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Invitro Obliteration of MDR *Pseudomonas aeruginosa* by Synergistic Action of *Amomum subulatum* Extract and Antibiotics.

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Abstract:
The pre-eminent role of *Pseudomonas aeruginosa* in hospital infection is due to its resistance to common antibiotics & antiseptics. Since primitive times, man has gone in different ways to search for cures and relief from various diseases by using several plants, plant products and plant-derived products. Recently, there is a scientific curiosity and certain popularity with regard to screening extracts from plants to use it medicinally all over the world mostly to take over problem of multi drug resistant (MDR) pathogens. In present study *Pseudomonas* isolated from the patients suffering from the ailments like respiratory tract infection, UTI, etc. and studying the antibiotic resistant pattern of these isolates and synergistic action of cold and hot water and methanol extracts of *Amomum subulatum*on the activity of antibiotics. Cold extracts in methanol and water was found to more effective than hot water extracts on many antibiotics. The results showed that *Pseudomonas* isolates not responding to antibiotics becomes sensitive to these antibiotics in presence of cold extracts in methanol and water of *Amomum subulatum.*

Keywords:
Pseudomonas aeruginosa, Amomum subulatum, multi drug resistant.

Introduction:

The ability of *P. aeruginosa* to survive on minimal nutritional requirements and to tolerate a variety of physical conditions has allowed this organism to persist in both community and hospital settings. In the hospital, *P. aeruginosa* can be isolated from a variety of sources, including respiratory therapy equipment, antiseptics, soap, sinks, mops, medicines, andphysiotherapy and hydrotherapy pools (Pollack M. 1995). Community reservoirs of this organism include swimming pools, whirlpools, hot tubs, contact lens solution, home humidifiers, soil and rhizosphere, and vegetables (Pollack M. 1995, Harris, A. A.*et. al.* 1984, Pitt, T. L. 1998). *P. aeruginosa* constitute a member of normal microbial flora of human beings. Approximate colonization rates in different sites in humans are 0 to 2% for skin, 0 to 3.3% for the nasal mucosa, 0 to 6.6% for the throat, and 2.6 to 24% for fecal samples (Morrison A. J. and R. P. Wenzel. 1984). Moreover colonization rates increases to more than 50% during hospitalization (Pollack M. 1995), especially among patients who have experienced trauma to or a breach in cutaneous or mucosal barriers by mechanical ventilation, tracheostomy, catheters, surgery, or severe burns (Blanc D. S., *et. al* 1998, Erol S. *et. al* 2004,

Ohara T. and Itoh. K.2003, Thuong M. *et. al* 2003, Valles J. *et. al* 2004). Immuno compromised Patients have higher risks for colonization by this organism (Morrison A. J., and Wenzel. R. P. 1984, Pollack M. 1995), and disruption in the normal microbial flora as a result of antimicrobial therapy has also been shown to increase colonization by *P. aeruginosa* (Blanc D. S., *et. al* 1998, Bonten M. J.*et. al* 2009,Takesue, Y., *et. al* 2002).

A new study published in the journal *Science* looked to develop a new class of antibiotics to specifically target *Pseudomonas* (Blanc D. S. *et. al* 1998). The authors produced peptidomimetic antibiotics that resemble peptide protegrin 1 (PG-1). PG-1 has a broad spectrum of activity against an array of pathogens and can cause hemolysis of red blood cells. The goal was to optimize PG-1 such that it was specific for *Pseudomonas*, but with less red blood cell lysis.

*Amomum subulatum*is mostly terrestrial, rhizomatous herb. *Ammomum* seeds are used as spices whereas their plant parts are used intraditional medicine for curative purpose of diarrhoea, toothache, dysentery, vomiting, rheumatism, and dyspepsia and lung diseases (Dutta *et. al.* 2000).

Development of drug resistance in *Pseudomonas* is worldwide and is real challenge to medical practitioners to deal with it. Study involves the isolation of *Pseudomonas* from the patients suffering from the ailments like respiratory tract infection, UTI, from the patients who are hospitalized for long period, etc. and studying the antibiotic resistant pattern of these isolates. Moreover study also involves the control of *Pseudomonas* by using synergistic action of antibiotic and herb (*Amomum subulatum*) extract. This study would help medical practitioners in effectively dealing the problem related to *Pseudomonas* infections.

