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Research Article

Extraction, Purification, characterization and antibacterial activity of phenoloxidase extracted from the Sea Squid

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Abstract

Serious morbidity and mortality resulted from the failure of traditional antibiotic treatment due to the rise of methicillin-resistant *Staphylococcus aureus* (MRSA). One possible mechanism for antibiotic resistance in *S. aureus* is the production of biofilms. Essential regulatory proteins for the development of *S. aureus* biofilms are *SarA, aur* and *nus*. In this work, we isolated and refined phenoloxidase and tested it against *S. aureus* to determine its antibiofilm capabilities. Using L-dihydroxyphenylalanine (L-DOPA) as the particular substrate, phenoloxidase (PO) from sea squid ink sacs was purified ion-exchange chromatography, and its biochemical and enzymatic characteristics were described. We assessed the MIC, eDNA inhibition, and gene expression of the purified PO as an antibacterial agent against *S. aureus*. Comparing the PO's MIC to the positive control, we discovered that it was 61µg/ml, a considerable difference. Both eDNA and extracellular polymer material (EPS) were inhibited. We discovered that whereas other regulatory proteins like nuc and sarA were increased, aur was downregulated based on the results of the real-time PCR. This validates the PO's possible role as an antibiofilm agent. To sum up, this study's findings demonstrated PO's anti-biofilm action on MRSA.

1. Introduction

A class of type-3 copper proteins known as polyphenol oxidases (PPOs) converts phenols to their equivalent quinones when molecular oxygen is present. PPO terminology distinguishes between catechol oxidase and tyrosinase. Monophenols can be hydroxylated to o-diphenols by tyrosinases, which then oxidize the o-diphenols to the equivalent quinone. Catechol oxidase, on the other hand, converts diphenolic substances into quinines [1]. The association between PPOs and plant defense mechanisms as well as PPOs' involvement in the browning phenomena have been the subjects of recent study on PPO function. The prior role has a lot of documentation. According to research, cyanidin-3-sophoroside, for instance, inhibits polyphenol oxidase activity, delaying the browning of fruit endocarp [2].

It is commonly known that the invertebrate defense system relies heavily on phenoloxidase (PO) which is derived from prophenoloxidase (proPO) [3]. PO attaches to infected cells in shrimps infected with the Taura sickness virus through a likely thiol ester-like motif, producing toxic phenol intermediates that may help stop viruses from entering the body cavity. Invertebrate hemocyte immune responses involve a melanogenic pathway called the "proPO activating system," which is a crucial cascade reaction [4]. ProPO is stimulated by invasive pathogens and then turns into PO. PO then oxidizes dopa to dopaquinone and hydroxylates tyrosine to dopa, which starts the synthesis of melanin [5]. Dopaquinone appears to convert quickly, without the use of enzymes, to dopachrome, which then gradually changes into melanochrome and ultimately eumelanin. When closely examined, it has been discovered that invertebrate PO is present in its inactive form (proPO), which gets activated by components of microbial cell walls [6].

One of the most significant infections for both people and animals is Staphylococcus aureus. Methicillin-resistant Staphylococcus aureus (MRSA) is a significant strain of *S. aureus* and a multidrug-resistant (MDR) pathogen, according to the World Health Organization.

According to [7], *S. aureus* biofilm is a significant contributor to long-term infections and medical device contamination. Mature biofilm is difficult to remove, and compared to planktonic bacteria, biofilm bacteria are more resistant to antibiotics [8]. Biofilms are resistant to antibiotics by a variety of strategies, including high rates of gene transfer, growth metabolism, modified permeability, and antibiotic neutralization. Anti-biofilm therapy is therefore a crucial tactic against *S. aureus* persistent infections [9].

SarA partially regulates protease and nuclease activities, which is regulates the biofilms in *S. aureus* [10]. The extracellular protease-mediated method is more significant in the SarA-regulated biofilm production than the PIA method, and SarA was not dependant on the agr during this process [11]. Proteins and eDNA are the primary components of the ica-independent pathway. A crucial part of protein-dependent biofilm development is played by extracellular proteases. Extracellular proteases can have their activity inhibited by SarA [12]. Early adhesion and development of ica is not dependent on *S. aureus* biofilms and are significantly influenced by eDNA. Thermostable nuclease *(Nuc)* controls the amount of eDNA in biofilms, and a number of studies have demonstrated that *SarA* may suppress Nuc expression [13].

While POs of different species of shrimp and shellfish have been thoroughly described, as of yet there is no data on the characteristics of squid PO. Purifying PO from Sea Squid and characterizing it biochemically and enzymatically and screening for its potential antibacterial activity against *Staphylococus aureus* was the main aim of the work.

2. Materials and methods

Sample collection

A medium-sized (18–22cm) squid ink (Loligo sp.) sample was taken in the Lampulo fish market in Banda Aceh. At the Insearch Biotechnology Laboratory in Bangalore, testing on several physicochemical parameters, including antibacterial activity, were conducted.

