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STUDY ON SYNERGISTIC ACTION OF *CINNAMOMUM ZEYLANICUM* (CINNAMON) AND ANTIBIOTICS AGAINST RESISTANT *PSEUDOMONAS SPECIES*

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Abstract

Background: Pseudomonas aeruginosa *has become an important cause of infection, especially in patients with compromised host defense mechanisms. Thus, it is a frequent cause of nosocomial infections. Development of drug resistance in Pseudomonas is worldwide problem and tough challenge to medical practitioners;*

Methods: *Present study involves the isolation of Pseudomonas 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 Cinnamomum zeylanicum on the activity of antibiotics by agar diffusion method;*

Results: *Out of 28 isolates of Pseudomonas isolates five isolates exhibiting wide resistance pattern were selected for study. The results showed synergistic effect of Cinnamomum zeylanicum on the antibiotics which are ineffective against the Pseudomonas isolates. Cold methanol and cold water extract showed good synergistic effect with antibiotics AK, CTX, CAZ, PI, G, CPZ, CPM, CB, NET, TCC, CTR, IPM, AT & AZ against Isolate P-19 making it susceptible to these antibiotics. Hot extract of both methanol and water showed no synergistic effect against Isolate P-19. All the extracts of Cinnamomum zeylanicum have shown no significant synergistic effect on the antibiotics against Isolate P-3, P-4, P-20 and P-26;*

Conclusions: *Cinnamom extract is effective in combination with antibiotic in dealing the drug resistance problem of Pseudomonas and such synergy is important in exploring the new options for drug resistance problem of many pathogens.*

Keywords: *Synergy, Cinnamomum zeylanicum,* Pseudomonas aeruginosa

Background

Development of multidrug resistance to the antibiotics is a major cause of concern particularly dangerous to cystic fibrosis patients and populations having weak immune system. Emergence of newer diseases causing pathogens and evolution of existing microorganisms are responsible for human morbidity and mortality [1]. Plants being a rich source of novel biologically active molecules and can serve as a natural remedy against pathogens. High cost of conventional antimicrobial treatments particularly in countries which are having low economic condition necessitates the use of plant based natural medicine. Phenomenon of pronounced effect of two different compounds than their individual activities is known as synergism and showing worsening effects in combination is called as an antagonism [2, 3].

There is a continuous and urgent need to discover new antimicrobial compounds with diverse chemical structures and novel mechanisms of action because there has been an alarming increasing need because of increasing incidence of new and re-emerging infectious diseases [4,5]. There is appearance of undesirable side effects of certain antibiotics as well as the increasing development of resistance to the antibiotics in current clinical use. Hospital born infections are important health problems in all over the world, because of their high mortality rate, and prolong time of hospitalization and increasing the cost of treatment [6]. *Pseudomonas aeruginosa* is recognized as one of the leading causes of nosocomial infection. *P. aeruginosa* exhibits high-level resistance for many antimicrobials and resistance can develop due to the frequent mono therapy treatment of infected patients [7,8]. Multiple combinations of antibacterial agents are proposed which provide larger spectrum antimicrobial effect and to prevent the rapid emergence of resistance in nosocomial infections. Combinations usually comprise an anti-pseudomonal beta-lactam and an aminoglycoside or a fluoroquinolone. Moreover, *P. aeruginosa* organism is resistant to many antibacterial drugs which are being used as a first line of treatment as they have developed several mechanisms for resistance which include antibiotic inactivation by enzymatic action, altering the efflux pump mechanisms, target mutation, and decreased uptake of antibiotics. Synergy is reported for β-lactams in combination with aminoglycoside antibiotics but the toxicity concerns with the aminoglycosides limit the use of these combinations. Also, fluoroquinolones in combination with potent anti-pseudomonal β-lactam agents are reported to prevent the development of resistance in *P. aeruginosa* [9, 10].

