Survey and experimental facts reports the escalating resistance of microorganisms to antibiotics across the globe. This tremendous expansion has stimulated the scientists to discover the alternative strategy that aim and obstruct the consequences of drug resistance. One approach to get rid of this problem could be the adoption of tactics of combination of plant extracts with antibiotics to increase their efficacy. The study was therefore, designed to determine the effects of combining the extract of *Piper nigrum* with selected antibiotics on MDR *Pseudomonas* sp. The *Pseudomonas* isolates were screened from different pathological laboratories of Nagpur (M.S.), India. Identified *Pseudomonas* isolates were then analysed to deduce antibiogram against total 18 antibiotics, most resistance 5 isolates were then evaluated for antibacterial activity of extract alone, and its antibiotic potentiating activity. The antimicrobial activity of each extract was assessed against selected microorganisms and varied results were obtained for each isolate. The hot and cold extracts of herb were prepared in solvents; Methanol and Water. Both hot and cold methanol extract showed very good synergistic effect on most of the antibiotics against Isolate Pseu 3 and water extract showed insignificant effect as compare to methanol extract. Almost all the extracts showed very good synergistic effect on all the antibiotics against Isolate Pseu 19. No significant effect was demonstrated against Isolate Pseu 20 & 26. The overall reults denotes the significant antibiotic potentiating activity of some extracts.
Keywords: Antibiotic potentiating activity, Antibacterial activity, Drug resistance. *Piper nigrum, Pseudomonas* sp.

Introduction:

The discovery of antibiotics was landmark discovery to eliminate the infections but the condition seems to ravage because of their indiscriminate use. Arbitrary practicing of antibiotic therapy by medical practitioners and public (self medication) has led to the development of multidrug-resistant (MDR) pathogens (Hemaiswarya S. *et. al.,* 2008). The antibiotic resistance not only made the bacteria resistant to commonly available antibacterials but also has acquired augmented virulence (Prabhavathi F. 2006). Researchers are concern about prevention of the emergence of new resistant strains and its spread (Okeke I. N. *et. al.,* 2005).

If infection acquire resistant to first line antimicrobials, then treatment procedure is shifted to second line antimicrobials then subsequently to third line antimicrobials. Second and third line drugs are often increases the cost of treatment (Sibanda and Okoh, 2007). The widespread resistance of first-line drugs in poor countries in developing nations increases the mortality and morbidity rates in population (WHO, 2002). The frightening challenge of MDR urges the development of alternative approach and discovery of new antimicrobial compounds (Sibanda and Okoh, 2007). The possible approach in the treatment of infectious diseases could be the use of agent that directly do not kill pathogenic bacteria but modify the bacterial phenotype that make MDR pathogen susceptible to the antibiotic (Taylor *et. al.* 2002). As the research outcomes have shown that active efflux is the main mechanism for the development of antibiotics resistance (Lin J. *et. al.,* 2002); inhibition of these active efflux pumps possibly decreases the rank of intrinsic resistance (Lomovskaya O., and Warren M. S., 2001). The duo of antibiotics and efflux pump inhibitors is an effectual approach to resolve the problem of MDR (Lomovskaya O. and Bostian K. A. 2006).

The benefit of plants to human life is diverse; as food, as sources of industrial substrate, as sources of medicinal component and many more (Azoro, 2004; Erturk *et. al.,* 2006). Medical trials and In vitro evidences proved that plants hold the capacity of combating the problem of antibiotic resistance. Phytomedicine practices have shown remarkable results in the treatment of many infectious diseases including viral infections (Cowan M. M. 1999). Since historic times, mono and poly herbal preparations have been exercised for the treatment of various diseases. In advance studies of phytomedicine, approach have been made to extract a range of natural products and screening of their antimicrobial activity (Abu-Shanab B. *et. al.* 2004). Phytochemicals give promising results in combination therapy which may act as multidrug resistance modifiers (Hemaiswarya S. *et. al.,* 2008). These phytochemicals can reverse the resistance by several mechanisms including blocking the efflux

pumps (Marquez B. *et. al.,* 2005; Adwan G. *et. al.,* 2011).

