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Studies on Inhibitory Effect of Phyto Extracts over Growth and Exotoxin Activity of Pseudomonas Aeruginosa Strain Pp76

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Abstract: *Pseudomonas aeruginosa is a common bacterium that can cause disease in animals and humans. The source of pathogenicity of this bacterium is a potent Pseudomonas exotoxin (PE). P. aeruginosa is naturally resistant to a large range of antibiotics. The objective of this present work project is to control Pseudomonas infection through therapeutic methods based on different medicinal plant extracts of turmeric, ginger, clove oil from clove, Euphorbia hirta, Fenugreek and Alangium. The main aim of this study is to inhibit exotoxin activity by using different phytotherapuetics. Exotoxin was extracted from Pseudomonas and to be purified. The domain regions of Exotoxin A, Exotoxin S and Exotoxin Y have to be analyzed and modeled by using Bioinformatics techniques. Docking of different plant derivatives with Exotoxins were done to determine the best phytotherapeutics to control toxin activity.*

Keywords: *Pseudomonas origins, Exotoxin A, Exotoxin S, Exotoxin Y, Phytotherapeutics, Bioinformatics, docking.*

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I. Introduction

There is a continuous need for new antimicrobial compounds with diverse chemical structures and novel mechanisms of action because there has been an alarming increase in the incidence of new and re-emerging infectious diseases, look of undesirable facet effects of bound antibiotics, moreover because the increasing development of resistance to the antibiotics in current clinical use. Despite the availability of specific antibiotics, Pseudomonas aeruginosa bacteria still cause troublesome infections in patients with a variety of illnesses such as extensive thermal injury, leukopenia from antineoplastic chemotherapy and other forms of immunosuppressive treatment, chronic pulmonary disease such as cystic fibrosis, or intravenous narcotic use.

Pseudomonas aeruginosa is a common bacterium that can cause disease in animals, including humans. The organism infect damaged tissues or those with reduced immunity. The symptoms of such infections are generalized inflammation and infection. If such colonization occurs in body organs, such as the lungs, the urinary tract, and kidneys, the results can be fatal [1]. *Pseudomonas aeruginosa* is a gram-negative, aerobic, rod-shaped bacterium with unipolar motility [2]. *P. aeruginosa* is also an opportunistic pathogen of plants[3].

Plants produce variety of bioactive molecules, making them rich source of different types of medicines. Approximately 20% of the plants found in the world have been considered for pharmaceutical or biological test and a sustainable number of new antibiotics introduced on the market are obtained from natural or semi synthetic resources.

According to World Health Organization [4] medicative plants would be the most effective supply to get a range of medication. The use of plant extracts and phytochemicals with notable antimicrobial properties is of never ending significance, within the past few years variety of investigations are conducted world wide to prove antimicrobial activities from medicinal plants[5,6]. For a long period of time, plants have been a valuable source of natural products for maintaining human health, especially in the last decade, with more intensive studies for natural therapies. Many plants aare used owing to their antimicrobial traits, that are due to compounds synthesized within the secondary metabolism of the plant.

In the present work, plant sources such as turmeric, ginger, clove oil from clove, Euphorbia hirta, Fenugreek and Alangium.

II. Material and Methods

Culturing and characterization of *Pseudomonas aeruginosa*

Pure culture of Pseudomonas aeruginosa was brought from NARI, Pune. The organism was subcultured in trypticase soy medium as slants and plates at 37ºC.

Streak plate method and spread plate method are used for the separating bacterial cell on trypticase soy agar surface to obtain the isolated colonies and then it is inoculated in petriplate. The plates are maintained at 37^0C and inoculated for 24 to 48 hours. The growth was observed

Extraction

Sample was collected, cleaned, shade dried and it was powdered . The basic principle is to grind the sample of plant material (dry or wet) finer, which increases the surface area for extraction thereby increasing the rate of extraction. Six different solvents such as water, ethanol, methanol, ethyl acetate, acetone and chloroform were used for the extraction. Powdered sample was mixed with the solvent in the ratio of 1:10(w/v). After homogenization using magnetic stirrer, the mixture was allowed to stand at room temperature for 24h. The mixture was then filtered and the filterate was concentrated by evaporation.

Well diffusion method

The antimicrobials present within the plant extract were allowed to diffuse out into the medium and interact in a plate freshly seeded with the test organisms. The ensuing zones of inhibition were uniformly circular as there will be a convergent lawn of growth. The diameter of zone of inhibition can be measured in millimeters.

33.9 g of the commercially available Muller Hinton Agar Medium (HiMedia) was dissolved in 1litre of distilled water. The test microorganisms were inoculated by the spread plate methodology. 10µ/ml l of the plant extract were loaded in the wells. The agar plates were then incubated at 37ºC. After sixteen to eighteen hours of incubation, each plate was examined. The diameters of the zones of complete inhibition were measured.

Exotoxin extraction

Pure culture of *Pseudomonas aeruginosa* was brought from National Institute of Immunology, Pune. A relatively simple, but completely defined media was developed in which *Pseudomonas aeruginosa* is capable of producing *Pseudomonas* Exotoxin in measurable quantity. The design of this media was based on environmental and nutrient conditions established for TSBD.

The organism was subcultured in trypticase soy medium as slants and plates at 37ºC. Stock cultures were transferred to Trypticase soy agar slants and incubated overnight at 37ºC. The resultant heavy growth was suspended in Trypticase soy broth dialyzate (TSBD) containing 15% (vol/vol) glycerol and stored in small volumes at -70 C. After overnight incubation at 37ºC, the growth was suspended in 1 ml of sterile saline and used to inoculate a TSBD culture (100 ml). After 6 h of growth at 37ºC with shaking, the TSBD culture was used as the inoculum. Exotoxin extraction of *Pseudomonas aeruginosa* strain PP76 was carried out based on method followed by Liu, P. V. 1964[7].

