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ANTIBACTERIAL ACTIVITY OF *STREPTOMYCES* ISOLATED FROM RHIZOSPHERIC SOIL OF KHAIRAGARH, CHHATTISGARH

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ABSTRACT

The present work was carried out to assess the antibacterial activity of *Streptomyces* isolated from rhizospheric soil of Khairagarh of Rajnandgaon district, Chhattisgarh; India. Seventeen *Streptomyces* with distinct characteristics were isolated on Starch casein nitrate agar media by serial dilution method. All *Streptomyces* isolates were screened for their antibacterial activity against *E. coli*, *B. subtilis*, *K. pneumoniae*, *P. aeruginosa*, *B. cereus*, *S. aureus*, *M. luteus*, *L. monocytogen*, *S. enterica*, *A. hydrophila*. Ten *Streptomyces* isolates exhibited antibacterial activity against at least one of the test organisms. Out of ten, one *Streptomyces* isolate (V5) exhibited broad spectrum activity.

Figures: 02 References:20 Tables:02
KEY WORDS: Antibacterial Activity, Rhizospheric Soil, *Streptomyces*.

Introduction

Wide range of bacteria and fungi are responsible for life threatening infection to humans and of which many are resistant to antibiotics inhibition [2,3]. In recent years antimicrobial resistance emerged as a serious problem. Some pathogens are resistant to many different types of antibiotics and are required to control immediately as they are responsible for the spread of single or multidrug resistance [6,14,19]. The main reason of antibiotic resistance is inappropriate prescription; irregular and improper use of antibiotics since their availability in the market[1,17]. Increasing antimicrobial resistance, occurrence of new diseases and toxicity in some antimicrobial compounds necessitates the discovery of new antimicrobial compound [18]. Soil harbors diverse range of microbial community, working as a nutritional medium for microorganisms. On the basis of soil texture and nutrition availability, soils hold many types of microbes in variable number and composition. *Streptomyces*, member of actinomycetes are widely distributed in various types of soils [9]. Earthy smell of soil is due to geosmin produces by *Streptomyces*, indicating that *Streptomyces* prevalently found in soils [8]. *Streptomyces* are aerobic, gram positive, mycelium producing bacteria, dominant genus of actinomycetes constituting more than 55% of G+C content in their DNA. Actinomycetes produces more than 50% of known antibiotics and 80% of this is produced by *Streptomyces* sp [4,5,13]. *Streptomyces* produce wide range of clinically important antibiotics and are broadly used in industries due to their bioactivities. Secondary metabolites produced by *Streptomyces* include antibacterial, antifungal, antiviral, antitumor, anti-hypersensitivity and immunosuppressive [11,15].

Material and Methods

Collection of soil sample:

Soil samples were collected from rhizosphere of plants in Khairagarh of Rajnandgaon district, Chhattisgarh; India. Soils were taken from up to depth of 25 cm, after removing 5 cm of surface soil. The soil samples were collected in sterile plastic bags [10].

Isolation of *Streptomyces*:
Soil sample were shaded dry for four days and 10 g soil sample dissolved in 100 ml of sterile distilled water and heat in water bath for 20 minutes at 60 °C the suspension was serially diluted and spread 0.1 ml aliquots of each serial dilution on Starch casein nitrate agar plate containing actidione to inhibit fungi, plates were incubated for 14 days. The pure cultures were stored in Starch casein nitrate agar and glycerol for further use.

Test organism: Gram positive and Gram negative bacterial were used to screened for antibacterial activity of isolates. Gram positive: *Staphylococcus aureus* (MTCC 7443), *Bacillus subtilis* (MTCC 1789), *Bacillus cereus* (MTCC 6909), *Listeria monocytogenes* (MTCC 1143), *Micrococcus luteus* (MTCC 7950). Gram negative: *Escherichia coli* (MTCC 3221), *Klebsiella pneumoniae* (MTCC 9544), *Pseudomonas aeruginosa* (MTCC 3163), *Salmonella enterica* (MTCC 3219) and *Aeromonas hydrophila* (MTCC 1739).

Screening of isolates for antibacterial activity:
Primary screening: primary screening of *Streptomyces* were done by using cross streak plate method on Starch casein nitrate agar. *Streptomyces* isolates were inoculated by a single straight line in the centre of Petri plates and after incubation of 7 days at 30 °C plates were inoculated with test organisms perpendicular to the *Streptomyces* isolates and incubated at 37 °C for 24 hours [12]. The bacterial inhibition was evaluated by measuring inhibition distance in mm between test organisms and *Streptomyces* straight line.

Secondary screening: isolates which exhibited primary activity were grown in 250 ml flask containing 50 ml of Starch casein nitrate broth (SCNB) and Glucose soybean meal broth (GSB) medium. Seven day old culture was used to inoculate the flask and incubated in a rotary shaker at 150 rpm at 30 °C for 14 days [10]. Biomass was separated from culture broth by centrifugation at 10000 rpm for 15 min and supernatant was used for antibacterial activity by agar well diffusion method [7]. The test microorganisms were swabbed on solidified Mueller Hinton agar (MHA) medium and wells were made with sterile cork borer (6mm). One hundred microliter of each supernatant of each *Streptomyces* isolate was loaded in well and kept in refrigerator for 1 hour for diffusion of antibacterial compound. After 1 hour Petri dish were placed in incubator at 37 °C for 24 hours.

Results and Discussion

Genus *Streptomyces* are responsible for the production of useful bioactive secondary metabolites compound, it is most valuable organism for pharmaceutical industries. After incubation, chalky, powdery and pigmented colonies were observed on SCA plate which possessed an earthy smell. 17 Streptomyces (V1-V17) were isolated from soil sample of Khairagarh and all isolates are Gram-positive [16].

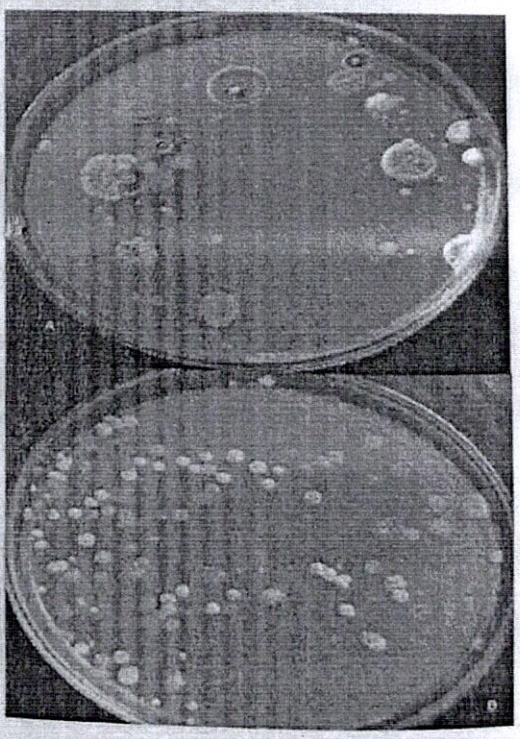

Fig. 1: Isolation of *Streptomyces* in SCA plates

TABLE- 1: primary screening of *Streptomyces* isolates for antibacterial activity by cross streak plate method.

	Gram-positive					Gram-negative				
	BS	BC	SA	LM	ML	KP	EC	PA	SE	AH
V1	++	-	+	-	-	++	-	-	-	-
V2	-	-	-	-	-	-	-	-	-	-
V3	-	-	-	-	-	-	-	-	-	-
V4	-	-	-	-	-	-	-	-	-	-
V5	+++	+++	+++	+++	+++	+++	+++	-	++	+++
V6	+++	+++	+++	++	++	+++	+	-	+	++
V7	-	-	-	-	-	-	-	-	-	-
V8	+	-	+	-	+	-	-	-	-	-
V9	+++	-	+++	++	++	+++	-	-	-	+++
V10	-	-	-	-	-	-	-	-	-	-
V11	+++	+++	+++	-	+++	-	-	++	-	++
V12	+++	+	+++	-	-	++	++	++	-	-
V13	++	-	++	-	-	-	-	-	-	-
V14	+	-	++	-	-	-	-	-	-	-
V15	+++	-	+++	-	-	-	++	-	-	++
V16	+	-	+	-	-	+	-	-	-	-
V17	+++	+++	+++	-	+++	-	-	++	-	+++

TABLE - 2: Secondary screening of isolates by well diffusion method.

Gram-positive					Gram-negative				
BS	BC	SA	LM	ML	KP	EC	PA	SE	AH
Zone inhibition in mm									
V1	15	-	16	-	-	-	-	-	-	-
V5	20	19	22	21	19	17	20	12	18	16
V6	15	11	18	17	17	18	16	-	10	17
V8	-	-	-	-	-	-	-	-	-	-
V9	20	16	19	16	15	12	-	-	-	16
V11	21	-	18	19	17	14	-	16	-	18
V12	18	-	19	-	18	-	16	16	-	-
V13	19	-	16	-	-	-	-	-	-	-
V14	18	-	15	-	-	-	-	-	-	-
V15	25	-	32	-	-	-	18	-	-	25
V16	-	-	-	-	-	-	-	-	-	-
V17	18	12	21	-	18	-	-	15	-	17

-: no activity, +: weak activity, ++: moderate activity, +++: good activity

Gram positive- BS: *Bacillus subtilis*, BC: *Bacillus cereus*, SA: *Staphylococcus aureus*, LM: *Listeria monocytogenes* and ML: *Micrococcus luteus*. Gram negative: KP: *Klebsiella pneumoniae*, EC: *Escherichia coli*, PA: *Pseudomonas aeruginosa*, SE: *Salmonella enterica* and AH: *Aeromonas hydrophila*.