Specifically spices have been used as a food component to enhance the flavor and aroma of foods, for food preservation and to utilize its medicinal value (Shelef, 1983; Giese, 1994). The antimicrobial properties of some spices, herbs, and their components have been documented since the late 19th century (Shelef, 1983; Zaika, 1988; Alzoreky and Nakahara, 2003; Kumral and Sahin, 2003; Park *et al.,* 2009). In Unani, Homeopathy and Ayurveda, in present times spices are used as aphrodisiac, stomachic drug preparations, carminative, antiseptic diuretic and for various other curative purpose. Phytochemical investigations have shown the presence of organic acids, gums, pectin, sugars, tannins, phenols, alkaloids, flavonoids, glycosides, sesquiterpenes and other unknown substances (Algohary M. E. M. *et. al.*, 1994).

Black pepper (*Piper nigrum* L.) is members of Piperaceae family. It is native to Malabar and Travancore coast of India but other than India, it is also cultivated in Brazil, Indonesia, Vietnam, Malaysia, China, Sri Lanka, and Thailand. The fruits contain 1.0-2.5% volatile oil, 5-9% alkaloids and a resin (Evans W. C. *et. al.*, 1997). Black pepper inhibits the growth of specific pathogens (Sagdic O. and Ozcan M, 2003). The phytocomponents shown to have antipyretic, CNS depressant, analgesic, antiinflammatory (Lee E. B. *et. al.*, 1984), hepatoprotective activity (Koul I. B. and Kapil A, 1993) and antioxidant (Khajuria A. *et. al.*, 1997).

The present study was aimed to evaluate the antimicrobial activity of *Piper nigrum* of hot and cold extract in water and methanol, and to search for the antibiotic potentiating activity of these extract against MDR *pseudomonas* sp. The exploration of idea of antibiotic potentiating activity of extract can lead to development of potent target drug for the treatment of infection of MDR *pseudomonas* sp.

Materials and methods:

Plant material and extract preparation,

The spice, *Piper nigrum* were purchased from local market of Nagpur (MS). The seeds were dried under shade, pulverized with hand mortar and pestle, filled in air tight bottle and stored in refrigerator till its use.

Preparation of herb extracts,

Hot extract (Soxhlet extract)

Herb (about 50g) was dried in oven with air circulation arrangement at 60 ^{0}C. Dried herbs are then grounded by mortar and pestle to fine powder.

5 g of this powder is than filled in sac of 400 mesh nylon cloth and placed in extractor of Soxhelt Extraction unit, 300 ml of solvent is added to round bottom (RB) flask and unit is assembled on heating mantle. Inlet of condenser is connected to tap (water) and water is continuously circulated in condenser till extraction is completed. Total 5 cycles of extraction were run for each sample. After 5 cycles, mantle is switched Off. Assembly is allowed to cooled with circulation of water is On in condenser. Sac of sample powder is removed from the extractor and unit is again assembled. Excess solvent is recovered by distillation in Soxhelt Extraction unit itself till thick consistency of sample extract is obtained in RB flask (Approximately to 50 ml).

Cold water extract

1 g of powder of *Piper nigrum* (seed) was added to 10 ml sterile distilled water in different 100 ml conical flask. These flasks were allowed to stand at 30^{0}C for 24 hours under shaking at 150 RPM. Solution was filtered to obtain a cold water extract.

Cold methanol extract

1 g of powder of *Piper nigrum* (seed) was added to 10 ml methanol in different 100 ml conical flask. These flasks were allowed to stand at 30^{0}C for 24 hours under shaking at 150 RPM. Solution was filtered to obtain a cold methanol extract.

Hot water extract

1 g of powder of *Piper nigrum* (seed) was added to 20 ml sterile distilled water in different 100 ml conical flask and boiled so that its volume reduced up to 10 ml. It was allow to cool, filter and stored in glass bottle.

Cold and hot concentrated extract was prepared in sufficient volume (50 ml) and preserved at 4^{0}C in sealed vials for further use to avoid batch to batch variations.

The extracts were abbreviated as; CMEM- Cold methanol extract of *Piper nigrum* (Black Pepper), HMEM- Hot methanol extract of *Piper nigrum* (Black Pepper), CWEM- Cold water extract of *Piper nigrum* (Black Pepper), HWEM- Hot water extract of *Piper nigrum* (Black Pepper).

Sterility testing of Plant extracts,

100 µl of hot and cold extract were spread on Nutrient Agar (NA) and Potato Dextrose Agar (PDA) plates, and checked for growth of bacteria in 48 hrs at 37^{0}C and at 25^{0}C and fungal contaminants after 1 week of incubation at room temperature to ensure absence of any microbial contamination.