Melanin-free ink

Fresh squids were bought from a local market in Bangalore and after bringing it to the lab, the squid's ink sac was cut off by severing the ink duct, and the ink was then squeezed out. Using cold deionized water (4°C), the squid ink was diluted ten times with de-ionized water. After that, it was centrifuged using a cooling centrifuge (REMI 25R) at 12000rpm for 30 minutes at 4°C. Melanin-free ink (MFI) was then stored at -20°C until further use [14].

Isolation of Polyphenol oxdiase (PO) from the ink

In 20mM of Tris–HCl buffer (pH 7.1), around 10ml of the MFI that was produced in the previous step was dissolved. The mixture was well combined, then centrifuged for one hour at 4°C (10000rpm). The supernatant obtained was then added to 67% saturated ammonium sulphate solution and left undisturbed over night at 4°C. Centrifugation at 10000rpm was done for 30min at 4°C to collect the precipitate. The precipitate obtained was then diluted in a minimum of 20mM Tris–HCl buffer (pH 7.1), desalted and then filtered. The resultant sample was lyophilized (PO) and stored as -20°C.

Purification of PO

With minor adjustments, column chromatography and purification were carried out using the methodology as described by [15]. Following a 1hr centrifugation for 10,000rpm at 4°C, the crude sample then was diluted in 0.2ml of 20mM Tris–HCl buffer (pH 7.1), and the supernatant was collected. A 1.5cm × 40cm Sephacryl S-100 gel-filtration column that had been previously equilibrated with a 20mM Tris–HCl buffer (pH 7.1) and eluted using the same buffer was used to load the supernatant. Fractions of exactly 2ml were collected (flow rate of 0.5ml / min) and at 280nm, ultraviolet absorption was observed. The fractions with activities were gathered and combined to form Sephacryl S-100 pure PO. The fractions' PO-like activities were assessed using 15mM L-dihydroxyphenylalanine (L-DOPA). Following application to a Q Sepharose rapid flow ion-exchange column (1cm × 30cm), the combined Sephacryl S-100 PO sample was eluted using a linear gradient from 0 to 0.5M NaCl in 20mM Tris–HCl buffer (pH 7.1). Fractions of 2ml were collected at a flow rate of 1.0ml / min and at 280nm, ultraviolet absorption was recorded. Using 15mM L-DOPA, the fractions' PO-like activities were assessed. After being desalted and pooled, the fractions exhibiting high PO-like activity were concentrated and were labelled as purified PO.

Enzyme assay

With a few modest adjustments, the PO-like activity was assessed using the methodology from . After dissolving 15 mM L-DOPA in 20 mM Tris–HCl buffer (pH 7.1), 10µl of each enzyme solution was added. Each sample received 280µl of ice-cold distilled water after being incubated for 40min at 28°C. The reaction mixture was then examined at a wavelength of 490nm using a UV-1800 spectrophotometer (Shimadzu, Tokyo, Japan). The rate of absorbance was used to measure the PO-like activity, and a rise of 0.001 per minute was considered to be one unit. Activity per unit was calculated with formula (U): Activity = $A_{490} \times 10^{-3}$/min.

SDS–PAGE

SDS–PAGE was carried out on 12.5% gels as described by [16]. Reducing SDS-PAGE, loading buffer containing and lacking β-mercaptoethanol was utilized. For two hours at 220V, the material was electrophoresed in an SDS gel using a 4.5% stacking gel (4.5%) and a resolving gel (12.5%). Proteins were stained with silver nitrate and 0.05% Coomassie brilliant blue R-250, following electrophoresis.

Bacterial culture

Staphylococcus aureus was kindly donated by Insearch Biotech laboratory, bangalore. The subculturing was done using the Trypticase soy broth (TSB). Trypticase soy broth (TSB) medium contained 0.5% glucose. *S. aureus* was cultured overnight and maintained at an OD_{600} of

0.1 (about 1×10^8 CFU/mL).

Antimicrobial Testing

The least inhibitory concentration (MIC) and agar diffusion methods were used to screen the antibacterial efficacy of the purified PO in conjunction with vancomycin, a positive control.

Agar Diffusion Method

The agar well diffusion approach was used in testing the antimicrobials on LB agar plates. To sum up, 100µL of the bacterial culture were evenly distributed across the agar plates. A sterile glass capillary tube was used to cut the wells, which had a diameter of roughly 3 mm, in the centre and on the periphery. About 50µl of PO at varying concentrations of 16, 32, 64 and 128µg/mL were applied to their respective wells. Throughout the investigation, vancomycin (64µg/mL) was utilized as a positive control. The plates were then incubated for 24 to 48 hours at 37°C. The growth inhibition zone's mean diameter (mm) was noted after incubation. Every test was conducted under the identical conditions and in duplicate.