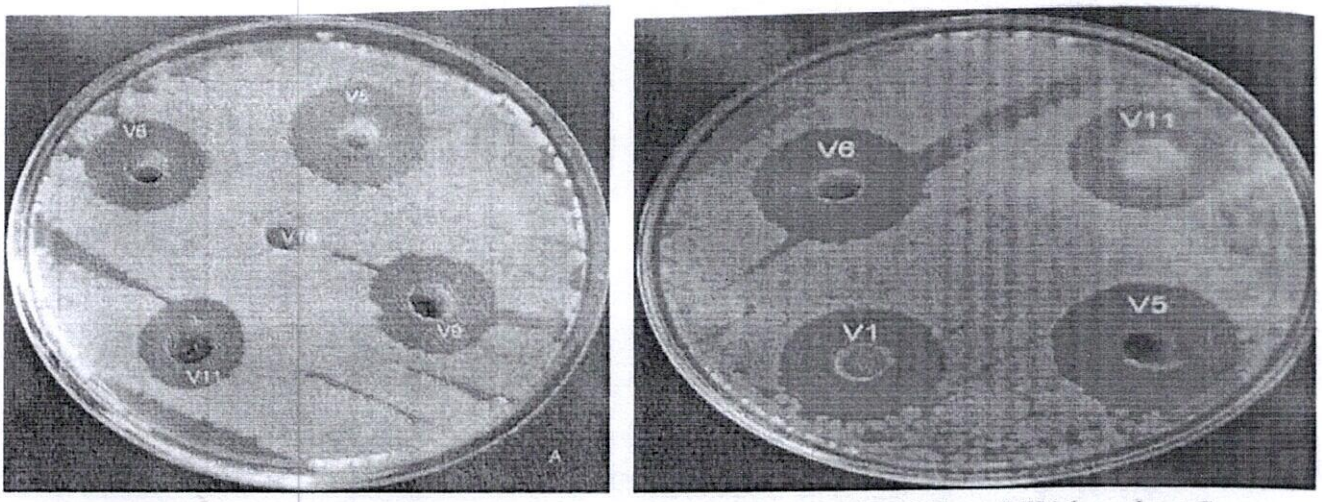

Fig. 2: Antibacterial activity of *Streptomyces isolates*, A: V5, V6, V9, V11 and V16 against *S. aureus*. B: V1, V5, V6 and V11 against *B. subtilis*.

Preliminary screening by cross streak method revealed that 12 *Streptomyces* isolates exhibited antibacterial activity against test organisms (table 1). Antibacterial isolates when subjected to secondary screening in broth using well diffusion method exhibited different activity from that of preliminary screening in Starch casein agar plate. Out of 12 only 10 *Streptomyces* isolates showed antibacterial activity while *Streptomyces* V8 and V16 were not effective in secondary screening (table 2). Similar results were observed in a study [20] in which 65 organisms showed antimicrobial activity in primary screening but in secondary screening only 52 isolates exhibited activity. V5 and V6 exhibited broad spectrum activity, V5 showed antimicrobial activity against all the test organisms and V6 inhibited all the organisms except *Pseudomonas aeruginosa*. Five isolates showed moderate activity and 3 isolates exhibited weak activity against test organisms.

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References

1. ALVAN, G., EDLUND, C. AND HEDDINI, A. (2011). The global need for effective antibiotics-a summary of plenary presentations. *Drug Resistance Updates*. **14**: 70-76.
2. AOUICHE, A., BIJANI, C., ZITOUNI, A., MATHIEU, F. AND SABAOU, N. (2014). Antimicrobial activity of saquayamycins produced by *Streptomyces* spp. PAL114 isolated from a Saharan soil. *Journal de Mycologie Medicale*. **24**: e17- e23.
3. ARASU, M.V., DURAIPANDIYAN, V., AGASTIAN, P. & IGNACIMUTHU, S. (2009). In vitro antimicrobial activity of *Streptomyces* sp. ERI-3 isolated from Western Ghats rock soil (India). *Journal de Mycologie Medicale*. **19**: 22-28.
4. BENTLEY, S.D., CHATER, K.F., CERDENO-TARRAGA, A.M., CHALLIS, G.L., THOMSON, N.R., JAMES, K.D., HARRIS, D.E., QUAIL, M.A., KIESER, H., HARPER, D., BATEMAN, A., BROWN, S., CHANDRA, G., CHEN, C.W., COLLINS, M., CRONIN, A., FRASER, A., GOBLE, A., HIDALGO, J., HORNSBY, T., RUTHER FORD, K., RUTTER, S., SEEGER, K., SAUNDERS,

D., SHARP, S., SQUARES, R., TAYLOR, AND HOPWOOD, D.A. (2002). Complete genome sequence of the model actinomycete *Streptomyces coelicolor* A3 (2). *Nature*. **417:** 141-147.
5. DEMAIN, A.L. (2006). From natural products discovery to commercial- lization: A success story. *Journal of Industrial Microbiology and Biotechnology*. 33: 486-95.
6. FISCHBACH, M.A. AND WALSH, C.T. (2009). Antibiotics for emerging pathogens. *Science*. **325**: 1089-1093.
7. GRAMMER A. (1976) antibiotic sensitivity and assay test. In: Collins, C.H. and P.N. Lyne, editors, Microbiological methods. London: *Butterworths*. 235.
8. GUST, B., CHALLIS, G.L., FOWLER, K., KIESER, T. AND CHATER, K.F. (2003). PCR-targeted *Streptomyces* gene replacement identifies a protein domain needed for biosynthesis of the sesquiterpene soil odor geosmin. Proceeding of The National Academy of Sciences of The United States of America, *100*, 1541-1546.
9. HODGSON, D.A. (2000). Primary metabolism and its control in *Streptomycetes*: a most unusual group of bacteria. *Advance in Microbial Physiology*, *42*, 47–238.
10. KUMAR, P.S., DURAIPANDIYAN, V. AND IGNACIMUTHU, S. (2014). Isolation, screening and partialpurification of antimicrobial antibiotics from soil *Streptomyces sp.* SCA7. *Kaohsiung Journal of Medical Sciences*, 1-12.
11. KUMARI, K.K, PONMURGAN, P. AND KANNAN, K. (2006). Isolation and characterization of *Streptomycetes* from soil samples for secondary metabolites production. *Biotechnology*, *5*, 478-480.
12. OSKAY, M. (2009).Antifungal and antibacterial compounds from *Streptomyces* strains. *African Journal of Biotechnology*. 8: 3007-3017.
13. PARADKAR, A.S., TREFZER, A., CHAKRABURTTY, R. AND STASSI, D. (2003). *Streptomyces* genetics: A genomic perspective. *Critical Reviews in Biotechnology*. **23**: 1–27.
14. PAYNE, D.J., GWYNN, M.N., HOLMES, D.J. AND POMPLIANO, D.L. (2007). Drugs for bad bugs: confronting the challenges of antibacterial discovery. *Nature Reviews Drug Discovery*. **6**: 29-40.
15. PROCOPIO, R.E.D.L., SILVA, I.R.D., MARTINS, M.K., AZEVEDO, J.L.D. AND ARAUJO, J.M.D. (2012). Antibiotics produced by *Streptomyces*. *The Brazilian Journal of Infectious disease*. **16**: 466–471.
16. RAMAZANI, A., MORADI, S., SOROURI, R., JAVANI, S. AND GARSHASB, M. (2013). Screening for antibacterial activity of *Streptomyces* species isolated from Zanjan Province, Iran. *International Journal of Pharmaceutical, Chemical and Biological Sciences*. **3**: 342-349.
17. SAHOO, K.C., TAMHANKAR, A.J., JOHANSSON, E. AND LUNDBORG, C.S. (2010). Antibiotic use, resistance development and environmental factors: a qualitative study among healthcare professionals in Orissa, India. *BMC Public Health*. **10**: 626-629.
18. SAISIVAM, S., BHIKSHAPATHI, D.V.R.N., KRISHNAVENI, J. AND KISHAN, V. (2008). Isolation of borrelidin from *Streptomyces californicus*-An Indian soil isolate. *Indian Journal of Biotechnology*. **7**: 349-355.
19. SPELLBERG, B., GUIDOS, R., GILBERT, D., BRADLEY, J., BOUCHER, H.W., SCHELD, W.M. (2008). The epidemic of antibiotic-resistant infections: A call to action for the medical community from the Infectious Diseases Society of America. *Clinical Infectious Disease*. **46**:155-164.
20. THAKUR, D., YADAV, A., GOGOI, B.K. & BORA, T.C. (2007). Isolation and screening of *Streptomyces* in soil of protected forest areas from the states of Assam and Tripura, India, for antimicrobial metabolites. *Journal de Mycologie Medicale*. **17**: 242-249.

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ASSESSMENT OF ANTIBACTERIAL AND SYNERGISTIC EFFECT OF *T. CORDIFOLIA* ETHANOLIC EXTRACTS WITH VARIOUS ANTIBIOTICS AGAINST THREE MTCC BACTERIAL STRAINS

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ABSTRACT

Present study focused on assessment of antibiotic resistance pattern of three MTCC bacterial strains *viz. E. coli, K. pneumoniae* & *P. aeruginosa* and analysis of synergistic potential of ethanolic extracts of *Tinospora cordifolia* with various classes of antibiotics. Antibacterial activity of antibiotics was assessed by disc diffusion method. Antibacterial effect of hot and cold ethanolic extracts was analysed by agar dilution method. Cold extraction was performed in 1:8 dilution while hot extraction was performed using 1:12 dilution of dried plant parts in ethanol. Synergistic activity of plant extracts was analysed using concurrent administration of extracts (by agar dilution method) and antibitics (Disc diffusion method). Zone of inhibition (mm) was calculated in triplicates and results were analysed by standard error, mean and ANOVA. In present study synergistic interaction among antibiotics and ethanolic extract of *T. cordifolia* Results are acceding previous findings and literature reported in Ayurveda & other primitive medicine system.

Figure: 02 References: 10 Table: 06
KEY WORDS: *E. coli, Klebsiella pneumoniae.* MDR, *Pseudomonas aeruginosa,* Synergism,

Introduction

Environmental bacterial strains are able to acclimatize themselves to unfavorable conditions & develop resistance among them. Among gram negative bacteria emergence of antibiotic resistance is increasing and thus multidrug resistance is accruing. This is a major concern of researchers now days. Prediction of antibiotic resistance among bacterial strains before they emerge at clinical settings is a novel approach for preventing super bugs and their spread endemically.