Collection of clinical samples,

Clinical samples of urine, pus, blood, stool, and secretion sample (coded as, U, P, B, S and Sc. respectively) were collected in sterile pathological sample collection bottle from different pathology laboratories of Nagpur (MS), India.

Isolation of pathogens from various clinical specimens,

A sample was immediately transferred to sterile nutrient broth for enrichment under aseptic condition and incubated at 37 ^{0}C for 48 hrs. After 48 hours, loopful of culture from enriched nutrient broth was plated on selective media so as to get well isolated colonies. Suspected

colonies showing typical cultural characteristics on selective media were picked up and were maintained on nutrient agar slant for further identification.

Identification of Isolates,

Isolates were identified on the basis of morphological, cultural & biochemical characteristics and the results were compared with Bergey's Manual of Determinative Bacteriology 9th edition.

Preparation of inoculums,

A loopful of culture from slants was inoculated in into the screw cap tube containing 5ml sterile nutrient broth and incubated at 37^{0}C for 24hrs. Again loopful of culture from same broth was transferred to fresh 5ml of sterile nutrient broth and incubated at 37^{0}C for 6-8 hrs. Turbidity was adjusted according to 0.5 McFarland standards which were then used as an inoculum which corresponds to size of1.5×10^8 CFU/ml. This suspension was used as inoculums.

Antimicrobial drugs,

Cefepime(CPM), Cefotaxime(CTX), Carbenicillin(CB), Ceftazidime(CAZ), Netillin(NET), Tobramycin(TB), Ticarcillin(TCC), Amikacin (AK) Piperacillin(PI), Ceftriaxone(CTR), Gentamicin(G), Imipenem(IPM), Norfloxacin(NX), Aztreonam(AT), Cefoperazone(CPZ), Meropenem(MRP), Ciprofloxacin(CIP), Aziocillin(AZ).

Antibiotic Susceptibility Test by Disk Diffusion Method,

Antimicrobial susceptibility testing was performed by the disc diffusion method with commercially available discs (HiMedia, Mumbai, India)

Selected antibiotic discs were placed over plates seeded with 100 ml broth culture (0.5 McFarlandstandards) over surface of Hi sensitivity test agar and plates were kept undisturbed in a refrigerator for 1 hour. Then plates were removed from refrigerator and shifted to incubator maintained at 37^{0}C for 18-24hrs. After incubation all plates were examined for zone of inhibition and zone of inhibition was noted down. Isolates were considered susceptible, intermediate, or resistant to a particular antimicrobial agent on the basis of the diameters of the inhibitory zones that matched the criteria of the manufacturer's interpretive table, which followed the recommendations of the performance standard for antimicrobial disk susceptibility test, CLSI (CLSI 2007) (formerly NCCLS)

Testing the antimicrobial activity by well diffusion method,

Hi sensitivity test agar was use to check antimicrobial activity by well diffusion method. Autoclaved medium was poured in to petriplates in the laminar air flow hood. After cooling of medium within petriplates, the microbial turbidity adjusted to 0.5 McFarland standards were spread then wells were made on the petriplates with the help of stainless steel borer of diameter 10 mm. Plates were kept undisturbed in a refrigerator for 1 hour. Then plates were removed from refrigerator and shifted to incubator maintained at 37^{0}C for 18-24hrs and zone of inhibition was measured with the help of zone measuring scale (HiAntibiotic Zone Scale™, HiMedia, Mumbai).

Sensitivity or resistance of isolate against different herbal extracts was interpreted from the diameter of zone of inhibition by referring the following Table 1 (Johnson T. and Case C., 1995

Table 1: Susceptibility testing of herbal extract

Activity	**Diameter of zone of inhibition (mm)**
Resistant	10 or less
Intermediate	11–15
Susceptible	16 or more