Dialysate of Trypticase soy broth (TSBD)with 1% glycerol and 50 mM monosodium glutamate was used as a starter medium for toxin extraction, was transferred in four 250 ml flasks and inoculated with six hours incubated inoculums . After incubation, the cells were removed by centrifugation at 12,000 X g for 20 min and filtration of the supernatant fluid (spent medium) through a membrane filter (0.45-µm porosity). Spent media were kept cold throughout all procedures and samples were stored at -20 or -70ºC.

Exotoxin purification

Toxin was precipitated with 90% saturation of ammonium sulpahate. The precipitate was allowed to settle overnight at room temperature. After removing supernatant, the precipitate was placed at 4^0 C for 30 to 60 min, and then allowed to settle for 24h at room temperature. It was found that storage at 4^0 C for short periods of time increased the speed of settling considerably. After removal of the supernatant the volume of the precipitate was approximately 5% that of the original culture volume. The precipitate was collected by centrifugation at 16,000g for 20 min at -5^0 C , dissolved in 10 ml of 5. 0 m tris HCI buffer (pH 7.8).

Toxin activation

For "activation" of Exotoxin, 10-µl samples at approximately 100 µg/ml were mixed with 10 µl of 10 M urea, 100 mM dithiothreitol, 0.03 M EDTA, 0.05 M Tris-hydrochloride, pH 8.1.

Test for proteins

Biuret test, Ninhydrin test, Nitrous oxide test were done to check the presence of proteins and the total protein content in exotoxin was estimated using Lowry's method.

Antiserum production

One albino rabbit was used for the production of antitoxin sera. Purified Exotoxin toxin (13 µg) in 0.25 ml of sterile saline was injected intravenously into each rabbit as a primary dose for the production of higly specific antitoxin. One week after the intravenous injection, each rabbit was subjected to a subcutaneous injection of 25 µg of toxin (2ml). After seven days, serum from the rabbits were collected and stored in small samples at -70^0 C until used. Ouchterlony assays were used to compare antigenic identity of toxin preparations [8].

Ouchterlony Double Diffusion Method

Ouchterlony double diffusion method is used to measure the titer of an antibody. In this test, antigen and antibody diffuse toward each other in a semisolid medium to a point in the medium where optimum concentration of each is attained.

In this method, Agarose gel was prepared by mixing 12 ml of 1.0% agarose (0.12 g) in 1X Assay buffer and heating slowly till agarose dissolves completely. The agarose gel was allowed to solidify for 15- 20 minutes. The 30 µl sample were loaded in the wells of agarose gel using 40 µl of antigen as standard. In the well, and side wells with of all the diluted samples of anti-serum. The unit was then incubated at 37°C for overnight or at room temperature for 24 to 48 hours. After 24-48 h, precipitation lines with highest dilution were formed, which indicated the titre value of the antiserum.

Application of Bioinformatics

Exo-A, Exo-S and Exo-Y sequences were obtained from ExPASy (Expert Protein Analysis System) servers that are a central resource for proteomics tools and databases.

Using FASTA and BLAST approach, modeling of Exo-A, Exo-S and Exo-Y was done ,it was then followed with "CASTp calculation link to upload the Modeled protein pdb file of Exo A and the output was checked . Then ligand selection was done using Drug bank.

Docking was done using Hex software. , The docked file was opened in pymol software and the docked receptor-Ligand complex in Pymol software was visualized.

III. Result

The growth of *Pseudomonas aeruginosa* was in the form of mucoidal colony with umbonate elevation with the production of diffusible green pigment.

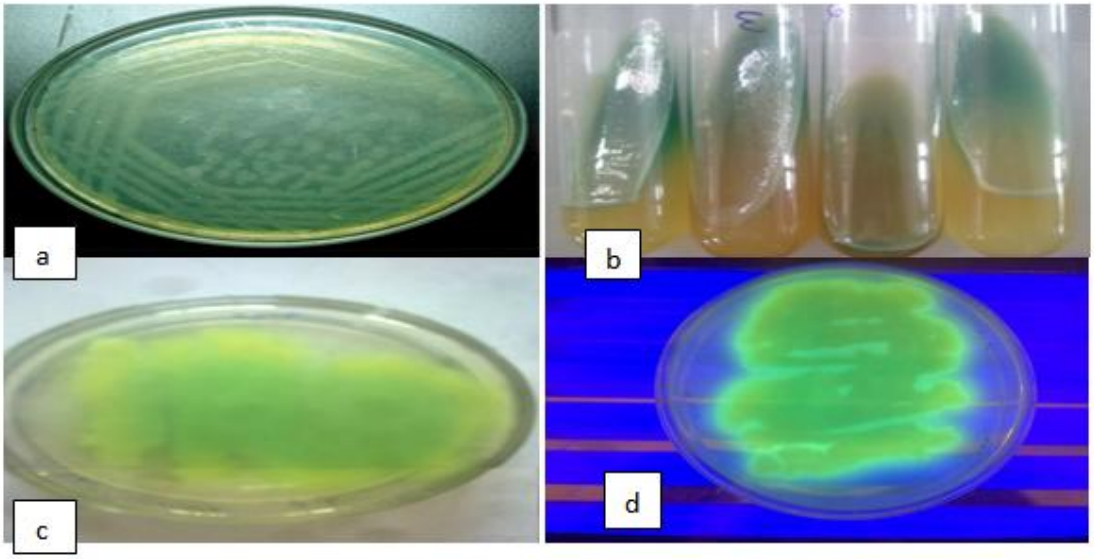

Fig.1: Pseudomonas aeruginosa (a. pure culture**,** b. slants for preservation, c. subcultured on TSA plates**, d.** subcultured plate under UV for fluorescence production)

Well diffusion method

To check the inhibitory effect over the growth of *Pseudomonas aeruginosa*, phyto extracts samples such as turmeric, ginger, euphorbia hirta, cinnamon, alangium and fenugreek with the of concentration 10 µg/ml were tested by well diffusion method. Cinnamon and clove oil showed moderate activity on growth of *Pseudomonas aeruginosa.* On this basis, further tests were carried out to check the action on activated toxin.

