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ANTIMICROBIAL ACTIVITY OF CELL FREE EXTRACT OF *PSEUDOMONAS AREOGINOSA* MTCC 741 TOWARDS OPPORTUNISTIC HUMAN PATHOGENS

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ABSTRACT

In this era of antibiotic resistance researchers have found that secondary metabolite of many microorganisms have antimicrobial activity and can be used in treating various diseases when chemotherapeutic agents fail to do so. Similarly, our investigation was on in vitro antimicrobial action of secondary metabolite of Pseudomonas areoginosa MTCC 741 (MTCC741) on various opportunistic pathogens. The antimicrobial assay showed 200 µl of cell free MTCC 741 has maximum antibacterial activity on all the test microorganisms, with maximum zone of inhibition on fungus, Aspergillus niger 31.0`±0.8 followed by bacterium Staphylococcus aureus 31.0±2.6. It is also found that 200µl of the extract inhibit E.coli (30.0`±0.8) and Staphylococcus epidermides MTCC 9041(25.33±2.73). The biochemical analysis of the sample showed the presence of 20.56± 2.3 BSA mg/ml protein, 23.24± 2.5 GAE mg/ml phenolics and flavonoids 30.0 ± 0.99 Q Emg/ml. Further, HPLC analysis of the sample confirm presence of 28.24± 2.5 GAE mg/ml phenolics , flavonoids 31.09 ± 0.99 QEmg/ml along with 2.67mg/ml of phycocyanin and 2.5µg/ml of phennazine. It is also see that this compound has antioxidant property with GSH 6.3 ± 0.33µM and 90.2% Radical Scavenging Activity. Thus, all the above compounds like phenolics, flavonoids, phycocyanin, phennazine along with GSH and Radical Scavenging Activity had aid to the antimicrobial activity of this compound and therefore can be used as an antimicrobial agent to these test microorganisms or any other pathogens when traditional chemotherapeutic agents fail to do so.

KEY WORDS

Antimicrobial, antioxidants, metabolite, bioactive, Pseudomonas areoginosa, HPLC

INTRODUCTION

In today's world most the clinical use of these antibiotics is major catalyst for the improvement of human diseases. This effectiveness started decreasing from late 90's and from the beginning of 21st century medical practitioner faced a new problem in treating infections was "antibiotic resistance."Antibiotic resistance is nothing but ability of a bacterium to destroy the effectiveness of an antibiotic and effectively survive in the human body[1]. Moreover, antibiotic resistance evolves naturally because of the morphological structure of the bacteria[2] or may be due random mutation of the genome[3] or may be due to ability the microorganisms to gain a morphological structure during a stress that resists access of antibiotic or drugs to destroy the microorganisms [4]. Notably, Methicillin-resistant *Staphylococcus aureus* (MRSA)and Vancomycin-intermediate *S. aureus* (VISA) strains have a thickened cell wall that is

believed to deplete the vancomycin available to kill the bacterium[5]; this mechanism of resistance would significantly impact the near future prospects of the current anti-MRSA therapies[6]. Moreover, drug resistance not only taking a toll of human life but also increased the cost of treatment not in the developing country like India but also it was seen that there was total cost per case of bacteremia that was caused by an antibiotic-resistant strain, including MRSA (50% of the cases), was US$ 88,445 [7].So, this has forced many researchers to have focused their research on natural antioxidant obtained from plant[8] or other microorganisms or no various medicine[9] that can enhance efficiency of various antibiotics. But, usage of antioxidants combined with antibiotics sometime found to decreases the efficiency of antibiotics[10]. This demanded the use of natural compounds or secondary metabolites obtained from microorganisms like Gram negative *Pseudomonas* and *Streptomyces.* Secondary metabolites are nothing but small organic compounds (molecular masses generally less than 3000 Da), which, as opposed to primary metabolites have no function in the life cycle of cells. These compounds can inhibit antibiotic degrading enzymes, as well as certain enzyme activities in human metabolism that cause illness [11]. This demanded isolation of antibacterial compound from these bacteria and test them on various drug resistance organisms. It is seen that the characteristic feature of *Pseudomonas aeruginosa* is the production phycocyanin pigment, a water soluble blue green nitrogen-containing tricyclic molecules phenazines compound with antibiotic, antitumor, and antiparasitic activities [12]. This prompted us to isolate cell free extract from *Pseudomonas aeruginosa* MTCC 741 and carried out its antimicrobial activities on various pathogenic test microorganisms.

MATERIAL AND METHOD

1.Collection of strain: A freeze dried subculture of clinical isolate of *Pseudomonas areoginosa* MTCC 741(MTCC741) similar to NCTC10662, ATCC25668, DSM46358 was obtain from Microbial Type Culture Collection and Gene Bank (MTCC),Institute of Microbial Technology Chandigarh.