Material and methods:

Collection of clinical samples:

A total of 28 isolates of *Pseudomonas aeruginosa* isolated from different clinical specimens of stool, urine, blood and pus from wound were collected from different pathology laboratories of Nagpur (MS), India.

Isolation of *Pseudomonas* sp. from various clinical Samples:

Collected clinical sample was immediately transferred to sterile nutrient broth for enrichment under aseptic condition and incubated at 37^0c for 48 hrs. Enriched nutrient broth was streaked on Pseudomonas isolation agar (PIA)to get well isolated colonies. Typical colonies were picked up and were maintained on nutrient agar slant. Gram negative coccobacilli observed after Gram staining were continued for further identification and study.

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:

Nutrient broth was inoculated with freshly sub-cultured bacteria and incubated at 37^{0}C for 6-8 hours corresponds to the turbidity of 0.5MacFerlandstandard (1.5×10^8 CFU/ml). Such prepared inoculum was used to spread onto Hi-sensitivity test agar (Himedia Laboratories Ltd., India).

Antibiotic Susceptibility Test:

The disc diffusion method was used to determine the antimicrobial activities. Fresh inoculums (0.5 McFarland standards) were inoculated on the surface of Hi-sensitivity test agar plates. The three to four sterile discs placed onto each agar plate containing microorganisms. Commercially available discs of (HiMedia, Mumbai, India) Amikacin(AK), Cefepime(CPM), Cefotaxime(CTX), Carbenicillin (CB), Ceftazidime (CAZ), Netillin (NET), Tobramycin (TB), Ticarcillin (TCC), Piperacillin (PI), Ceftriaxone (CTR), Gentamicin (G), Imipenem (IPM), Norfloxacin (NX), Aztreonam (AT), Cefoperazone (CPZ), Meropenem (MRP), Ciprofloxacin (CIP), Aziocillin (AZ) were used. Selected antibiotics placed over plates seeded with broth culture and were incubated at 37 ^{0}C for 24 hrs. After incubation, the zones of inhibition were measured and classified as 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 (formerly NCCLS) (CLSI, 2007).

Plant Material:

The herb *Amomum subulatum* were collected from market, dried, pulverized by a mechanical grinder and stored in airtight glass containers in dark until extraction.

Preparation of Solvent Extracts:

Hot methanol extract:

The extraction of compounds from *Amomum subulatum* by the soxhlet method was performed by using methanol as a solvent. The soxhlet procedure consisted of ground *Amomum subulatum* (50g) placed inside a thimble loaded into the soxhlet extractor installed over water bath maintained at 60 ^{0}C. The total extracting time was 6 hrs. and the totalamount of solvent was 300 ml maintained continuously refluxing over the sample. After the extraction the solvent was removed from the solute mixture by reduced pressure with rotary evaporator to obtain final volume of 50 ml.

Cold methanol extract:

1 g of powder of *Amomum subulatum* was macerated in20 ml methanol for 24 hours in orbital shaking incubator set at 30^{0}C with 250 RPM.Solution was

filtered to obtain a cold methanol extract and excess solvent evaporated in rotary evaporator to get final volume up to 10ml.

Cold water extract:

1 g of powder of *Amomum subulatum* was macerated in 20 ml sterile distilled water for 24 hours in orbital shaking incubator set at 30^{0}C with 250 RPM. Solution was filtered to obtain a cold water extract and excess solvent evaporated in rotary evaporator to get final volume up to 10ml.

Hot water extract:

1 g of powder of *Amomum subulatum* was added to 50 ml sterile distilled water in different 100 ml conical flask and boiled so that its volume reduced up to 10 ml.

All extracts was filtered and stored in amber colored glass bottle and stored in a refrigerator.