The use of plant extracts and phytochemicals, both with known antimicrobial properties, can be of great significance in therapeutic treatments. In the last few years, a number of studies have been conducted in different countries to prove such efficiency**.** Many plants have been used because of their antimicrobial traits, which are due to compounds synthesized in the secondary metabolism of the plant. These products are known by their active substances, for example, the phenolic compounds which are part of the essential oils, as well as in tannin [11, 12]. Phytochemical study of leaf extracts of *Senna obtusifolia* (L) revealed that the extracts contained phytoconstituents; saponins, tannins, alkaloids, flavonoids including other bioactive components like thiocynate, nitrate, chloride and sulphates, beside are known to be bactericidal, pesticidal or fungicidal in nature thus conferring the anti-microbial property to plants. [13]

The present research demonstrates *in vitro* bioactivity of methanolic extract of stem bark of *Cinnamomumum zeylanicum* (Cinnamon) against human pathogenic bacterial strains of *Pseudomonas* sp. which had shown multi-drug resistance against several commonly used antibiotics. The results direct the ethnomedicinal use of the plant species against several microbial ailments and also open up a path for use of ethanomedicine and modern day antibiotics, a possible remedy against multi drug resistance bacteria.

Material and Methods

Collection of clinical samples:

Clinical samples of urine, pus, blood and sputum sample were collected from different pathology laboratories of Nagpur (MS), India.

Isolation of Pseudomonas sp. from various clinical Samples:

A clinical sample was immediately transferred to sterile nutrient broth for enrichment under aseptic condition and incubated at 37^0c for 48 hrs. After 48 hrs loopful of culture from enriched nutrient broth was streaked on Pseudomonas isolation agar (PIA) so as to get well isolated colonies. On the basis of cultural characteristic on pseudomonas isolation agar 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 as well as confirmed by biochemical identification using Vitek 2 System [14].

Preparation of inoculums:

A loopful of culture from slants was inoculated in 5ml sterile nutrient broth and incubated at 37^0C for 24hrs. Again loopful of culture from same broth was transferred to fresh 5ml of sterile nutrient broth and incubated at 37^0C for 6-8 hrs. Turbidity was adjusted according to 0.5 McFarland standards which were then used as an inoculum which corresponds to size of 1.5×10^8 CFU/ml.

Antibiotic Susceptibility Test:

Antimicrobial susceptibility testing was performed by the disc diffusion method with commercially available disks (HiMedia, Mumbai, India) of 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) [15].

Selected antibiotics placed over plates seeded with broth culture (0.5 McFarland standards) were incubated at 37 °C for 24 h. 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 (formerly NCCLS) [16]

Plant Material:

The stem bark of Cinnamon was obtained from the market.

Preparation of Solvent Extracts:

Hot methanol extract:

Herb (about 50g) was dried in oven with air circulation arrangement at 60 ^{0}C for 6 hours. Dried herbs were then grounded in grinder to fine powder. Solvent extracts (in 300 ml of solvent) were prepared using 25 g of this powder in extractor of Soxhelt Extraction unit. Total 5 cycles of extraction were run for each sample. Excess solvent is recovered by distillation in Soxhelt Extraction unit itself till thick consistency of sample extract is obtained in RB flask (final volume made up to 50 ml).

Cold methanol extract:

1 g of powder of *Cinnamon* was added to 20 ml methanol in 50 ml conical flask. These flasks were allowed to stand at 30^0C for 24 hours under shaking at 250 RPM. Solution was filtered to obtain a cold methanol extract and final volume was made up to 10ml.

Cold water extract:

1 g of powder of *Cinnamon* was added to 20 ml sterile distilled water in 50 ml conical flask. These flasks were allowed to stand at 30^{0}C for 24 hours under shaking at 250 RPM. Solution was filtered to obtain a cold methanol extract and final volume was made up to 10ml.

Hot water extract:

1 g of powder of *Cinnamon* 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.

Antimicrobial Activity of Herbal Extracts:

Antibacterial activity of the extract was determined by agar well diffusion assay method. Bacterial isolates first grown in sterile nutrient broth (0.5 McFarland standards), of which 1ml of culture was spread on Hi-Sensitivity agar plates. In the inoculated Hi-Sensitivity agar plates, wells were made by using sterile 6mm SS borer. The wells were filled with 100µl of the plants extracts and methanol as a blank. The plates were refrigerated at 8-10 ^{0}C for 1 hour for proper diffusion of solvent before plates were incubated at 37 ^{0}C. Zone diameter was measured after 24 h incubation [17].