Well diffusion study indicated Alangium (clove oil) indicated positive for the solvent with the inhibition zone of 1.3 cm and cinnamon showed positive action with solvent methanol with the inhibition zone of 2.98cm.

Table no1: Effect of phytoextract on growth of *Pseudomonas aeruginosa* strain PP76

Plants/solvents	Water	Ethanol	Ethyl Acetate	Acetone	Chloroform	Methanol
Turmeric	-	-	-	-	-	-
Ginger	-	-	-	-	-	-
Euphorbia Hirta	-	-	-	-	-	-
Cinnamon	-	-	-	-	-	+
Alangium	+	-	-	-	-	-
Fenugreek	-	-	-	-	-	-

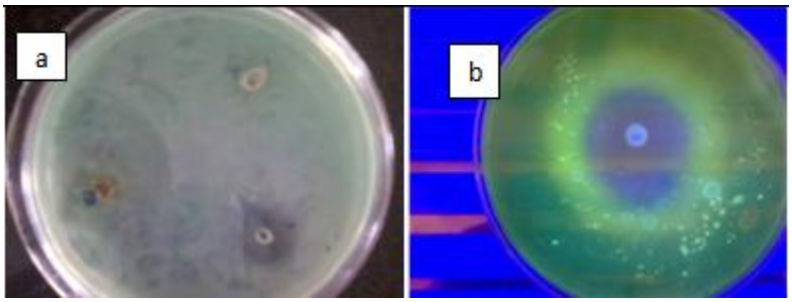

Fig.2: a. Inhibition zone produced for cinnamon extract in methanol and b. Inhibition zone produced for clove oil

Presence of proteins

Biochemical test indicated positive for biruet test with the formation of purple color, blue color for the ninhydrin test and with the libration of nitrogen gas for the nitrous oxide test.

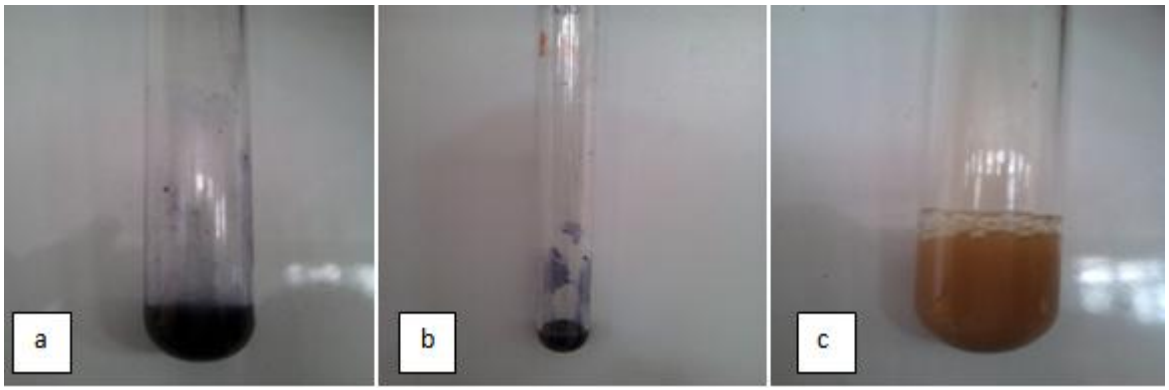

Fig. 3: Biochemical Test for Protein (a: biuret test b: ninhydrin test c: nitrous oxide test)

From Lowry's method , concentration of protein was estimated to be 90µg/ml

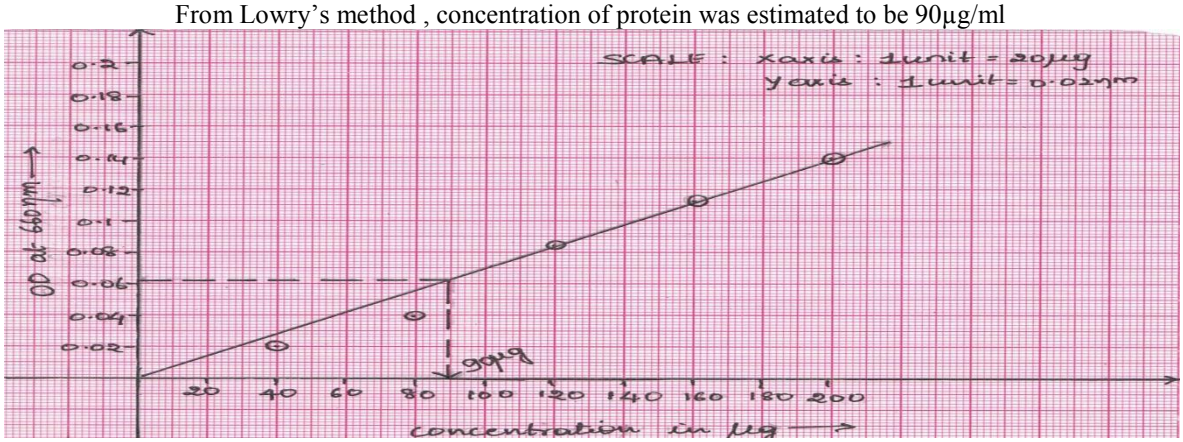

Fig. 4: Plot for Lowry's method of estimation of protein

Ouchterlony double diffusion method was carried out to check the immune activity of toxin against rabbit antisera. Ouchterlony double diffusion method performed with concentrated but unpurified *Pseudomonas aeruginosa* supernatant fluid. A line was formed in between Toxin and antisera but absence of line formation was observed in toxin titrated with Clove and Cinnamom.