Ancient civilians treat various ailments by their knowledge of herbal formulations. Back to nature is becoming necessity for researchers so that treatment of various ailments becomes more effective, less time taking and with lesser side effects.

As every treatment has some active principle behind their activity, researchers are trying to acquaint about active ingrediants/ principle behind antibacterial activity of medicinal plants. Present study is performed to understand antibiotic resistance pattern of MTCC *E. coli*, MTCC *P. aeruginosa* and MTCC *K. pneumoniae* for eleven antibiotics and synergistic potential of ethanolic extracts of leaf, fruit, root & stem of *Tinospora cordifolia* with seven antibiotics.

Material and Methods

Preparation of crude extracts:

Fresh leaf, fruit, root and stem were washed with running tap water followed by surface sterilization with $HgCl_2$ (0.01%) solution[6]. Leaves were shade dried and pulverized to

prepare crude extract using ethanol. Sequential extraction was performed using petroleum ether, chloroform, ethanol respectively. Cold extraction was performed using 25 g plant part in 200 ml solvent while hot extraction was performed using 25 g plant part in 300 ml solvent[4,5,7,9]. Hot extraction was performed at boiling point of the respective solvent to be tested. In present study, antibacterial effect of only ethanol extracts was analyzed.

Determination of antibiotic susceptibility

Antibiotic susceptibility test of three MTCC bacterial strains was performed by kirbey bauer disc diffusion method. Eleven antibiotics *viz.* cefotaxime (CTX 30mcg), ceftriaxone (CTR 30mcg), tetracycline (TE 30mcg), chloramphenicol (C 30mcg), nalidixic acid (NA 30mcg), gentamicin (GEN 10mcg), kanamicin (K 30mcg), ampicillin (AMP 10mcg), amoxicillin (AMX 25mcg), streptomycin (S 10mcg) and erythromycin (E 15mcg) were selected from seven classes of antibiotics for screening of resistance pattern of three MTCC strains.

Assessment of synergistic/Antagonistic activity

Synergistic/Antagonistic activity of plant extract with antibiotics was assessed by agar dilution method [8,10]. Plant extract was incorporated with MHA and culture was swabbed using sterile cotton swabs. Seven antibiotic discs *viz.* cefotaxime (CTX 30 mcg), ceftriaxone (CTR 30 mcg), chloramphenicol (C 30 mcg), gentamicin (GEN 10 mcg), kanamycin (K 30 mcg), nalidixic acid (NA 30 mcg) and tetracycline (TE 30 mcg) were selected for assessment of synergistic activity against three MTCC strains.

Percentage inhibition and activity index:

a) Percentage inhibition: Percentage inhibition of various antibiotics was calculated using the following formula:-

Percentage inhibition=

$$\frac{\text{Inhibition zone in mm}}{\text{Control}} \times 100$$

Control= growth zone of bacteria *i.e.* diameter of petri-plate as growth occur all around the petri-plate.

b) Activity index: Activity index of plant extracts is determined and calculated using formula given below

Activity index=

$$\frac{\text{(Inhibition zone of extracts+antibiotics)}}{\text{Inhibition zone of standard antibiotics}}$$

Results

1. Antibacterial activity

Among eleven antibiotics tested, maximum antibacterial activity was found with chloramphenicol against MTCC *K. pneumoniae*. Among three bacterial strains tested, MTCC *P. aeruginosa* was highest resistant bacteria strain, while MTCC *K. pneumoniae* was least resistant bacteria strain. Maximum antibiotics showed highest activity against MTCC *K. pneumoniae*. While maximum resistance for amoxicillin & ampicillin followed by erythromycin (Table 1) was observed in all bacterial strains tested. Chloramphenicol was the most effective antibiotic against all bacterial strains (Fig. 1). Maximum antibacterial activity at lower concentration was observed by hot ethanol leaf extract against MTCC *P. aeruginosa* (Table 5).

2. Percentage inhibition

Maximum percentage inhibition with ceftriaxone and cefotaxime was obtained for MTCC *K. pneumoniae* followed by MTCC *E. coli* and MTCC *P. aeruginosa*. Maximum percentage inhibition with tetracycline, chloramphenicol, gentamicin and nalidixic acid was obtained for MTCC *K. pneumoniae* followed by MTCC *E. coli*. While kanamycin showed maximum percentage inhibition for MTCC *E. coli* followed by MTCC *K. pneumoniae* (Table 3).

a) **Organism wise:** Maximum percentage inhibition among six bacterial strains & seven antibiotics tested was found against MTCC *K. pneumoniae* (for five antibiotics) while least percentage inhibition was found against MTCC *P. aeruginosa*.

b) **Maximum inhibition by individual antibiotic:** Maximum inhibition of ceftriaxone, cefotaxime, chloramphenicol,

nalidixic acid and gentamicin found for MTCC *K. pneumoniae*.

c) **Most effective antibiotic:** Most effective antibiotic tested was chloramphenicol followed by ceftriaxone with maximum zone of inhibition against MTCC *K. pneumoniae*. Maximum percentage inhibition for chloramphenicol found for MTCC *K. pneumoniae* followed by MTCC *E. coli*.

3. Activity index

1) Activity index of hot and cold ethanolic extracts of *T. cordifolia* against MTCC *E. coli*: Maximum activity index observed for cold ethanol root extract with gentamicin. Kanamycin showed maximum activity index against most the extracts tested. Highest activity index among all extracts tested against MTCC *E. coli* formed with cold ethanol root extract with gentamicin (Table 4).

a) **Leaf extracts:** Highest activity index formed with cold ethanol leaf extract with gentamicin when tested against MTCC *E. coli*. Maximum extracts showed higher activity index with gentamicin.
b) **Fruit extracts:** Highest activity index formed for hot ethanol fruit extract with kanamycin followed by gentamicin. Hot ethanol fruit extract showed higher activity index with all antibiotics except chloramphenicol and nalidixic acid. Higher activity index formed with gentamicin with fruit extracts.
c) **Root extracts:** Highest activity index against MTCC *E. coli* formed with cold ethanol root extract with gentamicin. Cold ethanol root extract possess higher activity index with all antibiotics except chloramphenicol and nalidixic acid.
d) **Stem extracts:** Higher activity index observed with hot ethanolic stem extract with kanamicin and cold ethanolic stem extract with gentamicin.

2) Activity index of hot and cold extracts of *T. cordifolia* against MTCC *K. pneumoniae*: Highest activity index formed by kanamycin with hot ethanol leaf extract among all parts tested against MTCC *K. pneumoniae*.

a) **Leaf extracts:** Highest activity index against MTCC *K. pneumoniae* was formed with hot ethanol leaf extract & kanamycin. Both extracts showed higher activity index with kanamycin.
b) **Fruit extracts:** Highest activity index against MTCC *K. pneumoniae* was formed by hot ethanol fruit extract with kanamycin. Both extracts showed higher activity index with kanamycin. Hot ethanol fruit extract possess higher activity index with all antibiotics except chloramphenicol and nalidixic acid.
c) **Root extracts:** Highest activity index against MTCC *K. pneumoniae* was formed with cold ethanol root extract & kanamycin. Both extracts showed highest activity index with kanamycin. Cold ethanol root extract showed higher activity index with all antibiotics except chloramphenicol.
d) **Stem extracts:** Highest activity index against MTCC *K. pneumoniae* was formed with hot ethanol stem extract & kanamycin. Both extracts showed higher activity index with kanamycin.

3) Activity index of hot and cold extracts of *T. cordifolia* against MTCC *P. aeruginosa*: Highest activity index against MTCC *P. aeruginosa* was formed by cold ethanol root extract followed by hot ethanol root extract with kanamycin.

a) **Leaf extracts:** Highest activity index against MTCC *P. aeruginosa* was formed by cold ethanol leaf extract with all antibiotics.
b) **Fruit extracts:** Highest activity index against MTCC *P. aeruginosa* was formed with hot ethanol fruit extract & kanamycin. Hot ethanol fruit extract showed significantly higher activity index with all antibiotics except ceftriaxone and cefotaxime.
c) **Root extracts:** Highest activity index against MTCC *P. aeruginosa* was formed with cold ethanol root extract & kanamycin.
d) **Stem extracts:** Highest activity index against MTCC *P. aeruginosa* was formed with hot ethanol stem extract & kanamycin. Lowest antibiotic index with no zone of inhibition formed, by concurrent administration of cold ethanol stem extract with chloramphenicol. Hot ethanol stem extract showed

significantly higher activity index with all antibiotics except chloramphenicol and nalidixic acid.

Synergistic/ Antagonistic effect of extracts with antibiotics:

Hot and cold ethanol leaf extract exhibited significant synergistic activity against all three bacterial strains. Cold ethanol fruit extract showed antagonistic activity against MTCC *K. pneumoniae*. Hot ethanol root and stem extract exhibited significant synergistic activity against MTCC *K. pneumoniae* and MTCC *P. aeruginosa*. Cold ethanol root extract showed significant synergistic activity against all three bacterial strains. While cold ethanol stem extract contains synergistic potential against MTCC *P. aeruginosa* only. Cold extraction methods showed higher antibacterial activity with antibiotics as compared to hot extracts. Maximum synergistic combination found against MTCC *P. aeruginosa* followed by MTCC *K. pneumoniae* with cold ethanol root extract and hot ethanol root extract respectively (Fig. 2).

