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ORIGINAL ARTICLE

Anti-virulence potential of *Allium Cepa* against multi- drug resistant biofilm forming Pseudomonas strains

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ABSTRACT
*Multidrug-resistant pathogen-associated infectious diseases are one of the major health threats. Search for alternatives to antibiotics is one thrust area of medicine, hence research is being intensified in this direction. The therapeutic effect of medicinal plants is mainly due to the presence of various bioactive components and extraction of such compounds in treatment against Antibiotic-resistant organism are found in be an alternative therapeutic regime. Allium species, especially onion which is used in the daily diet by the majority of the world's population found to have high therapeutic properties due to the presence of bioactive molecules present in them. The emergence of biofilm-associated pathogens with multi-drug resistance uropathogen associated with Catheter insertion is one of the major causes of Urinary tract Infections. In this study anti-virulence property of obtained plant material against multidrug-resistant catheter, isolates were carried out. Pulverized plant material was subjected to polarity-based cold extraction and all those extracts were pooled and dried to obtain the material used for anti-virulence testing. Detailed testing of the anti-virulence and anti-biofilm activity of sub-MIC levels of the obtained plant material was performed against multi-drug resistant catheter isolates. Strains **of** Pseudomonas aeruginosa treated with Allium cepa extract had exhibited significantly low virulence factor production in comparison with the untreated isolates. The plant extract was subjected to Antibiofilm activity, Protease activity, Inhibition on pyocyanin production, the Inhibition effect of Rhamnolipid production, and its effect on Extracellular Polysaccharide (EPS) production. The present study indicated that the plant material has potent anti-virulence and anti-biofilm activity against urinary catheter isolates and can be an alternative prospect to manipulate catheter-associated urinary tract infections.*
Keywords: *Multidrug resistance(MDR), Allium cepa, Antibiofilm, anti-virulence, Pseudomonas aeruginosa*

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INTRODUCTION

Antimicrobial resistance (AMR) is becoming an arising threat to the treatment of many pathogenic organisms in the world. Alternative therapeutic strategies are in great demand and therapeutic properties of medicinal plants and their purified constituents are used in this direction [1,2,3]. Phytomedicine and phytocompounds are good alternatives to synthetic chemical antimicrobial agents and antibiotics, because of the serious side effects. Inappropriate and overuse of antimicrobials also resulted in the emergence of new deleterious infections.

The therapeutic effect of such medicinal plants is mainly due to the presence of various bioactive components, such as antioxidants, including phenolics, vitamins, organo-sulfur compounds, volatile sulfur compounds, fatty acids, and tocopherols [2,4,5].

Allium species, especially onion used in the daily diet by the majority of the world's population found to have high therapeutic properties due to the presence of bioactive molecules present in them. The members of the genus are reported to have potential bioactive properties which are used to treat pathogenic infections, cancers, and cardiovascular diseases [3,6]. Allium species associated with bioactive constituents namely organo-sulfur compounds (alliin and γ-glutamylcysteine), volatile sulfur compounds (diallyl sulfide, diallyl disulfide, etc.), proteins (lectins), prostaglandins, fructan, vitamins (B1, B2, B6, C,

and E), polyphenols, fatty acids, and essential oils [3,7]. Bioactive polyphenols of Allium species have antioxidant, antimicrobial, antifungal, cytotoxic, and mutagenic activities [5,8]. Chemical constituents of Allium species are used in perfume and cosmetic industries as well.

Urinary Tract Infection (UTI) is one of the commonest infections that occurring in catheterized patients. [9] reported the Urinary tract infection (UTI) associated with the urinary catheter is one of the major types of nosocomial infection and needs utmost attention Colonization virulent and Multidrug resistance bacteria in the urinary tract and catheters results not only in infection but also various complications, such as blockage of catheters with crystalline deposits of bacterial origin, generation of gravels and pyelonephritis. [10]

Biofilms are an assemblage of surface-associated microbial cells with complex bacterial communities residing within an exopolysaccharide matrix that adheres to a surface. The presence of pathogenic bacterial communities embedded in a biofilm can cause chronic, nosocomial, and medical device-related infections Biofilm forming Uropathogenic organisms are associated with persistent and chronic inflammation leading to complicated and or recurrent UTIs. Biofilms act as a barrier for efficient antibiotic penetration in bacteria. Most of the biofilm formers are associated with Multidrug-resistant organisms (MDRO) and their treatment and management are hard. This Antibiotic-resistant nature of biofilms requires novel therapeutic approaches for its prevention and treatment [11,12].