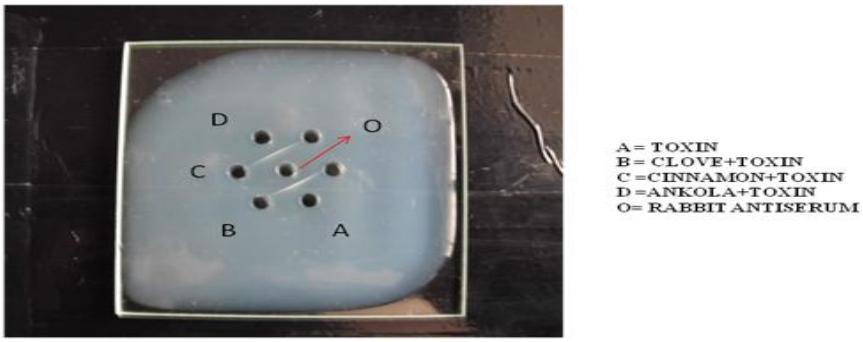

Fig. 5: Ouchterlony Double Diffusion Method

Bioinformatics studies on domain analysis of Exo-A,Exo-S and Exo-Y were carried out and the results were collected. Exo-A, Exo-S and Exo-Y toxin proteins were modeled by using swiss pdb software.

FASTA sequence for EXOTOXIN A collected from EXPASY:

>sp|P11439|TOXA_PSEAE Exotoxin A OS=Pseudomonas aeruginosa (strain ATCC 15692 / PAO1 / 1C / PRS 101 / LMG 12228) GN=eta PE=1 SV=2MHLTPHWIPLVASLGLLAGGSFASAAEEAFDLWNECAKACVLDLKDGVRSSRMSVDPAIADTNG QGVLHYSMVLEGGNDALKLAIDNALSITSDGLTIRLEGGVEPNKPVRYSYTRQARGSWSLNWLVPIGH EKPSNIKVFIHELNAGNQLSHMSPIYTIEMGDELLAKLARDATFFVRAHESNEMQPTLAISHAGVSVVM AQAQPRREKRWSEWASGKVLCLLDPLDGVYNYLAQQRCNLDDTWEGKIYRVLAGNPAKHDLDIKPT VISHRLHFPEGGSLAALTAHQACHLPLETFTRHRQPRGWEQLEQCGYPVQRLVALYLAARLSWNQVD QVIRNALASPGSGGDLGEAIREQPEQARLALTLAAAESERFVRQGTGNDEAGAASADVVSLTCPVAAG ECAGPADSGDALLERNYPTGAEFLGDGGDISFSTRGTQNWTVERLLQAHRQLEERGYVFVGYHGTFLE AAQSIVFGGVRARSQDLDAIWRGFYIAGDPALAYGYAQDQEPDARGRIRNGALLRVYVPRSSLPGFYR TGLTLAAPEAAGEVERLIGHPLPLRLDAITGPEEEGGRLETILGWPLAERTVVIPSAIPTDPRNVGGDLDP SSIPDKEQAISALPDYASQPGKPPREDLK

EXOTOXIN-A domain

Description:	Exotoxin A EC=2.4.2.-
Source organism:	Pseudomonas aeruginosa (NCBI taxonomy ID 287) View Pfam proteome data.
Length:	638 amino acids

Source	Domain	Start	End
sig_p	n/a	1	25
low_complexity	n/a	12	30
Pfam A	Exotox-A_bind	27	275
Pfam A	Exotox-A_target	277	409
Pfam A	Exotox-A_cataly	413	638

Fig.6: PFam result of Exotoxin A domain

FASTA sequence for EXOTOXIN S from EXPASY:

>tr|Q51451|Q51451_PSEAI Exoenzyme S OS=Pseudomonas aeruginosa GN=exoS PE=1 SV=1MHIQSLQQSPSFAVELHQAASGRLGQIEARQVATPSEAQQLAQRQDAPKGEGLLARLGAALMRP FVAIMDWLGKLLGSHARTGPQPSQDAQPAVMSSAVVFKQMVLQQALPMTLKGLDKASELATLTPEGL AREHSRLASGDGALRSLSTALAGIRAGSQVEESRIQAGRLLERSIGGIALQQWGTTGGAASQLVLDASP ELRREITDQLHQVMSEVALLRQAVESEVSRVSADKALADGLVKRFGADAEKYLGRQPGGIHSDAEVM

ALGLYTGIHYADLNRALRQGQELDAGQKLIDQGMSAAFEKSGQAEQVVKTFRGTRGGDAFNAVEEG
KVGHDDGYLSTSLNPGVARSFGQGTISTVFGRSGIDVSGISNYKNEKEILYNKETDMRVLLSASDEQGV
TRRVLEEAALGEQSGHSQGLLDALDLASKPERSGEVQEQDVRLRMRGLDLA

EXOTOXIN-S domain

Description:	Exoenzyme S
Source organism:	Pseudomonas aeruginosa (NCBI taxonomy ID 287) View Pfam proteome data.
Length:	453 amino acids