TABLE- 1: Antibiotic resistance pattern of three MTCC strains

S. No.	Antibiotics Class	Name of Antibiotics used	Antibiotics	MTCC *E. coli*	MTCC *K. pneumoniae*	MTCC *P. aeruginosa*
1	Cephalosporins	Cefotaxime	CTX 30	24.67	**26.67$**	13
2		Ceftriaxone	CTR30	24.33	**29.17$**	**19***
3	Tetracyclines	Tetracycline	TE 30	21	**23.33$**	10.17
4	Penicillins	Ampicillin	AMP 10	-	-	-
5		Amoxicillin	AMX 25	-	-	-
6	Others	Chloramphenicol	C 30	**25***	**30.67*$@**	14.67
7		Streptomycin	S 10	16	15	**17$**
8	Microlide	Erythromycin	E 15	-	**9$**	-
9	Quinolones	Nalidixic acid	NA 30	20.33	**22.33$**	6.33
10	Aminoglycoside	Gentamicin	GEN 10	14	**16.67$**	15
11		Kanamycin	K 30	**18.17$**	15.67	6.33

Where, * Organism wise highest activity, $ Antibiotic wise highest activity, @ Highest antibacterial activity among all antibiotics tested

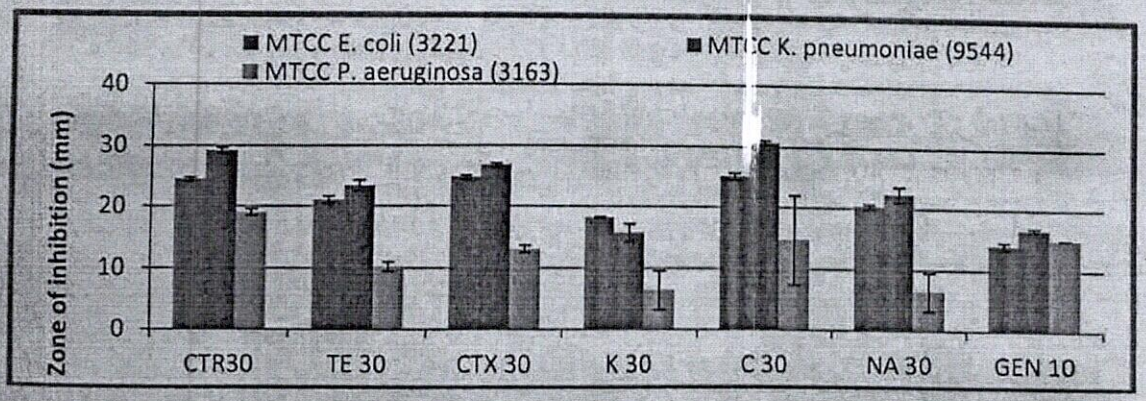

Fig. 1: Antibacterial activity (mm) and respective standard errors of seven antibiotics *viz.*

cefotaxime (CTX 30mcg), ceftriaxone (CTR 30mcg), tetracycline (TE 30mcg), chloramphenicol (C 30mcg), nalidixic acid (NA 30mcg), gentamicin (GEN 10mcg) and kanamycin (K30mcg) against three MTCC bacterial strains.

TABLE- 2: MIC of *T. cordifolia* ethanolic extracts against three MTCC bacteria

EXTRACTS	MEC	MKP	MPA
CEFE	0.50	0.50	0.13
CELE	0.13$	0.13	0.0019$
CERE	0.13$	0.13	0.13
CESE	0.50	0.50	0.016#&
HEFE	0.50	0.50	0.0078#&
HELE	0.0078$	0.0078$	0.00024*$
HERE	0.50	0.50	0.13#
HESE	0.063	0.016	0.0039#

Where, MEC= MTCC *E. coli*, MKP= MTCC *K. pneumoniae*, MPA= MTCC *P. aeruginosa*,
$ Indicates highest activity (against an organism), * Indicates highest activity among all extracts tested,

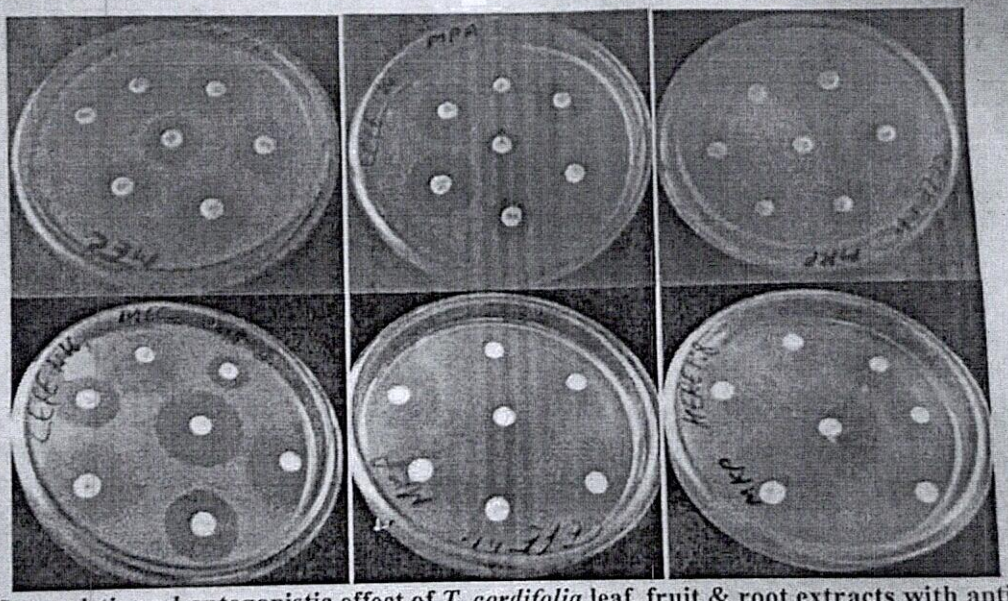

Fig.2: Synergistic and antagonistic effect of *T. cordifolia* leaf, fruit & root extracts with antibiotics against all three MTCC bacterial strains

TABLE - 3: Average percentage inhibition by antibiotics for six bacterial strains:

S. No.	Antibiotics	MEC	MKP	MPA
1	CTR30	12.17	14.58*	09.50#
2	TE 30	10.50	11.67	05.08
3	CTX 30	12.33	13.33*	06.50
4	K 30	09.08	07.83	03.17
5	C 30	12.5#	15.33*#^	07.33
6	NA 30	10.17	11.17*#	03.17
7	GEN 10	07.00	08.33*	07.50
TOTAL		73.75	82.25	42.25

Where, MEC= MTCC *E. coli* (3221), MKP= MTCC *K. pneumoniae* (9544), MPA= MTCC *P. aeruginosa* (3163), * Maximum percentage inhibition by individual antibiotic,# Maximum percentage inhibition of each bacterial strain, ^ Maximum percentage inhibition among all antibiotics tested.

TABLE - 4: Activity index of hot and cold extracts of *T. cordifolia* against MTCC *E. coli*, MTCC *K. pneumoniae* and MTCC *P. aeruginosa*

Organism	Extracts	CTR	TE	CTX	K	C	NA	GEN
MEC	HELE	1.16	1.31	**1.50$^{\sigma}$**	**1.68$^{\sigma}$**	1.03	0.89	**1.80$^{\tau}$**
	CELE	1.43	**1.37$^{\sigma}$**	1.17	1.66	1.16	0.97	**1.91$^{\sigma\tau\P}$**
	HEFE	**1.29$^{\sigma}$**	**1.23$^{\sigma}$**	**1.37$^{\sigma}$**	**1.57$^{\sigma\tau\P}$**	0.78	0.92	**1.55$^{\sigma}$**
	CEFE	1.11	1.02	0.78	1.05	0.79	0.74	**1.26$^{\tau}$**
	HERE	1.34	1.21	1.24	1.40	0.85	0.85	**1.63$^{\tau}$**
	CERE	**1.62$^{\sigma}$**	**1.29$^{\sigma}$**	**1.61$^{\sigma}$**	**1.47$^{\sigma}$**	0.95	0.90	**2.04$^{\sigma\tau\P\phi}$**
	HESE	0.99	0.98	1.02	**1.32$^{\tau}$**	0.72	0.76	1.09
	CESE	1.06	0.99	1.11	1.00	0.92	0.84	**1.27$^{\tau}$**
MKP	HELE	1.05	**1.66$^{\sigma}$**	1.12	**2.49$^{\sigma\tau\P\phi}$**	0.82	1.02	1.44
	CELE	**1.17$^{\sigma}$**	1.23	**1.26$^{\sigma}$**	**1.87$^{\tau}$**	0.92	1.05	1.54
	HEFE	**1.16$^{\sigma}$**	**1.14$^{\sigma}$**	**1.30$^{\sigma}$**	**1.47$^{\sigma\tau\P}$**	0.78	1.13	**1.36$^{\sigma}$**
	CEFE	0.54	0.89	0.63	**1.24$^{\tau}$**	0.71	0.79	1.12
	HERE	**1.17$^{\sigma}$**	1.23	1.31	**1.82$^{\tau}$**	0.90	1.04	1.54
	CERE	**1.17$^{\sigma}$**	**1.31$^{\sigma}$**	**1.35$^{\sigma}$**	**1.98$^{\sigma\tau\P}$**	0.96	**1.15$^{\sigma}$**	**1.68$^{\sigma}$**
	HESE	0.67	**1.71$^{\sigma}$**	**1.08$^{\sigma}$**	**2.31$^{\sigma\tau\P}$**	**1.06$^{\sigma}$**	0.96	**2.11$^{\sigma}$**
	CESE	0.91	0.94	1.04	**1.32$^{\tau}$**	0.73	0.85	1.09
MPA	HELE	0.84	**2.56$^{\tau}$**	1.13	2.53	1.41	2.33	1.53
	CELE	**1.83$^{\sigma}$**	**2.90$^{\sigma}$**	**2.61$^{\sigma}$**	**4.78$^{\tau}$**	**1.93$^{\sigma}$**	**3.16$^{\sigma}$**	**2.00$^{\sigma}$**
	HEFE	1.03	**2.72$^{\sigma}$**	2.16	**5.09$^{\sigma\tau\P}$**	**2.35$^{\sigma}$**	**3.55$^{\sigma}$**	**2.08$^{\sigma}$**
	CEFE	1.42	1.49	**2.38$^{\sigma}$**	**2.67$^{\tau}$**	0.00	0.00	1.55
	HERE	1.03	3.15	**2.46$^{\sigma}$**	**5.63$^{\tau}$**	2.62	**3.16$^{\sigma}$**	2.63
	CERE	**1.37$^{\sigma}$**	**3.37$^{\sigma}$**	2.12	**6.20$^{\sigma\tau\P\phi}$**	**2.68$^{\sigma}$**	2.92	**2.67$^{\sigma}$**
	HESE	**1.56$^{\sigma}$**	**3.42$^{\sigma}$**	**2.15$^{\sigma}$**	**5.06$^{\sigma\tau\P}$**	2.04	3.14	**1.95$^{\sigma}$**
	CESE	1.26	2.51	2.10	**3.10$^{\tau}$**	0.00	2.69	2.03