The Minimal Inhibitory Concentration (MIC)

As stated by [17], the microdilution method was used to estimate the MIC of the PO using 96 multi-well microtiter plates. To put it briefly, the dissolved PO were added to wells containing 180µl of TSB broth. The PO fraction was added at varying concentrations (0, 16, 32, 64 and 128µg / mL) to each well containing 180µL of broth. Finally, all of the wells—aside from the negative control—were filled with 10µL of the bacterial culture. As positive control, vancomycin was employed. For 18hr, the plates were incubated at 37°C. The minimum concentration (MIC) at which turbidity was observed was determined. The MIC was ascertained by analyzing the plates. After being incubated, the plates were screened for CFU / ml and their optical density was measured using a PLATE reader (Genetix Ltd.) at 600nm.

3. Biofilm Inhibition Assay

Biofilm inhibition assay was carried according to [18]. The plate previously used for broth dilution was used for the biofilm study after the readings were observed. Following incubation, the growth media was discarded and wells were washed thrice with phosphate-buffered saline (PBS). The wells were then stained with 0.1% crystal violet and then washed thrice with PBS (pH 7.4). The absorbance of the eluted solution was measured at 595nm using a Plate reader (Genetix Ltd.) after the cell-bound dye was eluted using around 200µL of ethanol:acetone (80:20).

Determination of biofilm by congo red method

In the *S. aureus* biofilm treated with varying doses of the purified PO, the synthesis of EPS was detected using a Congo red plate binding test. PO was applied to the strain at doses of 0, 16, 32, 64 and 128µg / mL. Lastly, the Congo red plate was vertically filled with 10µL of the bacterial culture, and it was cultured for 16 hours at 37°C. The colour shift indicated how the PO affected the synthesis of EPS.

Determination of the eDNA from the biofilm

UV spectrophotometry was used to identify the amount of eDNA secretion in *S. aureus* at various doses of PO. To a 96 well plate containing broth, varying concentration of 0, 16, 32, 64 and 128µg/ml were added. About 200µl of S. aureus suspension (1×10^8 CFU/mL) was added. The plate was incubated for twenty-four hours at 37°C. A spectrophotometer (Shimadzu, 1800) was used to measure the OD260 / OD280 ratio after Trizol was used to extract the eDNA. The amount of biofilm formed in a single hole of a 24-well plate was used to express the amount of eDNA per unit biofilm.

Real-time PCR of biofilm-related genes

In order to cultivate the *S. aureus* biofilm, the purified PO of 0, 32, 64 and 128µg/ml were used. The study included *aur, nuc,* and *sarA* as gene members, whereas *gyrB* was used as a housekeeping gene.

RNA extraction

A bacterial suspension of around 20µl was added to LB medium (50ml) and thoroughly mixed. About 10µl of varying concentrations (0, 32, 64 and 128µg/ml) were added to their respective tubes. Additionally, 10µl of the vancomycin was added to the positive control tube. The tube not treated served as control. The contents are correctly mixed and incubated for 24 to 48 hours at 37°C. The isolates following incubation were used for RNA extraction.

RNA extraction

In brief, the cultures (1×10^6) were spinned at 9000rpm for approximately 5min at 4°C. The pellet was added with 500µl of RLT and was aggressively vortexed for a duration of 5-10sec. After that, the contents were collected in a new tube and centrifuged for approximately 10 seconds at maximum speed. Pipetting was used to add an equal volume of 70% ethanol and completely mix it in. A 2 ml collection tube containing an RNeasy spin column was filled with about 700l of the resulting lysate. After spinning for 15sec at 9000rpm, the flow from the collection tube was discarded and about 700l of Buffer RW1 was added into the column.

Table 1: Table showing the list of primers used.

Name		length	Tm	GC%	Sequence	Product
aur	FW	22	60.04	54	CGCACATTCACAAGTTTATCGG	470
	RV	22	60.11	52	TTAGAGCGCCTGACTGGTCCTT	
nuc	FW	20	58.98	50	ATCGCTTGCTATGATTGTGG	490
	RV	19	59.09	50	TTCACCGTTTCTGGCGTAT	
sarA	FW	23	58.98	54	CAATTAGCTTTGAAGAATTCGCT	217
	RV	25	58.91	54	CGAAGTAATCTTCTTGAGATAAAAT	
gyrB	FW	20	59	52	CGCAGGCGATTTTACCATTA	382
	RV	21	59.97	50	TCGCTAGATCAAAGTCG	

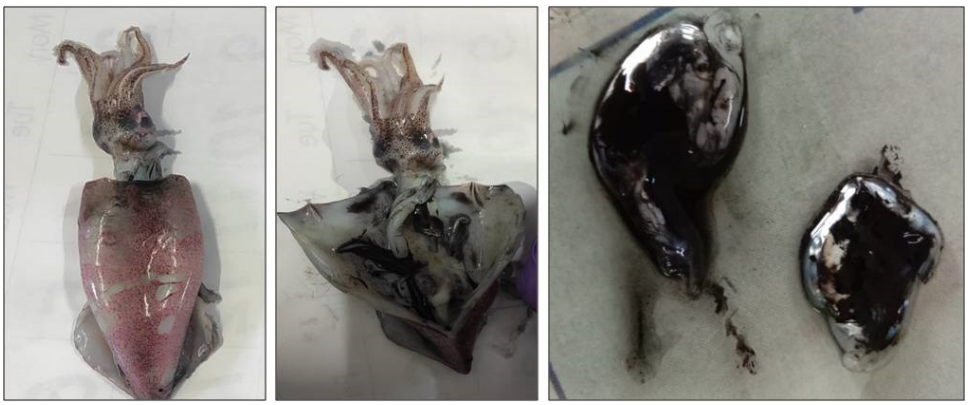

Figure 1: Photographic images of the sea squid, before and after dissection. Ink can seen from the last picture