Antimicrobial Activity of Herbal Extracts:

Antibacterial activities of *Amomum subulatum* extracts were evaluated by means of agar-well diffusion assay. Molten agar (45°C) were poured into sterile petri dishes and allowed it to solidify.1.5×10^8 CFU/ml (0.5 McFarland standard) cell suspensions were prepared and 100 µl was evenly spread onto the surface of the agar plates of Hi-sensitivity agar (HIMEDIA, India). Once the plates had been inoculated, 10 mm wells were punched into the agar with a sterile with the help of cork borer. 100 µl were placed into the wells and the plates were then placed to refrigerator for 1 hr and shifted to incubator maintained at 37°C for 24 hrs. Pure solvents were used as control.

Synergistic Study of Herb Extract on Antibiotic Activity

100 µL of herbal extract was transferred aseptically to sterile plate and moltenHi-sensitivity test agar medium (45-50 ^{0}C) was poured and rotated properly to ensure uniform distribution of herbal extract with medium. Solidified agar medium seeded with0.2 ml of 6-8 hours old inoculum was spread uniformly over agar surface with the help of L-spreader. 3 or 4 disc was place per plate and pressed gently. Plates were incubated at 35±0.5^{0}C and zone of inhibition was measured after 24 hrs.

Result and discussion:

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

These isolates were identified as belonging to genus *Pseudomonas* sp. on the basis of their biochemical characteristics. Antibiotic susceptibility of these isolates was carried out against common antibiotics used against *Pseudomonas.* Five isolates showing maximum antibiotic resistance were

selected 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.
The results showing synergistic effect of *Amomum subulatum* on the antibiotics which are ineffective against the *Pseudomonas* isolates are given in Table1.

Cold methanol extract and cold water extract showed good synergistic effect with AK against Isolate P-3, all the extract showed activity on NX, CPZ & NET and variable degree of effect of extracts was observed on rest of the antibiotics. Isolate P-4 showed no effect of any extracts on any of the antibiotics tested. Cold extracts of methanol and water was found to be more effective than hot water extracts on many antibiotics against Isolate P-19. Variable effect of different extracts on the some antibiotics was observed against Isolate P-20.No significant effect of extracts was demonstrated on most of the antibiotics except on TB, PI & AZ against Isolate P-26.

Since the time immemorial, natural plant products have been used in traditional medicine. Introduction of antibiotics therapy for bacterial and fungal infections introduce the emergence of MDR. Essential oil obtained from seed of *A. subulatum* showed antifungal activity against keratinophilic and dermophytic fungi (Jain P. C.*et. al.* 1976). Secondary metabolites of *A. subulatum* such as alkaloids, flavonoid, and tannins having antibacterial and antifungal activity. Acetone, methanol and ethanol extracts of *A. subulatum* showed antimicrobial inhibitory activity against dental isolates of *Streptococcus mutans* and *S. aureus*a nd fungi *Candida albicans* and *Saccharomyces cerevisiae* (Aneja K. R. and Joshi R. 2011, Hussain T. *et. al.* 2011). Charde V. N.*et. al* (2014) demonstrated polar and nonpolar solvent extracts of *Trachyspermum ammi* is effective in synergy with antibiotics *in vitro* management MDR *Staphyllococcus aureus*.

Methanolic extract of *A. subulatum* fruits showed significant antimicrobial activity against *Escherichia coli*. Cardamom extracts in petroleum ether showed antimicrobial activity against *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa* (Kumar U.*et. al.* 2010).Methanol extract of *A. subulatum* rind have antimicrobial activity against *Staphylococcus aureus* and essential oil showed inhibitory effect against *Pseudomonas aeruginosa, Staphylococcus aureus*, *Staphylococcus epidermidis*, , *Bacillus pumilus* and *Saccharomyces cerevisiae* (Agnihotri S. A. *et. al.* 2012).

This study provides a valid proof for use of *A. subulatum* in Indian traditional medicine and food products.The antibacterial activity and synergistic effect on antibiotics of the extracts may be due to presence of secondary metabolites such as flavonoids, alkaloids, steroids, terpenes, tannins and saponins. Cold extractives of *A. subulatum* in methanol and water have more potentiating effect with antibiotics in exhibiting antimicrobial activity against MDR *Pseudomonas* isolates.