Where,MEC= MTCC *E. coli* (3221), MKP= MTCC *K. pneumoniae* (9544), MPA= MTCC *P. aeruginosa* (3163), ¶ Highest activity index of a plant part among all extracts, $^{\tau}$ Highest activity index of an extract, $^{\sigma}$Highest activity of an antibiotic for a plant part, $^{\phi}$ Highest activity among all extracts against an organism

TABLE - 5: Minimum Inhibitory Concentration (MIC) of *T. cordifolia* used for assessment of synergistic activity with antibiotics

Extracts	MTCC Reference Strain		
	MEC	MKP	MPA
CEFE	0.50	0.50	0.50
CELE	0.13	0.13	**0.031$^{\#}$**
CERE	0.016	**0.0078$^{\#\P}$**	**0.0078$^{\#\P}$**
CESE	0.50	0.50	**0.016$^{\#}$**
HEFE	0.50	0.50	**0.0039$^{\#\&}$**
HELE	0.0078	0.0078	**0.000061$^{\$\sigma\#\&\P\dagger}$**
HERE	0.25	0.25	**0.0039$^{\#\&}$**
HESE	0.50	**0.0039$^{\$}$**	0.0020

Where, MEC= MTCC *E. coli*, MKP= MTCC *K. pneumoniae*, MPA= MTCC *P. aeruginosa*, $^{\$}$ Highest antibacterial activity of extracts with antibiotics (for an organism), $^{\#}$ Maximum activity of individual extract, $^{\&}$ Part wise (leaf, fruit, root, stem) maximum activity

TABLE - 6: Antibacterial activity of all parts of *T. cordifolia* extracts and antibiotics against six bacterial strains (One way ANOVA mean ± Standard error)

Extracts	MTCC Strains		
	E. coli	*K. pneumoniae*	*P. aeruginosa*
HELE	**27.46±1.21^{h}**	**30.00±1.64^{a}**	18.75±0.86
CELE	28.29±0.96	29.02±0.74	**29.52±0.92^{a}**
HEFE	25.64±1.15	27.14±1.19	**27.98±0.96^{a}**
CEFE	**19.89±0.78^{a}**	18.73±0.42	**16.16±2.20^{r}**
HERE	25.04±0.97	**29.02±0.87^{a}**	**31.04±1.47^{h}**
CERE	**29.07±2.07$^{\P c}$**	**30.68±0.64$^{\P c}$**	32.11±1.55$^{\P a c \dagger}$
HESE	20.39±0.77	**30.52±1.42^{ah}**	29.07±0.86
CESE	21.43±0.81	**22.36±0.77^{a}**	20.54±1.88

Where, MEC= MTCC *E. coli*, MKP= MTCC *K. pneumoniae* and MPA= MTCC *P. aeruginosa*, ^{a} Highest ZOI for each extract, ^{r} Least zone of inhibition among all organisms, † Highest ZOI among all combination, ¶ Highest ZOI against bacteria, ^{c} Cold extraction based highest ZOI, ^{h} Hot extraction based highest zone of inhibition

Discussion

Results of antibacterial activity revealed highest resistance to ampicillin and amoxicillin followed by erythromycin while least resistance was observed for ceftriaxone. Susceptibility for gentamicin and chloramphenicol was observed in all bacterial strains tested. MTCC *P. aeruginosa* showed highest resistance with antibiotic resistance to cefotaxime, kanamycin, nalidixic acid and tetracycline. Resistance commonly observed for penicilline for three organisms tested followed by microlides. Quinolones and tetracycline resistance was observed in MTCC *P. aeruginosa*.

Hot ethanol leaf extract exhibited highest significant inhibitory potential for all organisms tested. In present study, maximum percentage inhibition was found against MTCC *K. pneumoniae* (for five antibiotics) followed by MTCC *E. coli* and MTCC *P. aeruginosa* respectively.

Most effective antibiotic tested was chloramphenicol followed by ceftriaxone against MTCC *K. pneumoniae*. Maximum activity index was formed with kanamycin followed by cefotaxime, gentamicin and tetracycline.

Highest activity index among all extracts tested against MTCC *E. coli* was formed with cold ethanol root extract with gentamicin. Highest activity index was formed by kanamycin with hot ethanol leaf extract against MTCC *K. pneumoniae*. Highest activity index against MTCC. *P. aeruginosa* was formed by cold ethanol root extract with kanamycin.

Most of the ethanolic extracts exhibited synergistic activity with all antibiotics tested. Cefotaxime and kanamycin showed synergism with maximum extracts against MTCC *P. aeruginosa*. Kanamycin showed synergistic activity with most of hot extracts of *T. cordifolia* against MTCC *K. pneumoniae*.

References

1. CLSI STANDARDS (2013) M100-S23 performance standards for antimicrobial susceptibility testing; twenty-third informational supplement, 44-64.

2. CLSI STANDARDS (2012) Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically; approved standard 9th Edition, 13-16.

3. CLSI STANDARDS (2007) M100-S17 Performance standards for antimicrobial susceptibility testing; seventeenth informational supplement. Clinical and Laboratory Standards Institute, Pennsylvania, USA, 32-38.

4. DHANABALAN, R., DOSS, A., JAGADEESWARI, M., KARTHIC, R., PALANISWAMY, M. AND ANGAYARKANNI, J. (2008) Preliminary phytochemical screening and antimalarial studies of *Spathodea campanulatum P. Beauv* leaf extracts. *Ethanobotanical leaflets*. 7: 811-819.

5. JOHNSON, M., MARIDASS, M. AND IRUDAYARAJ, V. (2008) Preliminary phytochemical and antibacterial studies on *Passiflora edulis*. *Ethanobotanical Leaflets*, 425-432.

6. NAHUNNARO, H. (2008) Effects of different plant extracts in the control of Yam induced by *Rhizopus stolonifer* on stored Yam (*Dioscorea* Sp.) in Yola, Adamawa State Nigeria. *Agricultural Journal*, 382-387.

7. NAIR, R., KALARIYA, T. AND SUMITRA, C. (2005) Antibacterial activity of some selected Indian medicinal flora. *Turkish Journal of Biology,* 41-47.

8. SHANTHI, V. AND NELSON, R. (2013) Antibacterial activity of *Tinospora cordifolia* (Willd) Hook. F. Thoms on urinary tract pathogens. *International Journal of Current Microbiology and Applied Sci*ences, 190-194.

9. SHARMA, A. AND PATEL, V.K. (2009) *In vitro* screening of the antibacterial activity and identification of bioactive compounds from plants against selected *Vibrio* spp. pathogens. *Turkish Journal of Biology*, 137-144, 704-706.

10. ZHANG, S.X., RAWTE, P., BROWN, S., LOV, S., SIEBERT, H., PONG-PORTER, S., LOW, D.E. AND JAMIESON, F.B. (2011) Evaluation of CLSI agar dilution method trek sensititre broth microdilution panel for determining antimicrobial susceptibility of *Streptococcus pneumoniae*. *Journal of Clinical Microbiology*. 704-706.

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ISOLATION AND CHARACTERIZATION OF ANTIBACTERIAL ACTINOMYCETES FROM SOILS OF BASTAR BLOCK OF CHHATTISGARH

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ABSTRACT

In the present work, a total of 32 actinomycetes were isolated from soil samples of Bastar block of Chhattisgarh by serial dilution and spread plate method on starch casein agar & actinomycetes isolation agar. These actinomycetes were screened for their antibacterial activity by agar well diffusion method against 6 bacterial pathogens obtained from Microbial type Culture collection and Gene Bank (MTCC) IMTECH, Chandigarh. All actinomycetes isolates were morphologically distinct on the basis of colour of substrate and aerial mycelium and diffusible pigment. Among all actinomycetes isolates, thirteen isolates showed antibacterial. Out of 13 isolates, 8 isolates (61.5.%) exhibited antibacterial activity against at least one of the tested pathogenic bacteria, 4 isolates (30.7%) exhibited antibacterial activity against three gram positive bacterial pathogens and only 1 isolate had shown broad spectrum antibacterial activity against all the gram positive & gram negative bacterial pathogens used in this study. On the basis of morphological and biochemical characters, all actinomycetes were found belonging to *Streptomyces* genera.

Figures: 02 References:26 Tables:02
KEY WORDS: Actinomycetes, Antibacterial activity, Pathogens

Introduction

Actinomycetes are prokaryotic spore forming gram positive bacteria with high G + C content in their DNA ranging from 51% in *Corynebacteria* to more than 70% in *Streptomyces* and *Frankia*[22]. They are free living, saprophytic bacteria and due to their filamentous nature, branching pattern and conidia formation as similar as fungi they are also known as ray fungi[1]. They are most widely distributed group of microorganisms in nature and due to their diversity and ability for the production of most of the discovered bioactive secondary metabolites, mainly antibiotics [7,19], enzymes[11], enzyme inhibitors[16], immunosuppressive agents[5], holds a prominent position as targets in screening programs. The class Actinobacteria includes some of the resilient species which can easily adapt in various conditions making them capable of growing in extreme, hostile and polluted environment and because of this adaptability, they are renowned in medicinal and industrial fields[2]. There is great variation in selective antimicrobial activity of actinomycetes, both qualitative and quantitatively, which could be demonstrated through their antibiotic spectra[23]. The rate of discovery of new species and new compounds from common species of actinomycetes has declined, therefore, improved methodologies for isolating the uncommon and rare actinomycetes from the diverse habitats is required to avoid the re isolation of same strains and to improve the quality of natural products screened[5,20]. The present study is designed to isolate the potential actinomycetes from forest soils of Bastar region and screening them for their antibacterial activity against bacterial pathogens.