To wash the column, 700l of RW1 was poured to it, and spinned for 15sec at 9000rpm. The column was now washed by adding 500l of Buffer RPE, and the flow-through was disposed of after the spin column was centrifuged for 15 seconds at 9000rpm. After inserting the column into a fresh collection tube, 30–50l of RNase-free water was added. After centrifuging the contents for one minute at 9000rpm to extract the RNA, the RNA was stored at -20°C and checked for purity on spectrophotometer (1800, Shimadzu).

RT PCR

Using the RT PCR kit and SuperScript TMII Reverse Transcriptase, 200U/l (HiMedia), cDNA synthesis was carried out. To put it briefly, the initial reaction consisted of roughly 2.3g of the RNA that was produced in the preceding step. The obtained RNA concentration was approximately 1.23g/l. Thus, we utilized 1.45l of RNA, 1l of RT enzyme and primers. After carefully combining the components, they were incubated for 10 minutes at 25°C. The cDNA was obtained and incubated for 45 minutes at 70°C. It was then stored until it was needed for gene expression.

Real-time PCR

Primers were designed using primer3 and purchased from Sigm a-Aldrich, Mumbai. qPCR was then performed as described by [19] using SYBR Green Supermix (HiMedia). The total reacton volume was made to 12.5l.

Expression of gene members in samples

Expression of gene members in samples: Samples (control and treatment) were measured in RT PCR using the Corbett Research cycler (Bio-Rad). The PCR program employed *aur, nuc* and *sarA* primers at a concentration of roughly 600nM. Initially, 1.45l of the RNA products were employed, and the program was run for approximately 40 cycles, with an elongation for 45s at 72°C, 45s at 65°C, and 50s at 91°C. In order to derive a comparative analysis of the mRNA expression, the housekeeping gene gyrB and the corresponding gene of interest were amplified. By $\Delta\Delta^{Ct}$ method, the relative fold expression of mRNA were compared and quantified. The acquired Ct values were adjusted to the gene of interest's house keeping gene.

Statistical analysis

Each experiment was done via three independent experiments and the data was analysed with one-way analysis of variance (ANOVA). $P < 0.05$ being deemed statistically significant throughout the study. Microsoft Excel 2010 was used for all analyses during the investigation.

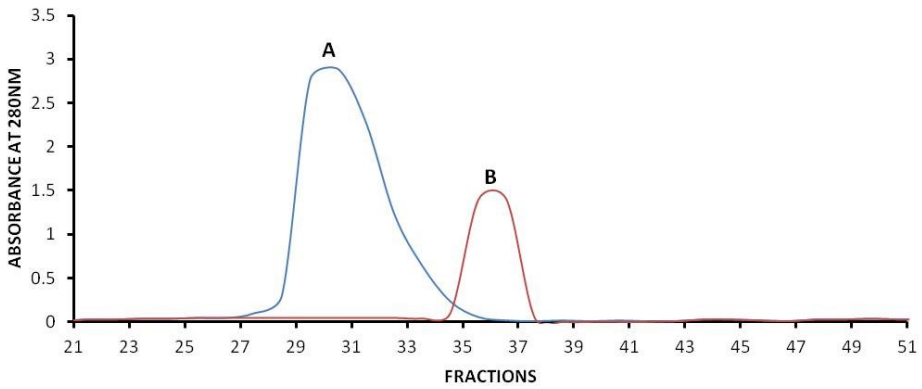

Figure 2: Abs280nm profiles of the fractions collected. A: Crude PO from Sephacryl S-100 gel-filtration (Fractions 28–33). (B) Filtered PO from DEAE Sepharose FF ion-exchange chromatography (Fractions 35-37)