Table. 1- Synergistic Action of *Amomum subulatum* and Antibiotic on *Pseudomonas* Isolates resistant to Antibiotics.
NI- No Inhibition

Sr. No	Isolate	Extract	AK	CTX	CAZ	TB	PI	G	NX	CPZ	CIP	CPM	CB	NET	TCC	CTR	IPM	AT	MRP	AZ
			Zone of Inhibition in mm																	
1	P-3	-	10		10				NI	7	NI	10	15	17	12	NI		12	10	NI
		CMEV	20		10				24	21	NI	15	24	29	25	31		12	10	20
		HMEV	NI		10				26	21	30	17	12	28	21	22		14	28	21
		CWEV	22		NI				26	18	24	10	16	30	NI	NI		11	25	23
		HWEV	NI		11				30	19	28	15	21	35	24	25		15	21	18
2	P-4	-		NI	NI	NI	24	NI	NI	NI	NI	NI	21		NI	NI	10	NI	NI	NI
		CMEV		21	11	17	25	15	NI	16	NI	12	23		18	17	32	12	12	22
		HMEV		22	10	18	22	17	20	17	11	11	23		22	17	25	12	11	22
		CWEV		19	10	22	22	17	29	16	31	11	22		18	15	30	20	NI	NI
		HWEV		18	11	14	26	17	24	15	22	13	26		19	17	26	12	12	17
3	P-19	-	NI	NI	NI	NI	10	NI		7		8	11	9	NI	NI	12	NI		8
		CMEV	16	22	13	18	25	15		15		10	29	22	24	34	33	24		18
		HMEV	15	22	12	18	24	26		32		33	23	21	19	22	36	26		22
		CWEV	11	13	NI	14	19	16		19		NI	13	12	14	11	21	12		16
		HWEV	12	20	13	17	26	12		16		12	31	27	32	33	28	15		18
4	P-20	-	13	17	NI	10	15	12	NI	13	11	NI	20	13	17	14	17	10	NI	17
		CMEV	13	19	NI	12	22	18	NI	13	9	10	21	18	17	16	26	11	10	21
		HMEV	14	17	10	17	22	16	NI	14	11	11	21	13	19	14	25	12	12	19
		CWEV	13	25	15	12	18	12	NI	12	30	24	21	16	18	12	22	NI	26	21
		HWEV	13	19	10	17	20	16	NI	19	8	10	21	13	21	19	23	12	10	21
5	P-26	-		17	NI	10	13	15	NI	10	NI	10	20	16	17	14		NI	NI	10
		CMEV		16	14	21	22	21	NI	10	NI	10	22	21	17	19		14	NI	20
		HMEV		17	NI	19	22	18	NI	12	NI	12	22	19	17	16		NI	10	21
		CWEV		22	17	19	24	18	22	23	28	18	17	21	17	14		NI	NI	17
		HWEV		18	11	21	23	18	NI	13	NI	12	23	19	17	17		NI	NI	20

Conclusion:

High availability and cheaper value complements the use of herbal drug at large scale. Despite of ancient knowledge and medical interest in plants, rare formulations are available in market world wide. The synergistic interaction of phytomedicine increases the efficacy of antibiotics with respect to its action. Encouraging results of purified and crude herbal extract provoked to study at high level. Plants, a sink of never-ending resource of natural drug which offers better remedy to deal with several pathogens even MDR which are effective either in alone or in combination. Several plants have been tested for antimicrobial properties and lots more yet to be investigated. Present study demonstrates the synergistic action of phytochemicals and antibiotics is a successful attempt for obliteration of MDR pathogens.

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References:

Agnihotri S. A., Wakode S. R. and Ali M. (2012) Chemical composition, antimicrobial and topical anti-inflammatory activity of essential oil of *Amomum subulatum* fruits. Acta Poloniae Pharmaceutica n Drug Research. 69(6): 1177-1181.

Aneja K. R. and Joshi R. (2011) Antimicrobial activity of *Amomum subulatum* and *Elettaria cardamomum* against dental carries causing microorganisms. Ethno bot. Leaflets.13: 840-849.