Material and Methods

Collection of soil samples

Three soil samples were collected randomly[25] from forest area of Bastar region, from 10 - 15 cm depth, transported to laboratory in sterile polythene bags, where the soil samples were meshed, sieved and air dried at room temperature for 7 days and treated by wet heating, dry heating method[8] and 1.5% phenol[9].

Isolation of Actinomycetes

Pre-treated soil samples were subjected to serial dilution in distilled water and spread on starch casein agar and actinomycetes isolation agar containing nystatin at 50 ug/ml [24] and incubated at 28° C for minimum three weeks. Plates were observed regularly till appearance of actinomycetes colonies. Morphologically distinct colonies were subcultured on respective media and pure cultures were maintained on starch casein agar slants and preserved at 4°C.

Screening of Actinomycetes isolates for antibacterial activity:

The actinomycetes isolates were grown in 250 ml Erlenmeyer flask containing 50 ml starch casein broth and incubated at 28°C for 7 days. After incubation, the mycelium was separated from the culture broth by centrifugation at 15,000 rpm for 30 min. The resulting supernatant was then used for antibacterial activity by agar well diffusion method[4] on Muller Hinton agar plates previously seeded with test organisms.

Test organisms

Antibacterial activity of actinomycetes were tested against 6 bacterial pathogens, three gram-positive bacteria such as, *Staphylococcus aureus* (MTCC 7443), *Bacillus cereus* (MTCC 6909) and *Bacillus subtilis* (MTCC 1789) and three gram-negative bacteria such as *Escherichia coli* (MTCC 3221), *Pseudomonas aeruginosa* (MTCC 3163) and *Klebsiella pneumoniae* (MTCC 9544) obtained from Microbial Type Culture Collection & Gene Bank (MTCC), IMTECH, Chandigarh. The antibacterial activity was observed after 24hr of incubation at 37°C, by measuring the size (diameter in mm) of zone of inhibition[12].

Morphological & biochemical characterisation of potential Actinomycetes isolates

The potent actinomycetes isolates showing antibacterial activity were studied for morphological, physiological and biochemical characteristics by following the methods described in International Streptomyces project[18]. The micro-morphology of isolates, mycelia Structure, spore arrangement were observed through light microscopy at 1000X resolution. The cultural characters, pigmentation of aerial mycelium, structure of sporophores were observed by cultivation the actinomycetes isolates on different ISP mediums such as ISP 2, ISP 4, ISP 5, ISP 6 & ISP 7[13]. Biochemical tests, such as, catalase test, nitration reduction, citrate utilization, starch hydrolysis, gelatine hydrolysis, lipid hydrolysis, urea hydrolysis, H_2S production, were performed for characterization of actinomycetes isolates.

Results and Discussion

Present study focuses on isolation of potent antibacterial soil actinomycetes. Total 32 distinct actinomycetes were isolated from forest soils of Bastar region of Chhattisgarh (Fig.1). Among them, only 13 isolates exhibited antibacterial activity. Out of these 13 isolates, 8 isolates exhibited antibacterial activity against at least one of the tested pathogenic bacteria, 4 isolates exhibited antibacterial activity against 3 tested bacterial pathogens and only 1 isolate exhibited antagonistic activity against all the 6 tested bacterial pathogens. The antibacterial activity analyzed by measuring the diameter of zone of inhibition by culture filtrate of 13 actinomycetes isolates is represented in Table No. 1. In the present study, results indicated that, the isolate BS 46 was most potent actinomycetes isolate which exhibited broad spectrum antibacterial activity against all the gram positive and gram negative pathogenic bacteria used in this study. The isolates, BS 23, BS 27, A 1, A10 & SCA 1 had shown antibacterial activity against all gram positive pathogens (Fig. 2.)

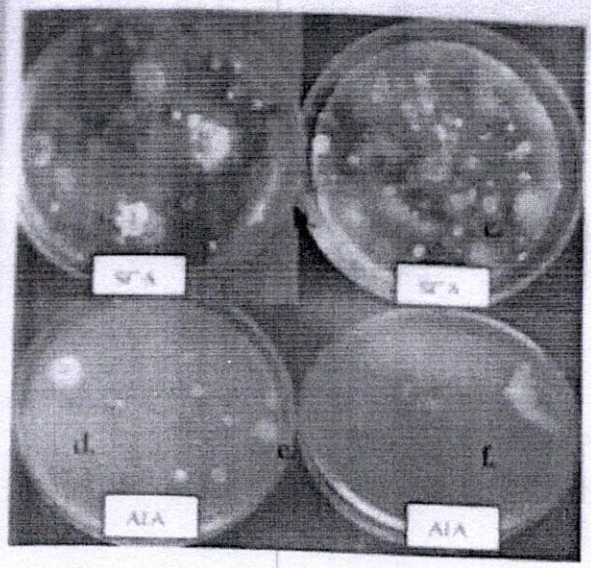

Fig.1: Isolation plates showing Actinomycetes colonies on Starch Casein Agar & Actinomycetes Isolation Agar

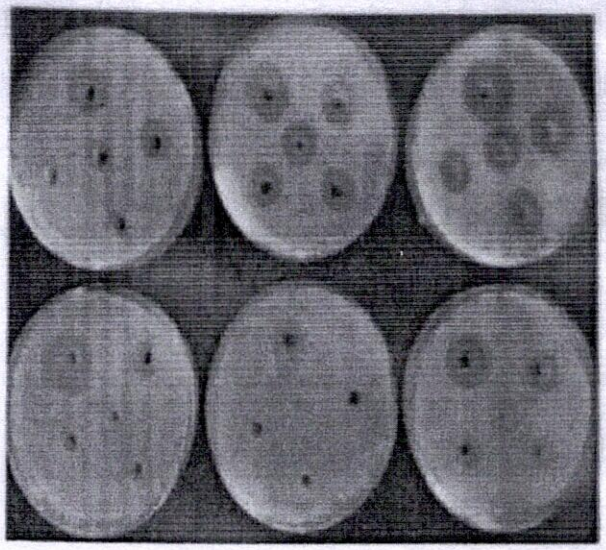

Fig.2: Antibacterial activity of actinomycetes by agar well diffusion method against bacterial pathogens a). *B.cereus*, b). *B. Subtilis*, c). *S. auerus*, d). *E. Coli*, e). *P. aeruginosa* f). *K. pneumoniae*.

Most of the actinomycetes isolates had shown good antibacterial activity against *Staphylococcus aureus* & *Bacillus subtilis*. The identification of actinomycetes isolates was carried out by using morphological and biochemical characters shown in Table No. 2. All Actinomycetes were gram positive when subjected to gram staining. In the microscopic observation at 1000X all isolates had shown filamentous, branched and long spore chain bearing structures. In spore staining, B2, B4, BS46, A1, B11, RG & SCDM had shown spiral shaped spore chain while the isolates BS23, BS27, BI3, A10, A11 & SCA1 were having rectiflexible spore chains. The isolates, BI 1, BI 4, A1 and SCDM had grey color aerial and substrate mycelium. The isolate RG had white colour aerial mycelium and red colour substrate mycelium. Diffusible pigments were produced by B2, B4, BS27, BS46 & A1 on starch casein agar plates. The pigment producing soil actinomycetes had been found efficacious producers of antimicrobial compounds against various Multi Drug Resistant pathogens[17]. On the basis of morphological and biochemical characters all actinomycetes isolates were found to be belonging to *Streptomyces* genera[18].

TABLE- 1: Antibacterial activity of Actinomycetes isolated from soils of Bastar block

Test Organisms	Actinomycetes isolates/ Zone of Inhibition (diameter in mm)												
	B2	B4	BS23	BS27	BS46	BI3	A1	A10	A11	SCA1	RG	SCDM	B11
B. cereus (MTCC	-	-	20	-	28	-	26	24	-	19	-	-	-
B. subtilis (MTCC 1789)	24	-	23	-	30	-	20	22	-	25	20	-	-
S. aureus (MTCC 7443)	-		22	-	32	-	21	27	-	24	-	19	22
E. coli (MTCC 3221)	-	-	-	20	27	-	-	-	16	-	-	-	-
P. aeruginosa (MTCC 3163)	-	-	-	-	19	-	-	-	-	-	-	-	-
K. pneumoniae (MTCC 9544)	-	20	-	-	24	22	-	-	-	-	-	-	-

TABLE- 2: Morphological & biochemical characteristics of potent actinomycetes

Characteristics	Actinomycetes isolates												
	B2	B4	BS23	BS27	BS46	BI3	A1	A10	A11	SCA1	RG	SCDM	B11
Aerial mycelium color	Grey	Grey	Cream	Cream	White	Grey	Grey	Black	Orange	White	White	Grey Black	Yellow Pink
Substrate mycelium color	Grey	Grey	Pink	Cream	Cream	Grey	Grey	Black	Cream	Cream	Red	Grey	Cream
Spore chain spiral	+	+	-	-	+	-	+	-	-	-	+	+	+
Spore chain Rectiflexible	-	-	+	+	-	+	-	+	+	+	-	-	-
Diffusible pigment	+	+	-	+	+	-	+	-	-	-	-	-	-
Gram stain	+	+	+	+	+	+	+	+	+	+	+	+	+
Catalase test	+	+	+	+	+	+	+	+	+	+	+	+	+
Citrate utilization	-	-	+	+	+	+	-	+	+	+	-	+	-
Nitrate reduction	+	-	-	+	+	-	+	-	-	+	+	+	-
Starch hydrolysis	+	+	+	+	+	+	+	+	+	+	+	+	+
Lipid hydrolysis	+	+	+	+	+	-	+	+	-	+	+	-	-
Gelatine hydrolysis	-	+	-	-	-	+	+	-	+	-	-	-	+

For the screening of potential Actinobacteria, less than one part of Earth's surface has been explored and approaches for exploration of random and exotic habitats would accelerate the production of bioactive substances from the Actinobacteria[3]. The present study revealed the presence of potential actinomycetes showing antibacterial activity in the soil samples of Bastar block of Chhattisgarh. The result of antibacterial screening revealed that most of the potential actinomycetes were effective against gram positive bacteria while few of them had shown activity against gram negative bacterial pathogens used in this study. One actinomycetes isolate (BS 46) had shown broad spectrum antibacterial activity. Improved isolation & screening methods on soils of diverse habitats should be applied to isolate potential bioactive actinomycetes[21]. Potent antibiotics from actinomycetes of diverse habitats could be beneficence to fight against antibiotic resistant pathogens[6]. There are several reports on dominance of *Streptomyces* genera in forest soils, but if they were properly screened and optimized under cultural condition, they could be prominent source for production & purification of antimicrobial compounds[10,15]. The presence of potential *Streptomyces* in soil largely depends upon texture and culture condition of soil[14].