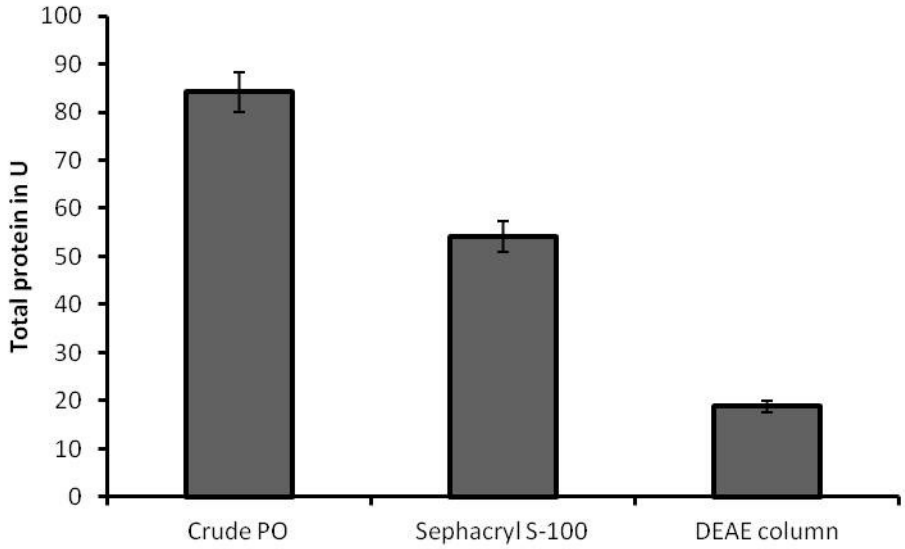

Figure 3: Total protein content of the fractions obtained from different columns. Content expressed in Units and is average of three independent experiments

4. Results and Discussion

Purification of PO

After dissolving about 16.2mg of crude PO precipitate in 0.5ml of 20mM Tris–HCl buffer (pH 7.1), the total L-DOPA oxidizing activity was determined to be 60.23U. The total protein content obtained from the crdue PO, sephacryl S-100 and DEAE column was found to be 22.34, 5.11 and 0.78mg/L. One Sephacryl S-100 gel-filtration column was loaded with approximately 0.5ml of the crude PO Figure 1. Peak fractions (28–33), which exhibited high PO activity, were gathered and combined. One DEAE Q Sepharose rapid flow ion-exchange column was laoded with 0.5ml of fraction. Peak fractions 35–37, which exhibited high PO-like activity, were gathered and combined. On the SDS–PAGE gel, the pooled fraction contained both 78.2kDa proPO and 75.5kDa PO molecules. The protein content obtained was estimated and expressed in Units (U). The content was found to be 84.23 ± 4.15, 54.16 ±3.12 and 18.79± 1.21 respectively from crdue PO, sephacryl S-100 and DEAE column was found to be 22.34, 5.11 and 0.78mg / ml. The specific enzyme activity obtained was estimated and expressed in U / mg. The content was found to be 5.203 ± 0.54, 13.92 ±2.13 and 85.4± 3.14 respectively from crude PO, sephacryl S-100 and DEAE column was found to be 22.34, 5.11 and 0.78mg/ml.

The majority of plants, microbes, and mammals have polyphenol oxidase (PPO), a copper-containing phenolase that is absent from *Arabidopsis thaliana, Brassica napus,* and green algae [20]. PPO in plants is encoded by many nuclear genome genes. In a single plant, different PPO genes are present. Potato was used to isolate nine PPO genes (StPOTP1, StPOTP2, StPOT32, StPOT33, StPOT72, and StuPPO5–StuPPO9) [21]. Bananas were used to clone four PPO genes, namely BPO1, BPO11, BPO34, and BPO35 [22] Cucumber only has one PPO gene, however tomatoes have seven PPO genes [5, 23]. With 26 members, *Salvia miltiorrhiza* has the highest number of PPO genes [24]. Using an SDS-Page electrophoresis, the molecular weight of the polyphenol oxidase enzyme isolated from the apricot fruit (Çelikezen, Fatih Çağlar , 2012% was determined in this study to be 64.8 kDa. Our study's findings are consistent with those reported in the literature [25, 26].

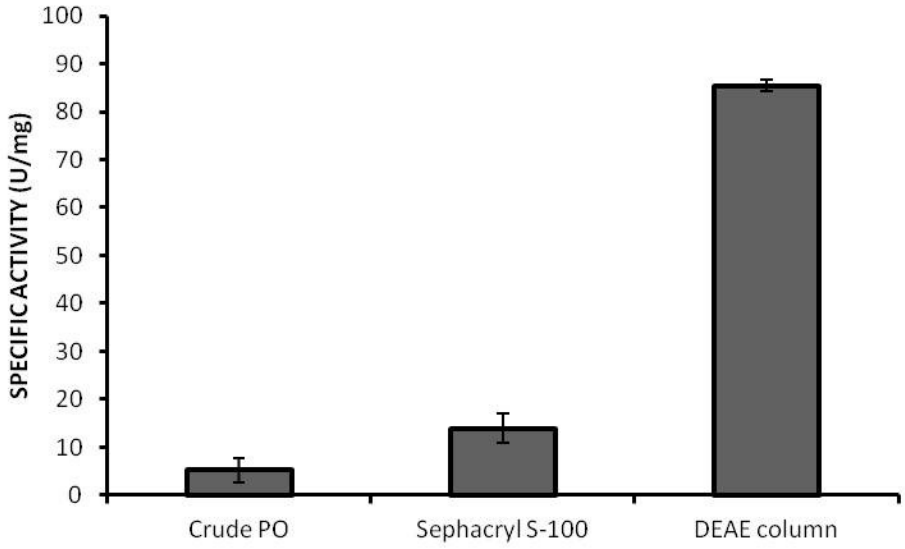

Figure 4: Specific enzyme activity of the fractions obtained from different columns. Enzyme activity expressed in U/mg and is average of three independent experiments