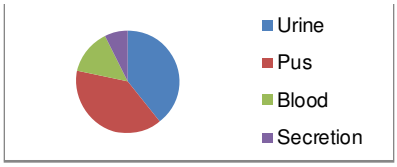
Testing of Synergistic activity,

100 µl of extract was transferred to sterile petri plates and 15ml of sterilized molten Hi sensitivity test agar maintained 55^{0}C in constant temperature water bath was then poured in a plate, then plate is rotated for about 30 to 35 seconds to ensure even mixing of extract with the agar medium. Agar medium was then allowed to solidify. 100 µl of inoculums was added on solidified Hi-sensitivity test agar and spread over agar medium with the help of sterile disposable L-spreader. Four or five antibiotic discs were placed over it equidistantly. Plates were kept undisturbed in a refrigerator for 1 hour. Then plates were removed from refrigerator and shifted to incubator maintained at 37^{0}C for18-24hrs. After incubation all plates were examined for zone of inhibition and zone of inhibition was noted down. Zone of inhibition is then measured and classified as susceptible, intermediate, or on the basis of manufacturer's interpretive table, CLSI standards.

Results and Discussion:

Total 28 isolates were screened from the different samples showing suspected colony on Pseudomonas isolation agar medium. The identification of these isolates was established on the basis of morphological, cultural and biochemical characteristics. Total 11 isolates were isolated from urine sample, 11 from pus, 4 from blood and 2 from secretion sample. The

prevalence of *pseudomonas* are represented in Graph 1.

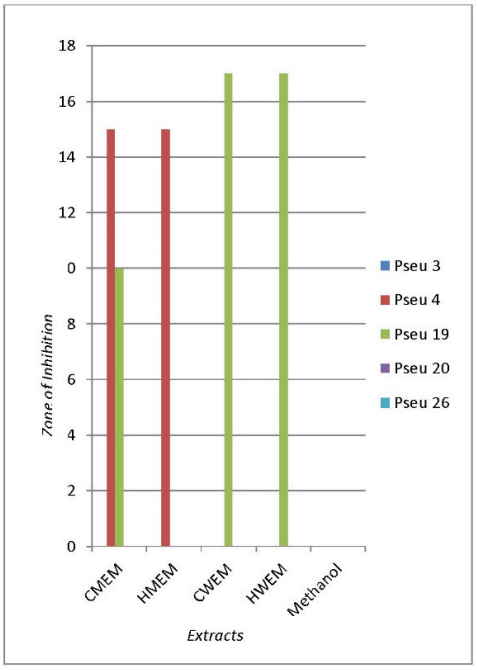


Graph 1: Prevalence of *pseudomonas* Sp. in various clinical samples.

Antibiotic susceptibility of these isolated was carried out against common antibiotics used against *Pseudomonas*. Total 18 antibiotics were tested against 28 isolates. Five isolates showing maximum antibiotic resistance were preferred for detail study. Isolate Pseu 3 showed resistance against AK, CAZ, NX, CPZ, CIP, CPM, CB, NET, TCC, CTR, AT, MRP & AZ. Isolate Pseu 4 was found resistant against CTX, CAZ, TB, PI, G, NZ, CPZ, CIP, CPM, CB, TCC, CTR, IPM, AT, MRP & AZ. Isolate Pseu 19 was resistant to AK, CTX, CAZ, TB, PI, G, NZ, CPZ, CPM, CB, NET, TCC, CTR, IPM, AT & AZ. Isolate Pseu 20 showed resistance to AK, CTX, CAZ, TB, PI, G, NZ, CPZ, CIP, CPM, CB, NET, TCC, CTR, IPM, AT, MRP & AZ. Isolate Pseu 26 was resistant to CTX, CAZ, TB, PI, G, NZ, CPZ, CIP, CPM, CB, NET, TCC, CTR, AT, MRP & AZ.

These five isolates of *pseudomonas* sp. are then tested for Antibacterial Activity against herbal extracts. Isolate Pseu 3 was found to be resistant to rest of the extracts. Isolate Pseu 4 was found to be intermediately susceptible to Cold methanol extract of *Piper nigrum*, Hot methanol extract of *Piper nigrum* and resistant to rest of the extracts. Isolate Pseu 19 was found to be susceptible to Cold water extract of *Piper nigrum* and Hot water extract of *Piper nigrum*. It is intermediately susceptible to Cold methanol extract of *Piper nigrum* and resistant to rest of the extracts. Isolate Pseu 20 was found to be resistant to all extracts. Isolate Pseu 26 was found to be resistant to all extracts. The results of antibacterial activity of different herbal extracts are represented in Graph 2.