Synergistic Study of Herb Extract on Antibiotic Activity:

100 µL of herbal extract was transferred aseptically to sterile plate using micropipette. Hi-sensitivity test agar medium maintained at 45-50 **^{0}**C was poured and mixed properly to ensure uniform distribution of herbal extract with medium. After solidification of agar medium, 0.2 ml of 6-8 hours old culture was inoculated on to the surface agar medium by micropipette using sterile tips under aseptic preparation and inoculums was spread carefully by sterile spreader to ensure uniform spread of organism on the agar surface. Discs of antibiotic showing resistance to antibiotic (3 or 4 disc per plate) were placed on agar surface and pressed gently. Plates were incubated at 35±0.5**^{0}**C and zone of inhibition was measured after 24 hrs.

Results 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 cultural, biochemical and morphological characteristics and identified as belonging to *Pseudomonas* sp. Total 11 isolates were isolated from urine sample, 11 from pus, 4 from blood and 2 from sputum sample.

All 28 isolates were investigated for study of antibiotic profile against commonly used antibiotics by disc diffusion method. After antibiotic susceptibility testing, five isolates encountered to be showing maximum antibiotic resistance and were selected for synergistic action study. P-3 showed resistance range against AK, CAZ, NX, CPZ, CIP, CPM, CB, NET, TCC, CTR, AT, MRP & AZ. Isolate P-4 was found resistant range against CTX, CAZ, TB, PI, G, NZ, CPZ, CIP, CPM, CB, TCC, CTR, IPM, AT, MRP & AZ. Isolate P-19 was resistant to AK, CTX, CAZ, TB, PI, G, NZ, CPZ, CPM, CB, NET, TCC, CTR, IPM, AT & AZ. Isolate P- 20 showed resistance range to AK, CTX, CAZ, TB, PI, G, NZ, CPZ, CIP, CPM, CB, NET, TCC, CTR, IPM, AT, MRP & AZ. Isolate P-26 was resistant range against CTX, CAZ, TB, PI, G, NZ, CPZ, CIP, CPM, CB, NET, TCC, CTR, AT, MRP & AZ.

Isolate no. 3, 4, 19, 20, 26 showed wide resistance profile towards selected antibiotics these isolates then carried for the synergistic effect of *Cinnamomum zeylanicum* on the antibiotics which are effective against the *Pseudomonas* isolates. Cold methanol extract showed good synergistic effect on antibiotics AK, CTX, CAZ, PI, G, CPZ, CPM, CB, NET, TCC, CTR, IPM, AT & AZ for isolate P-19, making it susceptible to these antibiotics. Similar effect was observed for cold water extract. Hot extract of both methanol and water showed no synergistic effect against Isolate P-19. The results of synergistic effect of herbal extracts and antibiotics are given in Table: 1. All the extracts of *Cinnamomum zeylanicum* have shown variable synergistic effect on the antibiotics against Isolate P-3, P-4, P-20 and P-26. Antibacterial, antifungal, anti-antropode, anti-helminthic, anti-cancer and many physiological defects

cardiovascular also been cured by use of phytochemicals [18, 19]. The antibiotic resistant profile of clinical isolate found to be resistant to commonly used antibiotics, ampicillin, tetracycline, rifampicin, amoxicillin, chloramphenicol, cefotaxime, erythromycin, penicillin, neomycin, streptomycin, trimethoprim and gentamycin and sensitive to ciprofloxacin only and have significant role of plasmid DNA in drug resistance [20]. The synergistic effect from the association of antibiotics with plant extracts against resistant bacteria leads to new choices for the treatment of infectious diseases. This effect enables the use of the respective antibiotic when it is no longer effective by itself during therapeutic treatment [21].

Essential oil of *Pelargonium graveolens* was reported to reduce the effective dose of norfloxacin against *Bacillus cereus, Bacillus subtilis, Escherichia coli, and Staphylococcus aureus* in combination [22]. Similarly essential oil of *Lantana camara* L. showed synergistic action on amikacin against *S. aureus* and *P. aeruginosa* [23]. Against *P. aeruginosa* the essential oil of *Croton zehntneri* leaves increase the effectiveness of gentamicin activity by 42.8% [24].

Phenolic fraction of *Cinnamomum zeylanicum* are supposes to be responsible for its anti-oxidant and free radical scavenging activity. Cinnamon extracts are known to have anti-inflammatory effects due up regulation of Tristetraprolins which destabilize of proinflammatory mRNA [25]. Hydrophobic essential oils to disrupt the bacterial cell membrane which confers the anti-microbial action and cause ion leakage. The column chromatography fraction's assay clearly indicated that cinnamaldehyde is the key compound responsible for antibacterial activity [26] and under biochemistry view trans-cinnamaldehyde, inhibits bacterial acetyl-CoA carboxylase [27].