2. Revival of MTCC 741

The freeze dried culture of MTCC **741** was revived under aseptic condition by following the protocol of MTCC with modification when required. At first, the ampoule was broken at the marked area and aseptically 500 µl of Nutrient broth was added to the ampoule and suspension of the culture were made and was transfer to 10ml of broth and 100 µl of the suspension was streak in a Muller Hinton Agar (HiMedia) and incubated overnight.

3. Extraction of secondary metabolites

The strain MTCC741 was inoculated in peptone free Nutrient broth (NB) at 10^6 CFU/ml and incubated for 72hours. Followed by the incubation culture was centrifuged at 10,000rpm for 20 minutes at 4^0 C [13]. The pellate formed after the centrifuged was discarded and the greenish color supernatant was collected for further analysis and stored was stored at 4^0 C for further use.

4. Assay of bioactive compounds

Total protein content

In this method crude extract and Bradford reagent [14] was mixed in the ratio of 1:1 and was kept for 5 minutes in dark and was observed at 595nm.and the total protein content was calculated from the standard curve by plotting Bovine serum Albumin (BSA 1mg/ml) by using the same procedure.

Total Phenolics content

Total Phenolics content was determined using Folin-Ciocalteau reagent [15]. The 100µl of this crude extract were taken in a test tube. To which 100 µl of 50% Folin-Ciocalteau reagent was added. The mixture was then kept for 3 minutes and to it 2ml of 2% sodium carbonate solution was added the volume was made up to 3ml with double distilled water. The mixture was kept for 1 minute in water bath and allowed to cool in darkness. The samples were then observed at720 nm in UV Vis spectrophotometer. The total phenolics content of the metabolite was calculated from standard curve of Gallic acid (1µg/µl) plotted by using similar procedure.

Total flavonoids contents

In this method [16] 750µl of these solutions were taken in a test tube. To which 150 µl of 5% $NaNO_2$ was added and was kept for 5 minutes in room temperature and to it 2.5ml of 10% $AlCl_3$ solution was added and kept in room temperature for 6 minutes and to it 1ml 1%NaOH was added and shaken vigorously for 5 minutes. The samples were then observed at 510 nm in UV Vis spectrophotometer. The total flavonoids contents of different extracts was calculated from standard curve of Quercetin (1mg/ml) plotted by using similar procedure.

5. Presence of enzymatic and non-enzymatic antioxidants

Glutathione Assay (GSH):, 0.5 ml of sample from each of three clusters was precipitated with 10% TCA (Trichloro acetate and centrifuged at 1000g. To the aliquot of supernatant, 2 ml of Phosphate-Buffered Saline (PBS) and 0.5 ml of DTNB (5, 5 -dithio-2-nitro benzoic acid) were added and final volume was made upto 5 ml with distilled water [17]. The yellow colored product's optical density was measured at 412nm and was calculated by standard curve of commercial glutathione and was expressed as µg/mg protein.

Radical scavenging activity

Antiradical activity was measured by a decrease in absorbance at 517 nm of DPPH (2,2-Diphenyl-1-Picrylhydrazyl) solution [18] brought about by this crude extract. In this assay DPPH acts as an indicator for "**Radical Scavenging Activity"** and changes its deep violet color to colorless or pale yellow in presence of antioxidant and help us to determine Radical Scavenging Activity (RSC) of the substances. Therefore**,** to determine RSC of the extracts a stock solution of DPPH(0.3mM) was prepared in carbinol and the crude extract concentration mixture taken in the test tubes was of 2ml out of which the extract were present in varied amount (30µl, 50µl, 100µl and 200µl) as per the concentration and rest was carbinol (1970µl, 1950µl, 1900µl and 1800µl) .Then to these test tubes, 1ml of DPPH solution was added to achieve the final volume of 3ml and kept for 20 minutes incubation in dark. After 20 minutes of incubation in dark the absorbance was measured at 517 nm. Decrease in the absorbance of the DPPH solution indicates an increase of the DPPH antioxidant activity and percentage of Radical Scavenging Activity (% RSC) was calculated by (Ao−As)/Ao x 100.[Ao = DPPH solution without the sample, As = DPPH solution with the sample].

6. HPLC Assay

High-performance liquid chromatography (HPLC) was performed on the dry green color extract to confirm the chemical nature of the sample [19].

7. *In vitro* Antimicrobial activity

The antimicrobial activity of this extract was determined on potent human pathogens; *Stapylococcus aureus , Staphylococcus epidermidis* MTCC 9041*, E. coli and Aspergillus niger.* All bacterial culture was inoculated in

sterile NB and was incubated for 24 hours at 37°C After this incubation 100 µl of inoculum was spread *on* Mueller Hinton Agar *(*HiMedia, Mumbai) to study anti bacterial effect by agar diffusion method by following the principle of Kirby Bauer[20]. Similarly, spore suspension of *Aspergillus niger* 10^8CFU/ml was prepared in sterile water and was spread on Potato dextrose agar (PDA) with streptomycin (10µg). To these plates 50-200 µl of the extracts were poured in different wells and their zone of inhibitions were measured after 24 hours in bacteria and 72 hours in fungus and was compared with water which was a negative control and antibiotics or antifungal as positive control.