The same five isolates are also tested for synergistic effect of *Piper nigrum* (Black Pepper) extract. The results showing synergistic effect of *Piper nigrum* (Black Pepper) on the antibiotics against the *Pseudomonas* isolates are given in Table 2. Methanol extract both hot and cold showed very good synergistic effect on most of the antibiotics against Isolate Pseu 3 and water extract showed insignificant effect as compare to methanol extract. Almost all the extracts showed very good synergistic effect on all the antibiotics against Isolate Pseu 19. No significant effect was demonstrated against Isolate Pseu 20 & 26.



Graph 2: Antibacterial Activity of Various Extracts of *Piper nigrum*.

Current and earlier studies reported the significant evidences of antimicrobial's combination therapy and the results are documented in various reports. On concluding the whole reports it is observed that, the instance where an antibiotic alone didn't worked well if it is administered in combination with either with other antibiotics or the plant extracts improves the antibiotic power to kill the particular pathogen. Prominently, in many illustrations the combinations of drugs/antibiotics and plant extracts work out to be significant in increasing microbicidal or microbistatic activity. The current report also substantiates the better results of combination of antibiotics and plant extracts. Potentiating activity of bioactive plant extracts on antibiotics is a novel concept in management of infections caused by MDR pathogens.

Synergy of antimicrobials have been reported in varied studies, one of the evidence have been given by Khan et al., 2009. In his study the synergistic activity of cefuroxime and penicillin G have been investigated against strains of *Staphylococcus aureus* and *Pseudomonas aeruginosa* where two antibiotics individually was totally ineffective (Khan S. S. *et. al.,* 2009).

Ethanolic leaf extract of *Vangueria spinosa* alone and in combination tested with two antibiotics; doxycycline and ofloxacin, gave promising result for the extermination of Gram positive bacterium (*Staphylococcus aureus*) and three Gram-negative bacteria (*Escherichia coli, Klebsiella pneumoniae*

and Pseudomonas aeruginosa) (Chatterjee S. K. *et. al.*, 2009). In another study, Tiwari *et al.,* (2005) demonstrated the synergistic antimicrobial activity of tea and various antibiotics against enteropathogens. Phytochemical, allicin display potentiating activity on Beta –Lactam antibiotics when tested against *Staphylococcus* spp. and *Pseudomonas aeruginosa* (Cai Yun *et al.,* 2007). Synergistic study of extracts of *T. ammi* seeds and antibiotics on susceptibility of resistant Staphylococcus isolates showed that most of the extracts exhibited potentiating effect on antibiotics (Chardeet *et al.,* 2014). The present study is in corollary with the reports of Chandler, R.F. *et. al.,* (1982) where the extracts of clove, jambolan, pomegranate and thyme displayed the synergistic effect over 19 different antibiotics which was previously ineffective against *P. aeruginosa*. Combination therapies is a boon to address antibiotic resistance and antibiotic use (Cottarel and Wierzbowski, 2007). Chaudhry and Tariq (2006) reported antibacterial potential of aqueous decoction of black pepper (*Piper nigrum* L.), bay leaf (*Laurus nobilis* L.), aniseed (*Pimpinella anisum* L.), and coriander (*Coriandum sativum* L.) have significant bactericidal activity against 176 bacterial isolates belonging to 12 different genera of bacterial population. *Foeniculum vulgare* seeds extract could turn susceptibility of antibiotics from resistant sensitive range when tested against Pseudomonas isolates (Charde *et al.,* 2014).

Ram Kumar Pundir *et al.,* (2010) proposed the antimicrobial potential of black pepper is due to the existence of essential oil present in it, and the peculiar aroma is due to sabinene, β-pinene and limonene. The mechanism of action of these components assumed to be due to disruption of membrane by the lipophilic compounds. The ethyl acetate and petroleum ether extract of *P. nigrum* does not have inhibitory effect on *P. aeruginosa* in contrast *P. aeruginosa* is susceptible to Ethanol extract of *P. nigrum* (Burhan M. *et. al.,* 2015). Hamdy *et al.*, (2013) proved potentiating activity of methanol black pepper extract over amoxicillin, doxycycline, gentamicin, erythromycin and cefotaxime against *E. coli* isolates. This reports the interactions between antibiotics and phytocomponents of spices.