Conclusion

From the present study it could be concluded that the soils of Bastar block of Chhattisgarh possess large group of antibiotic producing actinomycetes. The potent actinomycetes obtained in this study could be further studied and used as a source for production and purification of bioactive antibiotics.

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References

1. BALAKRISHNA, G., SHIVA SHANKAR, A. AND PINDI, P. K. (2012) Isolation of phosphate solubilizing actinomycetes from forest soils of Mahabubnagar district. *IOSR Journal of Pharmacy*. **2**: 271- 275.
2. BALLAV, S., DASTAGER, S. G. AND KEKAR, S. (2012) Biotechnological significance of Actinobacteriological research in India. *Recent Research in Science and Technology*. **4**: 31-39.
3. BALTZ, H. R. (2007) Antimicrobials from actinomycetes. back to future. *Microbiology*. **2**: 125-131.
4. BARRY, A. L. AND THORNSBERRY, C. (1985) Susceptibility tests: Diffusion test procedures. In : Lennette EH, Balows, A., Hausler Jr, W. J., Shadomy. H. J., eds. Manual of Clinical Microbiology. 4th ed, Washington, D.C.: *American Society Microbiology*, 978–987.
5. BERDY, J. (2005) Bioactive microbial metabolites. A personal view. *Antibiotics*. **58**: 1- 26.
6. BIZUYE, A., MOGES, F. AND ANDUALEM, B. (2013) Isolation and screening of antibiotic producing actinomycetes from soils in Gondar town, North West Ethiopia. *Asian Pacific Journal of Tropical Disease*. **3**: 375–381.
7. DASARI, V.R.R.K., MUTHYALAB, M.K.K., DONTHIREDDY, S.R.R. AND NIKKUA, M.Y. (2012) Novel Pyridinium compound from marine actinomycete, *Amycolatopsis alba* var. nov. DVR D4 showing antimicrobial and cytotoxic activities in vitro. *Microbiological Research*. **167**: 346-351.
8. HAYAKAWA, M., SADAKATA, T., KAJIURA, T. AND NONOMURA, H. (1991) New methods for the highly selective isolation of *Micromonospora* and *Microbispora* from soil. *Journal of Fermentation and Bioengineering*. **72**: 320–326.
9. HAYAKAWA, M., OTOGURO, M., TAKEUCHI, T., YAMAZAKI, T. AND IIMURA, Y. (2000) Application of a method incorporating differential centrifugation for selective isolation of
10. motile actinomycetes in soil and plant litter. *Antonie Van Leeuwenhoek*. **78**: 171–185.
11. HOBBS, GFRAZER, C.M., GARDNER, C.J.F. FLETT, D. AND OLIVER, S.G. (1990) Pigmented antibiotic production by *Streptomyces coelicolor* A3(2): kinetics and the influence of nutrients. *Journal of General Microbiology*. **136**: 2291–2296.
12. LAM KIN, S. (2006) Discovery of novel metabolites from marine actinomycetes. *Current Opinion in Microbiology* **9**: 245- 251.
13. MADIGAN, M. T., MARTINKO, J. M. AND PARKER, J. (1997) Antibiotics: isolation and characterization. In: Brock Biology of Microorganisms, 8th (eds), *Prentice-Hall International Inc. New Jersey*, 440-442.
14. PEELA, S., BAPIRAJU KURADA, V.V.S.N.B. AND TERLI, R. (2005) Studies on antagonistic marine actinomycetes from the Bay of Bengal. *World Journal of Microbiology & Biotechnology*. **21**: 583–585
15. RAI, M., BHATTARAI, N., DHUNGEL, N., AND MANDAL, P. K. (2016) Isolation of antibiotic producing Actinomycetes from soil of Kathmandu valley and a ssessment of their antimicrobial activities. *International Journal of Microbiology and Allied Sciences (IJOMAS)*. **2**: 22-26.
16. SANGHVI, G.V., GHEVARIYA, D., GOSAI, S., LANGA, R., DHADUK, N., KUNJADIA, P.D., VAISHNAV, D.J., AND DAVE, G.S. (2014) Isolation and partial purification of erythromycin from alkaliphilic *Streptomyces werraensis* isolated from Rajkot, India. *Biotechnology Reports*. **1**: 2-7.

17. SATHIYASEELAN, K. AND STELLA, D. (2012) Isolation and screening of α-glucosidase enzyme inhibitor producing marine actinobacteria isolated from Pichavaram mangrove. *International Journal of Pharmaceutical & Biological Archives*. **3**: 1142-1149.
18. SELVAMEENAL, L., RADHAKRISHNAN, M. AND BALAGURUNATHAN, R. (2009) Antibiotic Pigment from Desert Soil Actinomycetes; Biological Activity, Purification and Chemical Screening. *Indian Journal of Pharmaceutical Sciences*. **71:** 499–504.
19. Shirling, E. B., AND Gottlieb, D. (1966) Methods for characterization of *Streptomyces* species. *International Journal of Systemic Bacteriology*. **16:** 313-340.
20. SINGH, N., RAI, V., AND TRIPATHI, C.K.M. (2012) Oxytetracyclin production by immobilized cells of a new isolate of *Streptomyces rimosus* MTCC 10792. *Journal of Pharmacy Research*. **5**: 2477- 2480.
21. TAKAHASHI, Y., AND OMURA, S. (2003) Isolation of new actinomycete strains for the screening of new bioactive compounds. *The Journal of General Applied Microbiology*. **49**: 141-54.
22. VELHO- PEREIRA, S. & KAMAT, N. M. (2013) Actinobacteriological research in India. *Indian Journal of Experimental Biology*. **15**: 573-596.
23. VENTURA, M., CANCHAYA, C., TAUCH, A., FITZGERALD, G. F., CHATER, K. F., VENTURA, M. AND SINDEREN, VAN.D. (2007) Genomics of actino bacteria,tracing the evolutionary history of an ancient phylum genomics of actinobacteria. *Microbiology and Molecular Biology Reviews*. **71**: 495- 548.
24. WAKSMAN, S. A., SCHATZ, A. AND REYNOLDS, D. M. (2010) Production of antibiotic substances by actinomycetes. *Annals Of The New York Academy Of Sciences*, 1213 Issue: *Antimicrobial Therapeutics Reviews*. 112–124.
25. WILLIAMS, S.T. AND DAVIES, F.L. (1965) Use of antibiotics for selective isolation and enumeration of Actinomycetes in soil. *Journal of General Microbiology*. **38:** 251- 261.
26. WOLLUM, A.G. (1994) Soil sampling for microbiological analysis. In "Methods of Soil Analysis," Part 2, "Microbiological and Bio-chemical Properties." SSSA Book Series No.5. *Soil Science Society of America, Madison*, WI: 2-13.

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ISOLATION AND SCREENING OF LACCASE PRODUCING FUNGI FROM COCONUT WASTE SOIL

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ABSTRACT

Laccase (p-diphenol: Oxygenoxireductase EC 1.10.3.2) enzyme degrade many phenolic compounds. It is found in fungi, higher plants, bacteria and insects. Deuteromycetes, Ascomycetes, Basidiomycetes and many white-rot fungi contain laccase. This study describes the isolation and screening of laccase producing fungi from coconut waste soil sample. Thirteen fungi were isolated from soil sample by serial dilution technique on Potato Dextrose Agar (PDA) and qualitatively screened for their laccase producing ability on guaiacol containing potato dextrose agar medium. Of these, three isolates were laccase producers. Guaiacol is a compound indicator for laccase producing fungi. Three isolates had produced laccase qualitatively by forming reddish brown zone around colony and two isolate had shown maximum quantitative laccase production in Potato dextrose broth.

Figures: 02 References:13 Table:01
KEY WORDS: Guaiacol, Indicator, Laccase, Qualitatively, Quantitatively

Introduction

Laccase was discovered in the effluence of Japanese lacquer tree *Rbus vernicifera* (Yoshida, 1883). It is broadly distributed in fungi, higher plants, bacteria and insects. Deuteromycetes, Ascomycetes, Basidio-mycetes and many white-rot fungi contains laccase. It is divided into three types, type I, type II and type III. Each type of laccase plays an important role in the degradation of lignin[7]. Laccase belong to multi-copper oxidase family[1,8]. Laccase oxidizes only lignin model compounds (with free phenolic group). It is first ligninolytic enzyme secrete by fungi surrounded in the medium. ABTS [2, 2-azino-bis-(3- ethylbenzothiazoline-6-sulphonic acid)] are firstly described laccase mediator [4,5]. Fungal laccase are multinuclear, extracellular, monomeric glycoproteins with 10-20% of carbohydrate contents [11]. It requires oxygen as a second substrate for enzymatic action. It is capable to catalyze the ring cleavage of aromatic compounds[6]. Over 60 fungal strains of phyla especially Basidiomycota, Ascomycota and Zygomycota show laccase activities[3,9].