Source	Domain	Start	End
low_complexity	n/a	50	61
Pfam A	YopE	129	198
Pfam A	ADPrib_exo_Tox	242	430

Fig.7: PFam result of Exotoxin S domain

FASTA sequence for EXOTOXIN Y from EXPASY:

>tr|O85345|O85345_PSEAI Adenylate cyclase OS=Pseudomonas aeruginosa GN=exoY PE=4 SV=1MRIDGHRQVVSNATAQPGPLLRPADMQARALQDLFDAQGVGVPVEHALRMQAVARQTNTVFGI
RPVERIVTTLIEEGFPTKGFSVKGKSSNWGPQAGFICVDQHLSKREDRDTAEIRKLNLAVAKGMDGGA
YTQTDLRISRQRLAELVRNFGLVADGVGPVRLLTAQGPSGKRYEFEARQEADGLYRISRLGRSEAVQV
LASPACGLAMTADYDLFLVAPSIEAHGSGGLDARRNTAVRYTPLGAKDPLSEDGFYGREDMARGNITP
RTRQLVDALNDCLGRGEHREMFHHSDDAGNPGSHMGDNFPATFYLPRAMEHRVGEESVRFDEVCVV
ADRKSFSLLVECIKGNGYHFTAHPDWNVPLRPGFQEALDFFQRKV

EXOTOXIN-Y domain

Description:	Adenylate cyclase ExoY
Source organism:	Pseudomonas aeruginosa (NCBI taxonomy ID 287) View Pfam proteome data.
Length:	378 amino acids

Source	Domain	Start	End
Pfam A	Anthrax_toxA	28	207

Fig.8: PFam result of Exotoxin Y domain

Protein Modelling

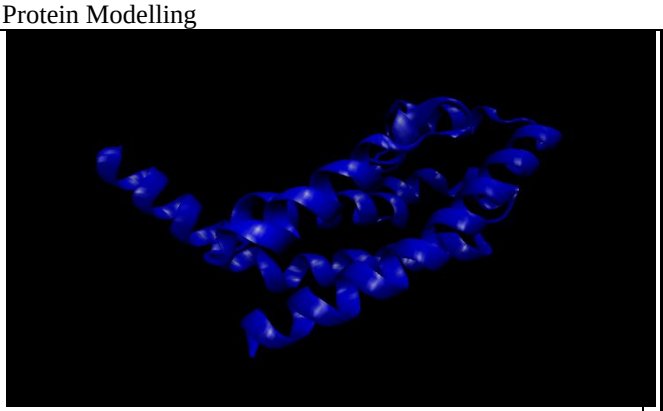

Fig. 9: Modeled Exo-A Protein

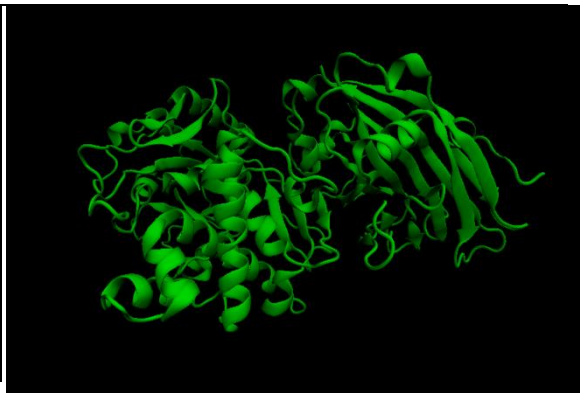

Fig. 10:Modeled Exo-S Protein

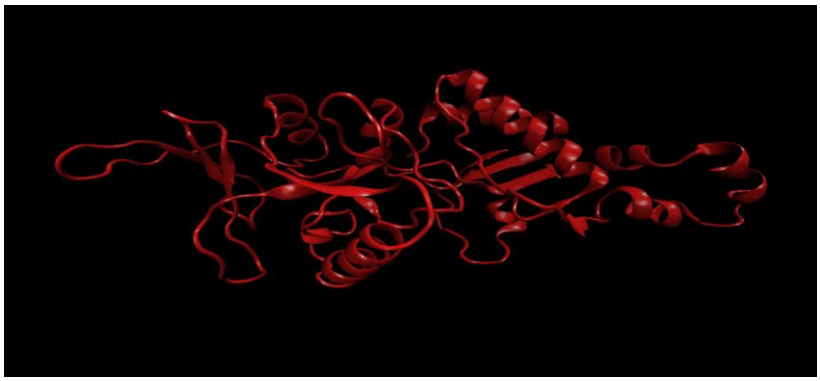

Fig.11: Modeled Exo-Y Protein

Fig. 12, Fig.13 and Fig.14 indicates the active site prediction using CASTp SERVER