Resistance of microorganism to antibiotics is very serious and worldwide problem. The present investigation gives light on alternative strategy to deal with MDR *Pseudomonas* species. The development of new antibiotic is very costly and time consuming affair. Thus, combination of phytocompounds and antibiotics can be a possible strategy as the experimental results of current study supports cold and hot extracts of *Piper nigrum* (Black Pepper) in methanol and water have potentiating effect on antibiotics against *Pseudomonas* isolates. Though, effective *in-vivo* practice is still far from reality.

Table 2- Synergistic Action of *Piper nigrum* (Black Pepper) and Antibiotic on *Pseudomonas* Isolates resistant to Antibiotics.

Sr. No.	Isolates	Extracts	AK	CTX	CAZ	TB	PI	G	NX	CPZ	CIP	CPM	CB	NET	TCC	CTR	IPM	AT	MRP	AZ
			Zone of Inhibition in mm																	
1	Pseu 3	Antibiotics	10	00	10	00	00	00	00	7	00	10	15	17	12	00	00	12	10	00
		CMEM	22	00	21	00	00	00	25	23	27	23	13	20	00	00	00	12	25	19
		HMEM	21	00	22	00	00	00	25	22	00	12	22	22	18	26	00	21	24	17
		CWEM	10	00	00	00	00	00	00	00	00	10	17	16	00	00	00	11	14	00
		HWEM	22	00	22	00	00	00	00	17	18	27	20	00	20	00	00	11	14	00
2	Pseu 4	Antibiotics	00	00	00	00	24	00	00	00	00	00	21	00	00	00	10	00	00	00
		CMEM	00	22	00	19	26	17	10	16	12	10	30	00	21	13	28	26	28	28
		HMEM	00	22	00	19	28	17	00	17	00	10	28	00	22	12	29	00	12	22
		CWEM	00	22	30	21	33	18	16	15	28	22	27	00	22	23	37	28	29	26
		HWEM	00	22	11	18	28	16	00	17	00	11	26	00	22	17	31	12	11	24
3	Pseu 19	Antibiotics	00	00	00	00	10	00	00	7	00	8	11	9	00	00	12	00	00	8
		CMEM	17	22	20	19	27	19	00	29	00	28	33	19	23	34	33	20	00	24
		HMEM	18	22	23	24	29	22	00	15	00	22	28	21	23	22	26	20	00	21
		CWEM	19	19	18	18	25	17	00	14	00	17	27	17	17	18	21	19	00	21
		HWEM	18	20	19	19	26	21	00	16	00	28	24	24	22	18	32	14	00	24
4	Pseu 20	Antibiotics	13	17	00	10	15	12	00	13	11	00	20	13	17	14	17	10	10	17
		CMEM	13	00	00	14	17	14	00	00	11	00	19	17	00	12	12	00	00	17
		HMEM	15	17	00	17	24	16	00	13	11	00	22	18	20	17	24	00	24	17
		CWEM	20	16	15	20	27	21	20	17	26	17	19	17	19	16	31	20	22	17
		HWEM	13	14	00	17	24	16	00	13	11	12	23	17	18	16	26	00	00	21
5	Pseu 26	Antibiotics	00	17	00	10	13	15	00	10	00	10	20	16	17	14	00	00	00	10
		CMEM	00	14	00	19	22	20	00	10	00	00	22	21	17	00	00	10	00	19
		HMEM	00	15	00	20	24	20	00	12	00	11	21	19	17	00	00	00	00	14
		CWEM	00	00	00	16	11	00	00	10	00	16	21	20	19	16	00	00	00	17
		HWEM	00	19	00	19	23	19	00	12	00	00	22	20	16	12	00	12	00	16

Conclusion:

The major profit of choosing plants derived medicine is that they are often comparatively safer than synthetic drugs and antibiotics even they offer low cost treatment therapy. Holding the present scenario of emergence and persistence of MDR pathogens, the current time demands an improved therapy to manage such infections.

Very promising results are obtained in the present study. Cold and hot extracts of *Piper nigrum* (Black Pepper) in methanol and water turned many *Pseudomonas* isolates susceptible to antibiotic which otherwise were resistant to these antibiotics. Furthermore herbs selected in study are part of Indian cuisine hence their toxicity is not an issue. The present study gives broad vision for further studies that includes; detail investigation of other herbs with greater antibiotic potentiating activity, evaluation of phytocompounds present in herb, formulation studies and clinical trials. These further investigations of the concept are needed to practice this strategy in reality.

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