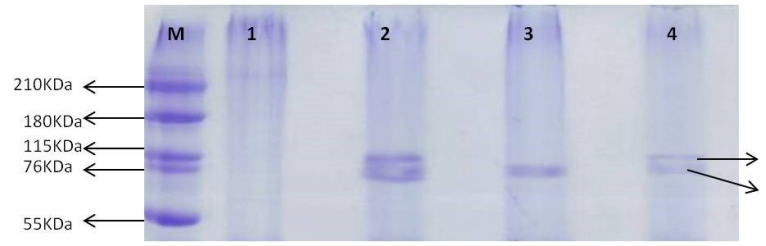

Figure 5: SDS–PAGE of PO fractions from Sea squid Reducing SDS–PAGE 12.5% gel was used. M: Molecular marker; 1: Crude PO; 2: Sephacryl S-100 fraction; 3: DEAE Sepharose Fast Flow fraction; 4: DEAE Sepharose Fast Flow fraction but stained with $AgNO_3$.

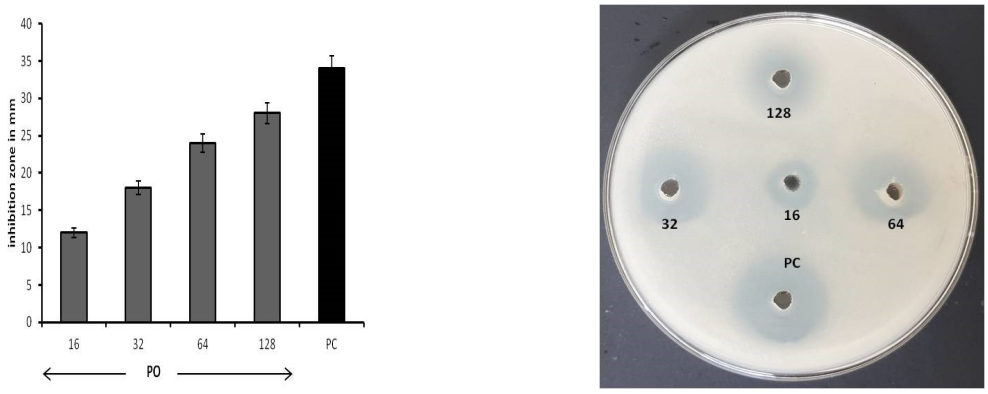

Figure 6: Left: Histogram showing the diameter of the inhibition zones in mm. Right: Agar plate showing the clear zones

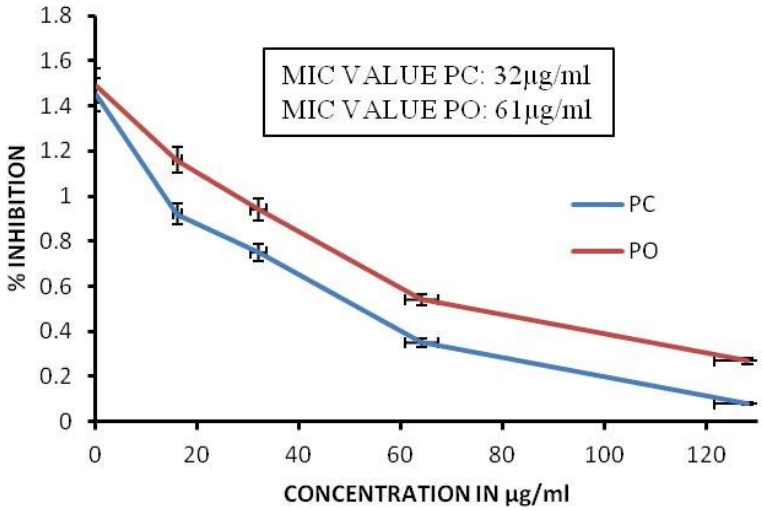

Figure 7: Percent inhibition of the *S.aureus* by broth dilution method: PO: purified fraction; PC positive control. MIC values can be seen in the graph (embedded)

Agar well diffusion

Vancomycin was used as positive control and demonstrated high significant levels of inhibition against *Staphylococcus*. PO showed significant inhibition zones when compared to positive control. The mean diameter of the zone of PO was found to be dose dependant ($P<0.05$). The inhibition zones were found to be 12±0.15, 18±0.85, 24±1.11 and 28±0.31 for 16, 32, 64 and 128µg/ml respectively. On the other hand, PC showed 34 ±2.11 inhibition zone. [27] reported antibacterial activity of five POs extracted from Morus notabilis (mulberry) against *Escherichia coli, Pseudomonas aeruginosa*.

Nan Wang, et al [28] demonstrated antibacterial activity of the POs extracted from flaxseed against *Pseudomonas*. [29] reported similar antibacterial activity of the polyphenol oxidase from Barbados cherry *(Malpighia glabra L.)* against gram negative bacteria. [30, 31] reported similar findings of the phenol oxidase as antibacterial.