Blanc D. S., Petignat C., Janin B., Bille J. and FancioliP. (1998) Frequency and molecular diversity of *Pseudomonas aeruginosa* upon admission and during hospitalization: a prospective epidemiologic study. Clin. Microbiol. Infect. 4:242-247.

Bonten M. J., BergmansD. C., Speijer H. and StobberinghE. E.(1999) Characteristics of polyclonal endemicity of Pseudomonas aeruginosa colonization in intensive care units. Implications for infection control. Am. J. Crit. Care Med. 160:1212-1219.

Charde V. N., Bezalwar P. M. andGomashe A. V. (2014) Synergistic Activity of Antibiotics and *Trachyspermum ammi* (Ajwain) Extract on MDR *Staphylococcus aureus.* Indian Journal of Applied Research. 4(11):450-454

CLSI (2007). Performance standards for antimicrobial susceptibility testing: 17th Informational supplement. Approved standard M100-S17, Wayne, USA: Clinical and Laboratory Standards Institute.

Erol S., AltoparlakU., AkcayM. N., CelebiF. and ParlakM. (2004) Changes of microbial flora and wound colonization in burned patients. Burns 30:357-361.

Harris A. A., Goodman L. and LevinS.(1984) Community-acquired Pseudomonas aeruginosa pneumonia associated with the use of a home humidifier. West. J. Med. 141:521-523.

Hussain T., Arshad M., Khan S., Hamid S. and Qureshi M. S. (2011) *In vitro* screening of methanol plant extracts for their antibacterial activity. Pak. J. Bot. 43(1): 531-538.

Jain P. C. and Agarwal S. C. (1976) Activity of some plants extracts against some keratinophilic species of Nannizzia". Ind. Drugs. 23(12): 25-26.

Kumar U., Kumar B., Bhandari A. and Kumar Y. (2010) Phytochemical investigation and comparison of antimicrobial screening of Clove and Cardamom. Inter. J. Pharmaceu. Sci. Res. 1(12): 138-147.

Morrison A. J., and Wenzel R. P. (1984) Epidemiology of infections due to Pseudomonas aeruginosa. Rev. Infect. Dis. 6(Suppl. 3):S627-S642.

Ohara T. and Itoh K. (2003) Significance of *Pseudomonas aeruginosa* colonization of the gastrointestinal tract.Intern. Med. 42:1072-1076.

Pitt T. L. (1998) Pseudomonas, Burkholderia, and related genera, 1109-1138.In B. I. Duerden (ed.), Microbiology and microbial infections, vol. 2.Oxford University Press Inc., New York, NY.

Pollack M. (1995) *Pseudomonas aeruginosa*, p. 1820-2003.*In* G. L. Mandell, R. Dolan, and J. E. Bennett (ed.), Principles and practices of infectious diseases. Churchill Livingstone, New York,NY.

DuttaS., Ahmed R. and Pathak M. G. (2000) Essential oil composition of *Amomum linguiforme* Benth. from north-east India, *Indian Perfum.* 44:11–13.

Srinivas, N.; et, al. (2010). *Science.* 327: 1010-1013.

Takesue Y., Yokoyama T., Akagi S., Ohge H., Imamura Y., Murakami Y. and Sueda T. (2002) Changes in the intestinal flora after the administration of prophylactic antibiotics to patients undergoing a gastrectomy. Surg. Today 32:581-586.

Thuong M., Arvaniti K., Ruimy R., de la Salmoniere P., Scanvic-Hameg A., Lucet J. C. and Regnier B. (2003) Epidemiology of *Pseudomonas aeruginosa* and risk factors for carriage acquisition in an intensive care unit. J. Hosp. Infect. 53:274-282.

Valles J., Mariscal D., Cortes P., Coll P., Villagra A., Diaz E., Artigas A. and Rello J. (2004) Patterns of colonization by *Pseudomonas aeruginosa* in intubated patients: a 3-year prospective study of 1607 isolates using pulsed-field gel electrophoresis with implications for prevention of ventilator-associated pneumonia. Intensive Care Med. 30:1768-1775.