The methanol leaf extract of *C. odorata* contains potential efflux pump inhibitors, the synergistic effect of the extract was observed on both Gram-Positive and Gram-negative bacteria [28]. The ethanol extracts of *Corchorus olitorius* leaf extract in *in-vitro* interaction with ciprofloxacin, gentamycin, streptomycin, erythromycin and ampicillin/cloxacilin mixture against Methicillin sensitive *Staphylococcus aureus* and Methicillin resistant *Staphylococcus aurues* (MRSA), the extract synergized the activities of streptomycin and ciprofloxacin and antagonized the activities of gentamycin, erythromycin and ampicillin/cloxacilin mixture on MRSA. [29]

Phytochemical analysis revealed the presence of saponins, flavonoids and naphthoquinone in the crude extract. Alcoholic extracts of *Eugenia jambolana* (EJ) seeds and *Elephantopus scaber* (ES) whole plant, inhibit the growth of vancomycin resistant *Enterococci* (VRE) [30]. *Escherichia coli*, *Pseudomonas aeruginosa* and *Proteus mirabilis* resistance to streptomycin, chloramphenicol, tetracycline, amoxicillin, rifamycine became sensitive extract of (phenol fraction and flavonoid content) *Melissa officinalis* in synergism with antibiotics [31]. MDR Gram-negative bacteria with over expressing active efflux pump phenotypes became sensitive to methanol extracts of *Citrus medica*, the bulbs of *Allium sativum* and *Allium cepa*, the seeds of *Carica papaya*, *Cola acuminata*, *Buchholzia coriacea*, *Garcini kola*, and *Garcinia lucida*, the seeds and fruits of *Picralima nitida* some Cameroonian medicinal plants showed synergism with currently used antibiotics on multidrug resistant. The association of phenylalanine arginine *β*-naphthylamide (PA*β*N or efflux pumps inhibitor) to different extracts modifies their activities at typical concentration [32].

Use of Cinnamom extract is effective in combination with antibiotic in dealing the drug resistance problem of *Pseudomonas.* Such synergy is important in exploring the new options for drug resistance problem of many pathogens.

Table 1- Synergistic effect of *Cinnamomum zeylanicum* (Cinnamom) 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	P-3	Antibiotics	9 (R)	10 (R)	10 (R)				NI	7 (R)	NI	10 (R)	15 (I)	17 (S)	12 (R)	NI		12 (R)	10 (R)	NI
		CMEK	10 (R)		11 (R)				NI	10 (R)	8 (R)	12 (R)	16 (I)	NI	10 (R)	NI		13 (R)	12 (R)	11 (R)
		HMEK	10 (R)		13 (R)				NI	7 (R)	6 (R)	12 (R)	14 (I)	NI	NI	NI		13 (R)	10 (R)	NI
		CWEK	10 (R)		11 (R)				NI	NI	NI	11 (R)	12 (R)	NI	8 (R)	NI		13 (R)	10 (R)	9 (R)
		HWEK	10 (R)		11 (R)				NI	10 (R)	NI	10 (R)	14(I)	NI	8 (R)	NI		13 (R)	12 (R)	10 (R)
2	P-4	Antibiotics		NI	NI	NI	24 (S)	NI	NI	NI	NI	NI	21 (S)		NI	NI	10 (R)	NI	NI	NI
		CMEK		NI	NI	NI	16 (R)	NI	NI	11 (R)	8 (R)	15 (I)	23 (S)		NI	NI	10 (R)	9 (R)	NI	NI
		HMEK		NI	NI	9 (R)	16 (R)	NI	NI	NI	NI	NI	10 (R)		NI	NI	10 (R)	8 (R)	10 (R)	10 (R)
		CWEK		NI	NI	NI	13 (R)	NI	NI	NI	NI	14	14 (I)		NI	NI	10 (R)	8 (R)	NI	NI
		HWEK		NI	NI	NI	14 (R)	NI	NI	NI	NI	NI	16 (I)		NI	NI	10 (R)	NI	10 (R)	NI
3	P-19	Antibiotics	NI	NI	NI	NI	10 (R)	NI		7 (R)		8 (R)	11 (R)	9 (R)	NI	NI	12 (R)	NI		8 (R)
		CMEK	18 (S)	26 (S)	14 (R)	NI	28 (S)	18 (S)		16 (I)		15 (I)	27 (S)	19 (S)	22 (I)	19 (I)	30 (S)	14 (R)		23 (S)
		HMEK	NI	NI	NI	NI	14 (R)	15 (S)		NI		NI	12 (R)	15 (S)	NI	NI	NI	12 (R)		NI
		CWEK	16 (I)	21 (R)	11 (R)	NI	10 (R)	NI		31 (S)		11 (R)	33 (S)	25 (S)	NI	NI	8 (R)	NI		27 (S)
		HWEK	NI	NI	NI	7 (R)	12 (R)	NI		9 (R)		NI	12 (R)	NI	NI	NI	13 (R)	NI		9 (R)
4	P-20	Antibiotics	13 (R)	17 (R)	NI	10 (R)	15 (R)	12 (S)	NI	13 (R)	11 (R)	NI	20 (S)	13 (I)	17 (I)	14 (I)	17 (I)	10 (R)	10 (R)	17 (R)
		CMEK	14 (R)	20 (R)	NI	16 (S)	13 (R)	15 (S)	NI	12 (R)	9 (R)	NI	19 (S)	17 (S)	19 (I)	17 (I)	20 (S)	NI	NI	19 (S)
		HMEK	14 (R)	17 (R)	NI	12 (R)	15 (R)	16 (S)	NI	14 (R)	12 (R)	NI	18 (S)	17 (S)	19 (I)	17 (I)	24 (S)	12 (R)	NI	20 (S)
		CWEK	12 (R)	16 (R)	NI	15 (S)	15 (R)	17 (S)	NI	15 (R)	12 (R)	NI	19 (S)	17 (S)	18 (I)	17 (I)	22 (S)	10 (R)	NI	18 (S)