Broth dilution

From the data, we found the PO to be equally competent to inhibit the growth of the culture. The OD value at 128µg / ml for PC and PO was found to be reduced to 0.08 and 0.27 from 1.45 (control). This was highly significant when compared to the positive control. The MIC which is lower is considered effective and the MIC values of the PO and PC 32 and 61µg / ml respectively.

Congo red assay

The primary polysaccharide in the *S. aureus* biofilm matrix, EPS has a structural purpose. On a Congo red plate, *S. aureus* was co-cultured using MIC doses of PO. When 64µg/ml of PO was added, the hue progressively diminished in comparison to the control, indicating a decrease in EPS synthesis.

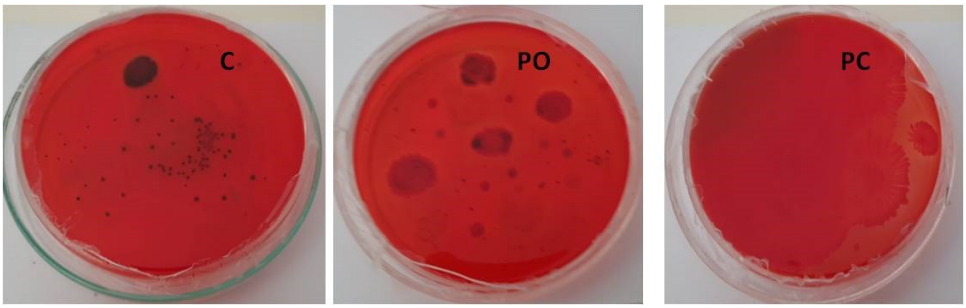

Figure 8: Congo red assay: Biofilm formation: Black colonies: Biofilm producers; Brownish / Pinkish: Non biofilm producers. C: control (without treatment); PC: positive control

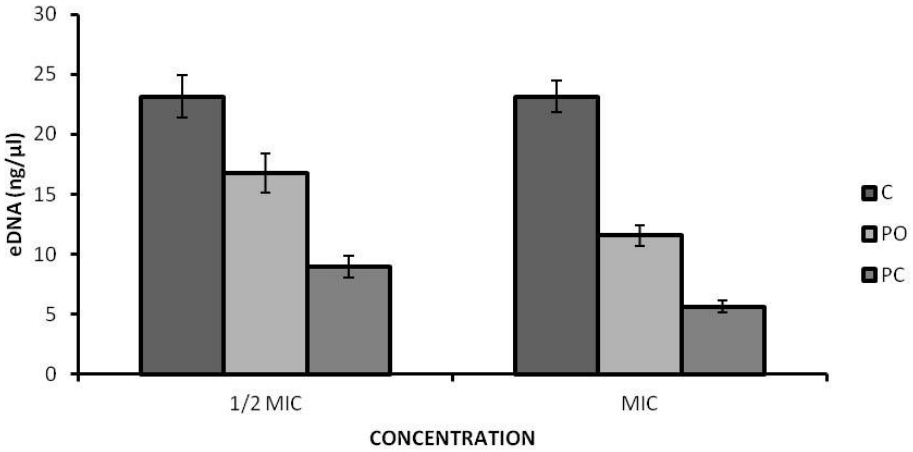

Figure 9: eDNA determination: Histogram showing the inhibition of eDNA secretion in ng/µl. C: control (without treatment); PC: positive control

Estimation of eDNA inhibition

Another matrix element of biofilm is eDNA, which is mostly derived from DNA released during bacterial autolysis, sometimes referred to as the ”binder” of biofilm. The secretion of eDNA was dramatically suppressed at ½ MIC and MIC concentrations in the biofilm cultivated. From the data obtained, we found a significant decrease in the secretion of eDNA under the treatment of PO at both ½ MIC and MIC concentrations. The eDNA was found to get suppressed to 11.54 and 16.78 at MIC and ½ MIC concentrations respectively from 23.14 (control). Our results are highly significant when compared to positive control.

Gene expression

The study found that *SarA* transcription levels decreased with PO, while transcription levels of *Aur* and *Nuc* negatively regulated genes and increased. The expression of the selected genes *aur, sarA, nuc* and the normalizer *gyrB* was quantified with real-time RT–PCR. As shown in the figures. the expression of the gene members from the Ct depicts the under and over expression of the gene members. Gene member *aur* post treatment was down-regulated to 0.42 when compared to the control (1 or 100%). Gene member *nuc* after treatment was up-regulated to 1.87 when compared to the control (1 or 100%). Gene member *aur* after treatment was up regulated to 3.16 when compared to the control (1 or 100%). On the other hand, the positive control showed similar trends as like our PO fraction. Positive control showed downregulated to 0.37 in case of *aur* expression. Positive control showed an upregulation to 2.31 and 4.53 for *nuc* and *sarA* expression.