RESULT

1. Calculation of total protein ,phenolic and flavonoid content of extracts:

The biochemical analysis of the sample showed the nature of it to be protein which contains phenolics and flavonoids (Table1)**.**

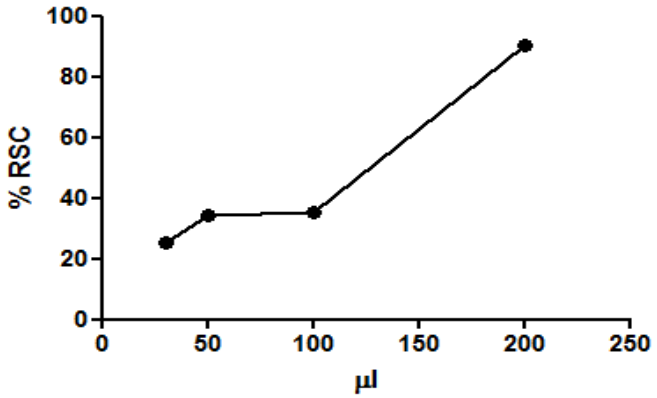
Protein (mgBSA/ml)*	Phenol (mgGAE/ml)*	Flavonoid content (mgQE/ml)*
20.56± 2.3	23.53 ± 2.5	30± 0.99

*Results on the basis of three replicates/treatment

Table 1:- Result for Total protein , phenol flavonoid and GSH content

2. Calculation of enzymatic and non-enzymatic antioxidants

Antioxidant property is determined by GSH and DPPH assay. The GSH is found to be 6.3±0.34 µM Whereas, Percentage of RSC (Figure1) is determined by DPPH assay at different concentrations for the extracts of which 2o0µl of extract showed maximum 90.2 % of RSC.



*Results on the basis of three replicates/treatment

Figure1 : Radical Scavenging Activity of the metabolite

3. HPLC Assay

HPLC analysis (Figure2) of the sample after confirming with standards also shown the presence of 28.24± 2.5 GAE mg/ml phenolics, 31.09 ± 0.99 QEmg/ml flavonoids and in similar procedure indentifies the protein to be phycocyanin with concentration around 2.67± 0.09 mg/ml of the sample. Whereas, it also confirmed the presence of around 2.5± 0.001 µg/ml of phennazine compounds.