		HWEK	15 (I)	19 (R)	NI	16 (S)	13 (R)	15 (S)	NI	14 (R)	12 (R)	NI	19 (S)	14 (I)	19 (I)	16 (I)	22 (S)	NI	NI	19 (S)
5	P-26	Antibiotics		17 (R)	NI	10 (R)	13 (R)	15 (S)	NI	10 (R)	NI	10 (R)	20 (S)	16 (S)	17 (I)	14 (I)		NI	NI	10 (R)
		CMEK		17 (R)	NI	19 (S)	13 (R)	NI	NI	14 (R)	8 (R)	10 (R)	20 (S)	18 (S)	13 (R)	16 (I)		NI	NI	16 (R)
		HMEK		19 (R)	NI	18 (S)	13 (R)	15 (S)	NI	NI	10 (R)	10 (R)	21 (S)	17 (S)	17 (I)	16 (I)		10 (R)	10 (R)	16 (R)
		CWEK		NI	NI	12 (R)	12 (R)	13 (S)	NI	10 (R)	9 (R)	NI	11 (R)	17 (S)	19 (I)	13 (R)		11 (R)	11 (R)	17 (R)
		HWEK		21 (R)	NI	13 (S)	16 (R)	20 (S)	NI	12 (R)	8 (R)	NI	19 (S)	17 (S)	18 (I)	13 (R)		8 (R)	11 (R)	17 (R)

C- Cold, H- Hot, W- Water, M-Methanol, E- Extract, K- *Cinnamomum zeylanicum,* NI – No Inhibition, (S)- Sensitive and (R)- Resistant

Conclusion

Synergistic interaction of phytomedicine with antibiotics can exhibit efficacy with respect to its action. Varied action of purified extract and crude one reports the need of study at high level. Abundance and cheaper value complements the use of herbal drug at large scale. Despite of increased interest in medicinal plant research very few formulations are available in market worldwide as compared to chemical based chemotherapeutic formulations. Plants, a mine of inexhaustible source of natural drug offers better options in dealing several MDR pathogens either alone or in combination with existing line of treatment. Numerous plants have been tested for antimicrobial properties and lots more yet to be investigated. Synergistic action is of key importance in phytomedicine, to study the efficacy of apparently low dosage of active component in herbal product. Synergistic action of phytochemical and antibiotics have shown mark positive effect, in *in-vitro* study. Synergistic action of phytochemicals and antibiotics is therefore a successful attempt for annihilation of MDR pathogens.

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