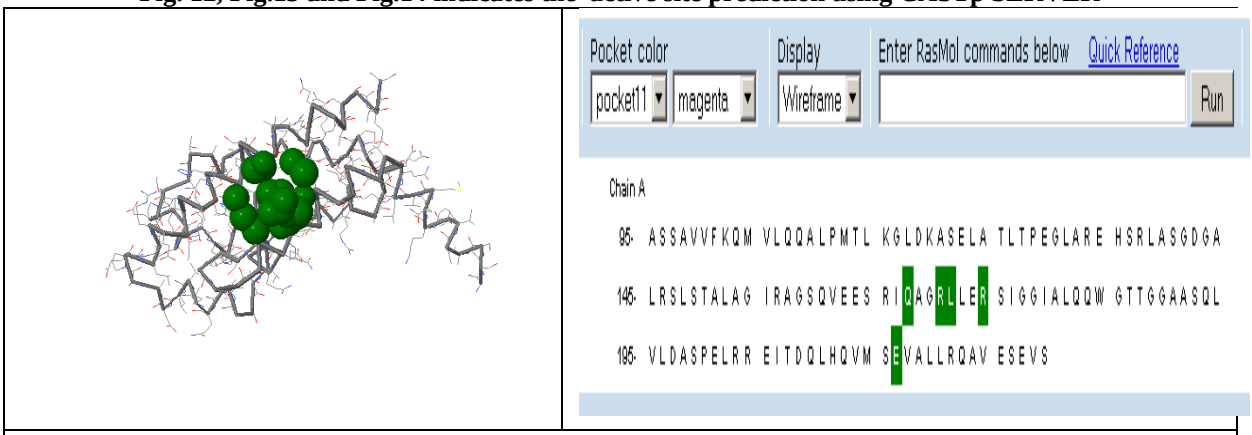

Fig.12:Active site prediction of Exo-A

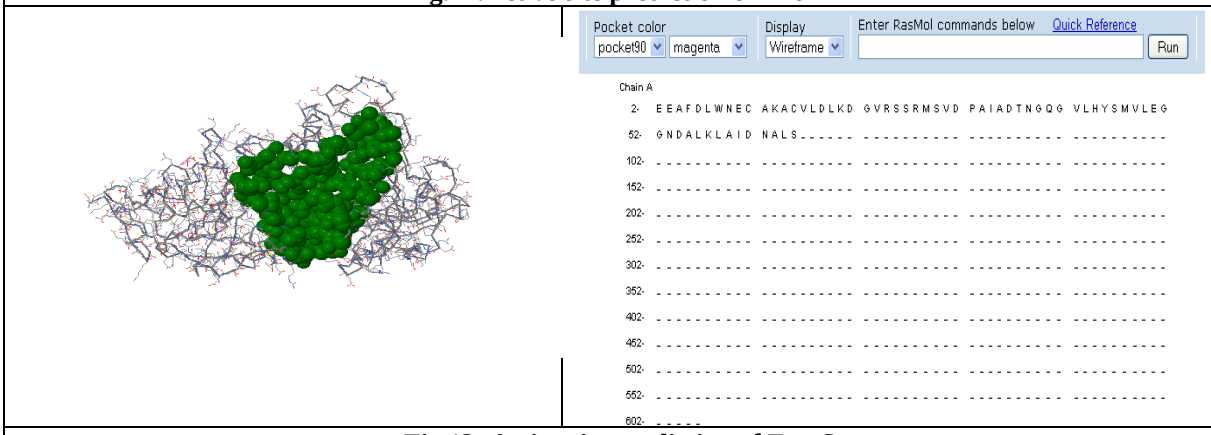

Fig.13: Active site prediction of Exo-S

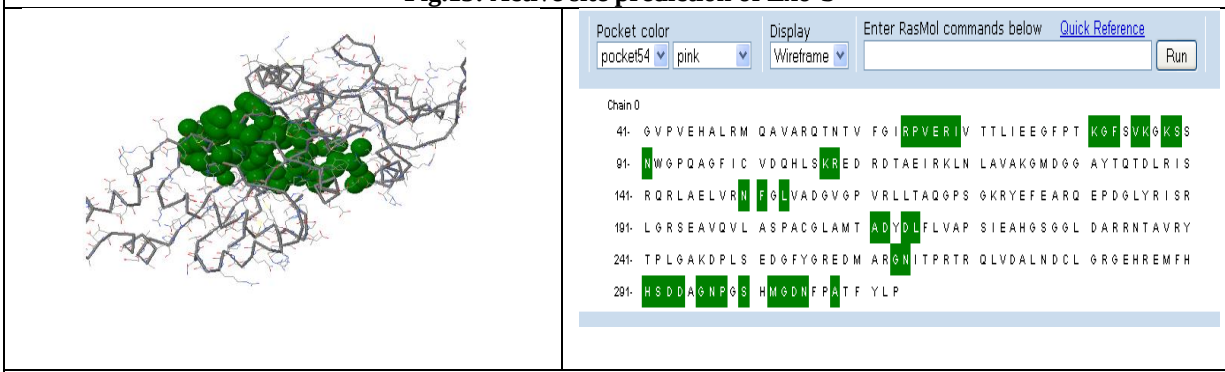

Fig.14:Active site prediction of Exo-Y

Eugenol ,a phenylpropene, is an allyl chain-substituted guaiacol. It's appearance is a clear to pale yellow oily liquid extracted from certain essential oils especially from clove oil, nutmeg, cinnamon, basil and bay leaf. It is slightly soluble in water and soluble in organic solvents. Eugenol is responsible for the aroma of cloves. It is the main component in the essential oil extracted from cloves, comprising 72–90% of the total Eugenol. Clove oil had inhibitory effect over toxin activity based on Ouchterlony double diffusion method.

Molecular structure of Eugenol had been collected from Drug bank. By using Hex software modeled Exo-A was docked with euginol. Similarly Docking studies were carried out over Exo-S and Exo-Y.