5. Conclusion

One effective tactic against a persistent Staphylococcus aureus infection is anti-biofilm. *SarA* is a positive regulator of S. aureus biofilm development. We found that PO, the *SarA* inhibitor, was effective against *S. aureus* in both our antibacterial and antibiofilm investigations. We report for the first time, using a purified fraction of PO against MRSA strains. Various POs derived from diverse sources were evaluated as antibacterial agents. The inhibitory activity of PO on the biofilm regulatory proteins was demonstrated by this investigation. In conclusion, our research elucidated the mechanism by which PO suppresses the transcriptional regulator *SarA* of *S. aureus* to influence the production of biofilms. Moreover we report for the first time, extraction purification of phenol oxidase and directing towards oral bacteria to detect novel antimicrobial peptides.

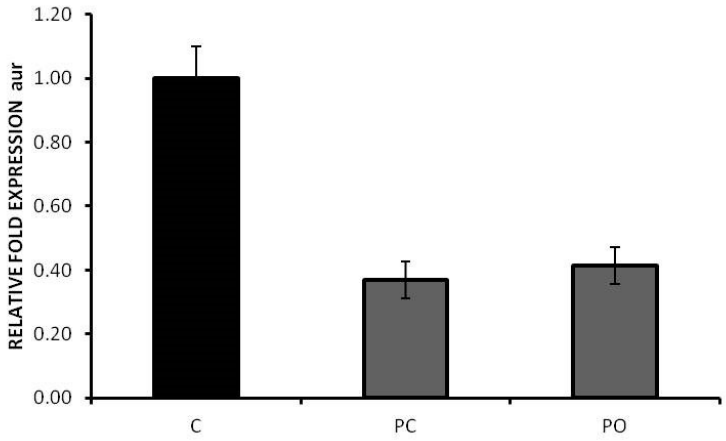

Figure 10: Histogram showing the relative fold expression of the gene member *aur*. *gyrB* was used as normalizer. All of the study was done in triplicates

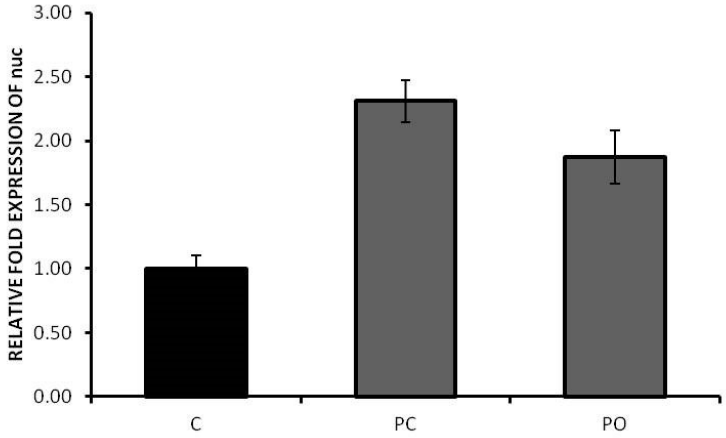

Figure 11: Histogram showing the relative fold expression of the gene member *nuc*. *gyrB* was used as normalizer. All of the study was done in triplicates

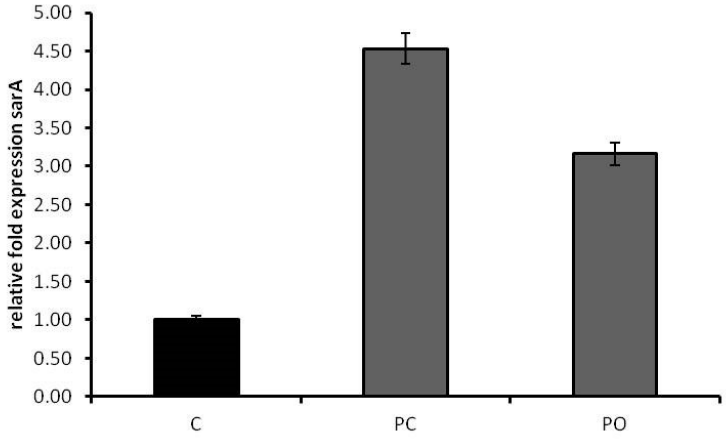

Figure 12: Histogram showing the relative fold expression of the gene member *sarA*. *gyrB* was used as normalizer. All of the study was done in triplicates

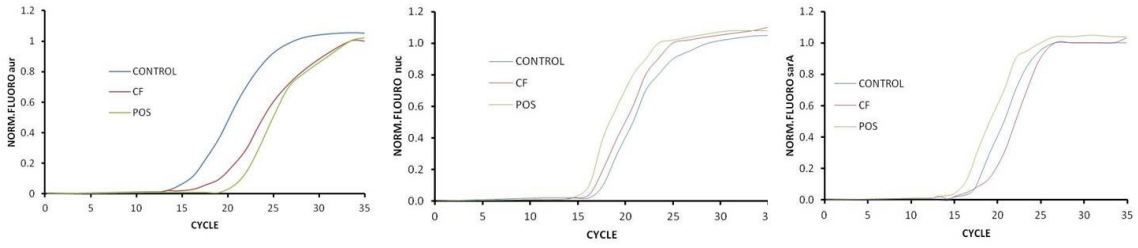

Figure 13: Real time quantitative curves of the selected genes *(aur, nus, sarA)*. *gyrB* was used as normalizer. All of the study was done in triplicates

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