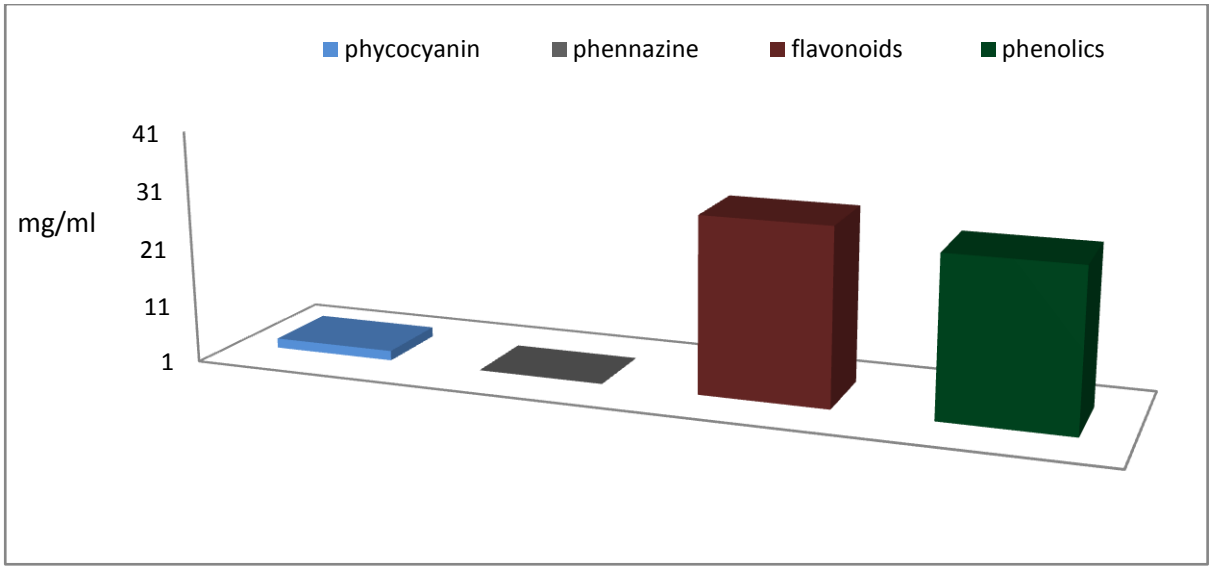


Figure 2 HPLC Analysis of Sample

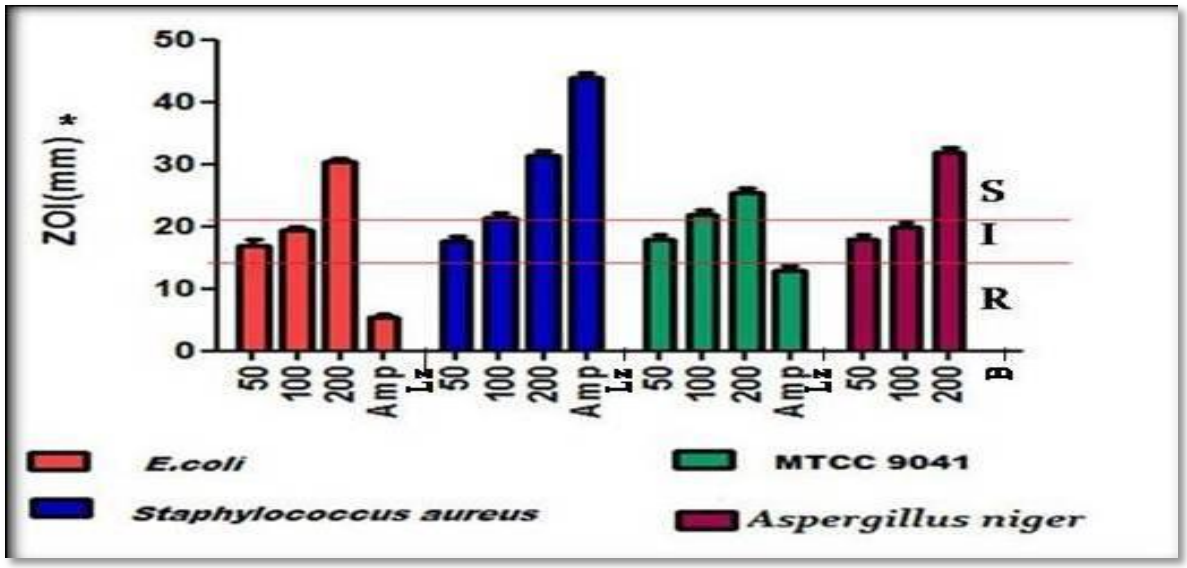
4.Antimicrobial Activity of the extract

Antimicrobial susceptibility test is done by measuring the diameter of the zone of inhibitions[ZOI] obtained from extract solutions (Figure3) and the antibacterial activity is classified[21] into the following types:

>21 mm zone of inhibition is sensitive (S)

16-20 mm zone of inhibition is intermediate (I)

<15 mm zone of inhibition is resistant (R) and is **compare with antibiotic as positive control and water as negative control.**



*All values are expressed as Mean ± SD of three observations

Antibiotics : Ampicillin(AMP)& Linezolid (LZ) **Antifungal:** Copper oxy chloride (B)

Figure 3 Comparison Of Antimicrobial Activity Of The Extract With Antimicrobial Agents

DISCUSSION

In recent year, secondary metabolites have been a great use to treat drug resistance bacterial and fungal diseases[22].Similarly, the secondary metabolite of MTCC 741 showed a similar result by inhibiting the growth of *Aspergillus niger, S.aureus, .E.coli* and MTCC9041. This antagonism is due to presence of Pyocyanin and phenazines. It is seen that pyocyanin inhibit and control nucleic acid and protein synthesis [23] and phenazines [24] interacts with DNA topoisomerases, anti-oxidants or charge-transferring molecules. Moreover, cell free extract though have only phenolics, flavonoids and antioxidant property which have aid to its antimicrobial property[25]. It has also being seen that these bioactive compounds have aid to antimicrobial action on microbes. This antagonism action of various bioactive compounds of MTCC 741 may be helpful to treat diseases caused by the tested pathogens or drug resistance strain of these pathogens and help us to fight the battle against antibiotic resistance. Thus, in future this compound may be a good alternative to treat and control of infections caused by MRSA and VISA and help human to live healthy life.

CONCLUSION

Thus, this extract can be used as a chemotherapeutic agent in this era of antibiotic resistance. This antimicrobial activity may be due to presence of phenolics, flavonoids, GSH and %RSC . However, further tests especially *in vivo* tests are required to analysis antimicrobial on other pathogens and mode of action of this extract on fungus especially *Aspergillus* spp. Moreover, harmful effects of this extract are need to be observe with special attention.

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