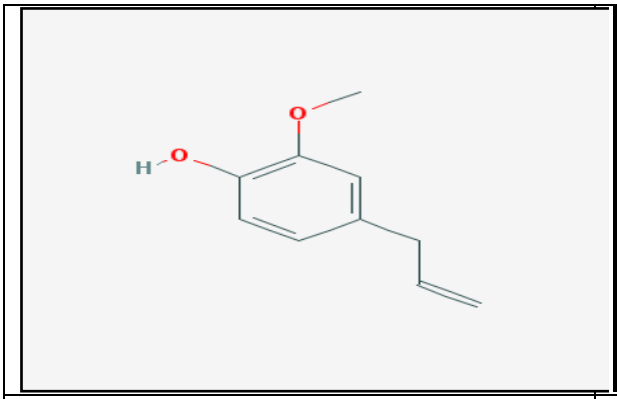

Fig. 15:Molecular structure of Eugenol

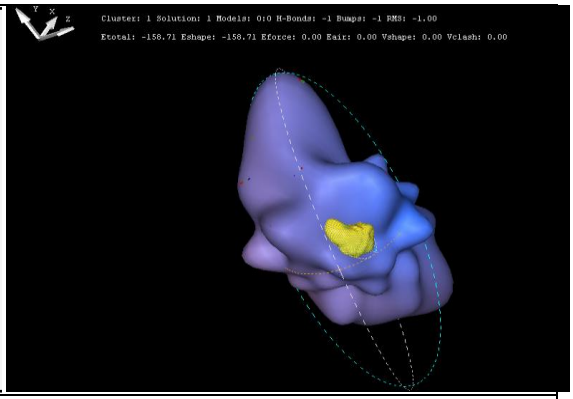

Fig.16: Docking result of Exo-A with Eugenol

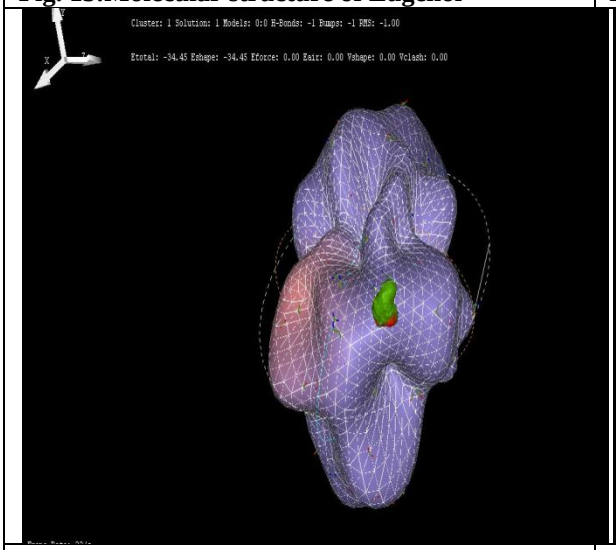

Fig. 17:Docking result of Exo-S with Eugenol

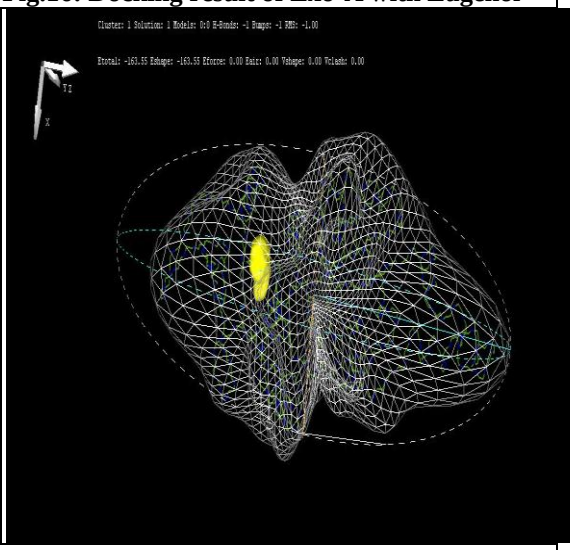

Fig.18: Docking result of Exo-Y with Eugenol

Fig. 19, Fig. 20 and Fig.21 indicated the PYMOL view of Exo-A, Exo-S, Exo-Y with Eugenol