Material and Methods

The reagent grade chemicals Potato Dextrose Agar, Potato Dextrose Broth, Guaiacol, Sodium Acetate Buffer and Streptomycin were procured from Hi-Media, Mumbai.

Collection of soil sample and isolation of fungi

Sample of coconut waste soil was collected in sterile plastic bag from DPS Chowk Bhilai, District Durg (C.G.) and subject to isolation of

fungi by serial dilution method. 1 gram of soil sample was added into 10ml sterile water and mixed. The suspension was serially diluted 10^{-1} to 10^{-5} dilution. After dilution 1ml of each dilution was spread on sterile plate and adds Potato Dextrose Agar medium which containing 0.01% streptomycin poured in plates and plates rotate clock-wise and anti-clock wise.
Streptomycin was used for inhibition of bacterial contaminants. After solidification of medium plates were incubated at 28°C for 7 days[12].

Qualitative screening
The fungal strains were inoculated in 0.01% Guaiacol containing Potato Dextrose Agar plates. The plates were incubated at 30°C for 7 days. After 7 days the laccase producing fungal strain showed reddish brown color zones around the fungal colony. Positive fungal culture was taken for the quantitative estimation[10].

Quantitative screening
For quantitative screening positive fungal cultures were carried out in Erlenmeyer flask (250ml) which containing 100ml Potato Dextrose Broth. Flasks were incubated at room temperature for 15 days.

Extracellular enzyme activity
After incubation crude extract were filtered in Whattman no 1 filter paper. Laccase activities were assayed by using 10mM Guaiacol and 100mM sodium acetate buffer (pH 5.0) at room temperature. The reaction mixture contains 1ml Guaiacol, 3ml sodium acetate buffer and 1ml enzyme source. Reaction mixtures were incubated at 30°C for 10 minutes. Laccase activity is measured U/ml that define the amount of enzyme production one micromole colored product in per minute per ml. The absorbance of reaction mixture was monitored at 470 nm by using UV Spectrophotometer[1,2].

$$\text{Volume activity (U/ml)} = \frac{\Delta A470nm/min \times 4 \times Vt \times \text{dilution factor}}{\epsilon \times Vs}$$

Calculation
Where,
Vt = final volume of reaction mixture (ml) = 5.0
Vs = sample volume (ml) = 1
ϵ = extinction co-efficient of Guaiacol = 6,740/M/cm
4 = derived from unit definition & principle

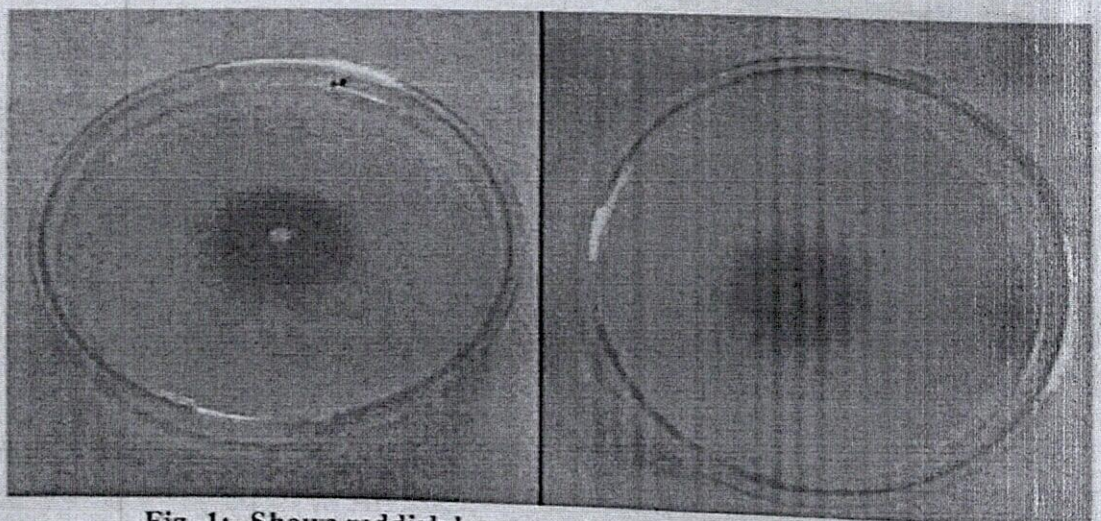

Fig. 1:- Shows reddish brown color zone around the plates

TABLE - 1: Qualitative screening of the isolated fungal strains

S. No.	Isolates	Guaiacol oxidation
1.	CWA	-
2.	CWB	-
3.	CWC	-
4.	CWD	++
5.	CDE	-
6.	CWF	+++
7.	CWG	+++
8.	CWH	-
9.	CWI	-
10.	CWJ	-
11.	CWK	-
12.	CWL	-
13.	CWM	-

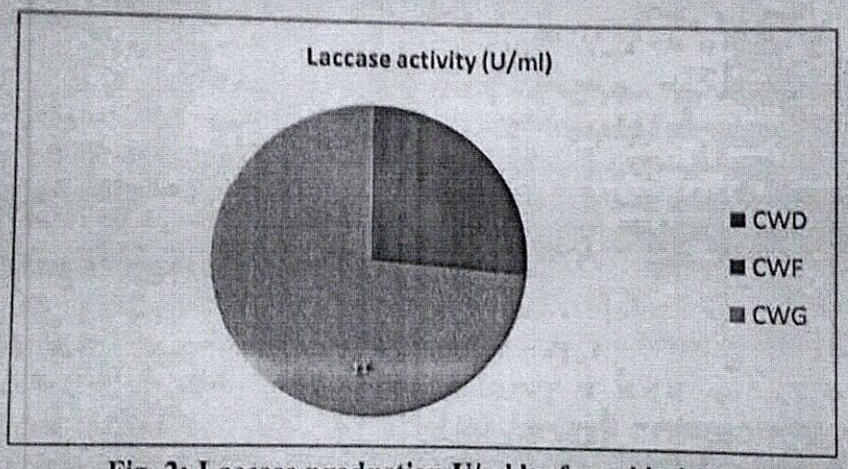

Fig. 2: Laccase production U/ml by fungal isolates

Result and Discussion

The present study mainly targeted to laccase producing fungi from coconut waste soil sample. Thirteen fungal colonies were isolated from soil sample. Potato Dextrose Agar medium were used for maintain of fungal isolates. They were screened for their potential laccase producing ability using Guaiacol as an indicator. All isolates were inoculated in PDA plates which contain 0.01% Guaiacol. Plates were incubated at 30^{0}C for 7 days. After 7 days of incubation reddish brown colored zone was produced around the fungal colony in the medium. It shows positive reaction for production of laccase enzyme[10]. Three fungal isolates shows positive reaction with Guaiacol. Screening test results are shown in the Table No1. Positive cultures were taken for quantitative screening of laccase. All positive fungal cultures were inoculated in Potato Dextrose Broth. After 15 days of incubation, culture filtrate was taken from flask for analyzing their laccase activity. CWF and CWG showed maximum laccase activity 0.289 U/ml and 0.983 U/ml respectively by spectrophotometrically analysis of culture filtrate with Guaiacol (substrate) and sodium acetate buffer solution[1]. Quantitative laccase activity results are shown in Graph No. 1.

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References

1. ADIVEPPA, B. V. AND BASAPPA, B. K. (2015) Isolation, screening and identification of laccase producing fungi. Inter. *Journal of Pharma and Bio Science*. **6**: 242-250
2. ALCALDE, M. (2007) Laccase: biological functions, molecular structure and industrial applications In: Industrial enzymes: structure, function and applications Netherlands-Springer **26:** 461-476
3. BALDRIAN, P. (2006) Fungal laccase-occurrences and properties. *FEMS Microbiology Reviews*. **30:** 215-242.
4. BOURBONNAIS, R. AND PAICE, M. G. (1990) Oxidation of non-phenolic substrates: An expanded role of laccase in lignin biodegradation. *FEBS Letters*. **267**: 99-102.
5. CALL, H. P. AND MUCKE, I. (1997) History, overview and applications of mediated lignolytic systems especially laccase-mediator-systems (Lignozyme ® process) *Journal of Biotechnology*. **53**: 163-202.
6. CLAUS, H. (2004) Laccases: Structure, reaction, distribution. *Micron*. **35**: 93-96.
7. DEDEYAN, B. KLONOWSKA, A. TAGGAR, S. TRON, T. LACAZIO, G. GIL, G. AND PETIT, J. L. (2000) Biochemical and molecular characterization of a laccase from *Marasmius quercophilus*. *Applied and Environmental Microbiology*. **66**: 925-929.
8. HOEGGER, P. J. KILARU, S. JAMES, T. Y. THACKER, J. R. AND KUES, U. (2006) Phylogenetic comparison and classification of laccase and related multicopper oxidase protein sequences. *The FEBS Journal*. **273**: 2308-2326.
9. KIISKINEN, L. L. RATTO, M. AND KRUUS, K. (2004) Screening for novel laccase-producing microbes *Journal of Applied Microbiology*. **97**: 640-646.
10. MANIKANDAN, D. AND SHOBA, F. G. (2013) Isolation and microbial depiction of laccase producing stains from various geographical areas in Tamil Nadu, India *International Journal of Advances in Interdisciplinary Research*. **1**: 21-27.
11. MAYER, A. M. AND STAPLES, R. C. (2002) Laccase: New functions for an old enzyme. *Phytochemistry*. **60**: 551-565.
12. WAKSMAN, S. A. (1922) A method of counting the number of fungi in the soil. *Journal of Bacteriology*. **7**: 339-341.
13. YOSHIDA, H. (1883) Chemistry of lacquer (Urusbi) part 1. *Journal of the Chemical Society*. **43**: 472-486.