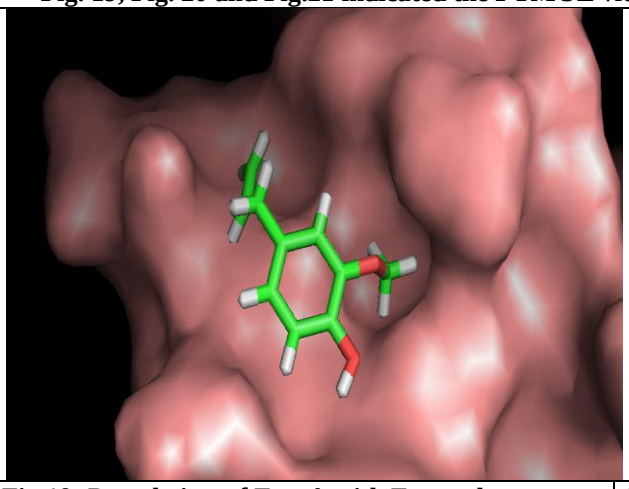

Fig.19: Pymol view of Exo-A with Eugenol

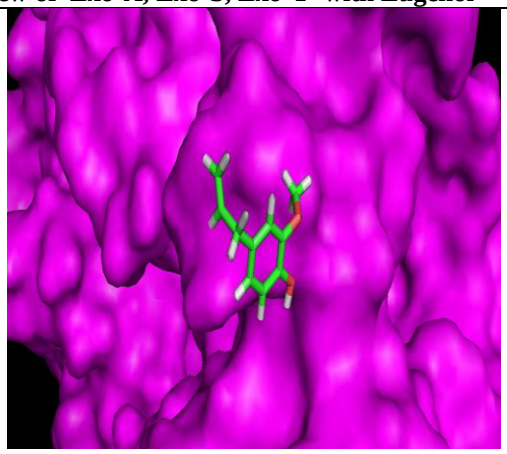

Fig.20: Pymol view of Exo-S with Eugenol

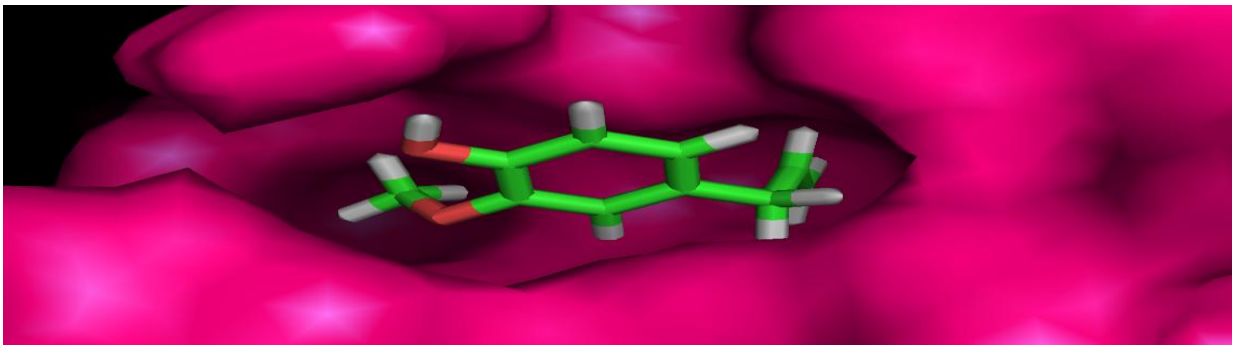

Fig.21: Pymol view of Docking result of Exo-Y with Eugenol

IV. Discussion

Plant based antimicrobial compounds have enorrmous therapeutical potential as they can serve the purpose without any side effects that are often associated with synthetic antimicrobials. The methanol, ethanol, ethyl acetate and chloroform and aqueous extracts of turmeric, Ginger, Euphorbia hirta, Cinnamon, Alangium and fenugreek were tested for antimicrobial activity against of Pseudomonas aeruginosa strain PP76. Methanol extract of Cinnamom and chloroform leaves extracts of Alangium exhibited pronounced activity against Pseudomonas aeruginosa strain PP76. Clove oil had shown more antimicrobial activity and inhibition zone of 2.98 cm was observed in well diffusion method.

A potent toxin Pseudomonas exotoxin A (PE) was secreted by Pseudomonas aeruginosa and may have an important role in the pathogenesis of Pseudomonas infections. PE is made up of a single polypeptide chain with a molecular mass of 66,000 daltons. Crystallographic studies have shown that PE contains three distinct domains. Domain I was responsible for cell recognition, domain III was responsible for translocation across membranes and domain III for ADP ribosylation of elongation factor 2, the cytoplasmic target of the PE molecule. ExoS is a bifunctional cytotoxin with two active domains, a C-terminal ADP-ribosyltranferase domain and an N-terminal Rho GTPase-activating protein (GAP) domain. ExoS a bifunctional cytotoxin with two active domains, a C-terminal ADP-ribosyltranferase domain and an N-terminal Rho GTPase-activating protein (GAP) domain. Previous studies revealed that these three toxins namely Exo-A,Exo-S and exo-Y are potential therapeutic target. In the present study, phytoextracts were tested against exotoxin to inhibit their activity[9]. Biochemical test like Biuret test, Ninhydrin test,Nitrous test clearly indicated the presence of proteins and the concentration of proteins using lowry's method confirm the presence of Exotoxin.

Ouchterlony double diffusion method was carried out to check the immune activity of toxin aginst rabbit antisera. A line was formed in between Toxin and antisera but absence of line formation was observed in toxin trated with Clove and Cinnamom. From the above result, it is proved that clove oil and cinnamon has inhibitory effect over activated toxin of Pseudomonas aeruginosa.

Bioinformatics studies on domain analysis of Exo-A,Exo-S and Exo-Y were carried out and the results were collected. Exo-A, Exo-S and Exo-Y toxin proteins were modeled by using swiss pdb software.

The sequences and domain of various virulence factors of Pseudomonas aeruginosa (Exo A, Exo S and Exo Y) were collected and was checked for the compatibility with eugenol compound.Hex software modeled Exo-A, Exo-S and Exo-Y were docked with eugenol. The Etotal of Exo-A,Exo-S and Exo-Y were -158.71, -34.45 and -161.35. Our studies revealed that Eugenol had more inhibitory effect over Exo-Y and Exo-A.

V. Conclusion

The study aimed at extraction of plant based antimicrobial compounds with therapeutical potential without any side as exploration of plant-derived antimicrobials has become necessaity. Study describes the inhibitory effect of phyto extracts over the growth and exotoxin activity of Pseudomonas aeruginosa strain PP76. The chemical compound eugenol present in clove and cinnamon showed inhibitory effect over the growth and Exotoxin activity of Pseudomonas aeruginosa. Cinnamon and clove oil showed moderate activity on growth of Pseudomonas aeruginosa. On this basis, further tests were carried out to check the action on activated toxin.

Ouchterlony double diffusion method performed with concentrated but unpurified Pseudomonas aeruginosa supernatant fluid and proved that clove oil and cinnamon has inhibitory effect over activated toxin of Pseudomonas aeruginosa strain PP76. Through molecular docking it was observed that Eugenol compound was more compactly docked with the Exotoxin Y.

Further, research is essential to identify the antibacterial compounds from these plants considered for study and also to determine their full spectrum of efficacy. However, the present study on inhibitory effect of

phytoextract over growth and exotoxin activity of Pseudomonas aeruginosa strain PP76 forms a primary platform for further phytochemical and pharmacological studies to discover new antibiotic drugs against Pseudomonas aeruginosa infection.

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