To overcome the challenges of multidrug-resistant uropathogens an attempt was made to investigate the anti-virulence and anti-biofilm activity of sub-MIC levels of plant extract of *Allium cepa* was performed against multidrug-resistant biofilm-forming catheter isolate

MATERIAL AND METHODS

Plant Material

The aerial parts and bulbs of *Allium cepa*, naturally growing plants belonging to the Liliaceae family were collected from Kerala agriculture farm of Pathanamthitta. Kerala In the laboratory, the plant samples were air-dried at room temperature for later analysis.

Preparation of the *Allium cepa* extract

Ethanol and water extraction of *Allium cepa* sprouts airdried powder was carried out using Soxhlet apparatus with a final volume of ethanol/water (1:1, v/v). The extracts were stored under refrigerated conditions until used.

Strain and culture condition

The strain used in this study was obtained from the culture collection of *P. aeruginosa* (RRLP1, GenBank ID: KR149278 and RRLP2, GenBank ID: KT309033). These isolated were reported to have multidrug resistance and produce various virulence factors (VFs) and were obtained from urinary catheters of patients having Catheter-Associated Urinary Tract Infection s (CAUTIs) and were maintained in the laboratory of Pushapagiri Medical College. ((13.) These strains were further sub-cultured to achieve an optical density (OD) of 0.08 at a wavelength of 620 nm and used for the study

MIC determination and sub mic determination

The MIC was assessed against the test organisms (PAO1, RRLP1, and RRLP2), following the micro-dilution method according to the Clinical and Laboratory Standards Institute guidelines (14). All further experiments in the study were performed at sub-MIC levels of the plant material. Dose-response growth curve analysis of the test organisms was performed at sub-MIC levels of the plant material.

Allium cepa Antibiofilm effect

Biofilm inhibition assay of the plant extract was carried out as described previously (13). Biofilms induced by test organism carried out in microtiter plates (MTP) Sample with and without extracts tested for without (Control) for biofilm formation at sub-MIC levels of plant material. LB broth culture 1×10^6 CFU ml-1 of test organism were incubated at 37°C for 48 hours. Planktonic cells were removed and wash the cells with sterile water and dried. MTP well with biofilm was further stained with 0.4% (w v−1) crystal violet, washed, and solubilized with 95% (v v−1) ethanol. Absorbance at 570 nm (ELx- 800 absorbance reader, BioTek®, Mumbai, India) and the percentage biofilm inhibition was calculated using the formula:% of inhibition = [(Control OD570−Test OD570)/×Control OD 570] × 100

Effect of sub-mic levels of Allium cepa on the virulence of tested strains, *in Vitro* Estimation of Extracellular polysaccharide (EPS)

For the estimation of EPS production Biofilm production on the coverslips(1 × 1 cm) was estimated in the with or without sub-MIC levels of *Allium cepa* extract and was incubated. Total carbohydrate assay was carried out for the quantification of EPS as per the procedure defined(15). Biofilm was washed in (0.9%) NaCl and incubated with H_2SO_4 and incubated for 1 h and absorbance was measured in dark at OD 490nm(ELx-800 absorbance reader, BioTek®).

Estimation Pyocyanin production

The test organism with treated and untreated plant extracts was estimated for the amount of Pyocyanin production using a standardized procedure (16). All the test organisms were grown without and with various concentrations of plant extract (23µg/ml,50µg/ml,75µg/ml, 100µg/ml and 125µg/ml) and was incubated for 24 h. After centrifugation cell-free supernatants were collected, chloroform, and acidified. The absorbance of this acidified chloroform layer was measured

Staphylolytic activity Estimation of protease estimation LasA

To determine the staphylolytic activity *Staphylococcus aureus* ATCC 25923 was boiled for 10 min and centrifuged for a further 10 min at 14,000 rpm. The pellet was resuspended in 10 mM phosphate buffer, pH 7.5, to an optical density at 595 nm of 0.8. Determination of LasA protease activity was done by measuring the staphylolytic activity (17) of the extract-treated(extract with various concentration) cell-free culture supernatants as test and untreated as a control. 900 µl of boiled *Staphylococcus aureus* culture cells were mixed with 100 µl of treated and untreated culture supernatant and incubate for 60 min. The OD value was measured at the wavelength of 600 nm and was compared. The activity was measured as the change in the OD 600 h−1 µg−1 protein.

Estimation of elastase (LasB)

The elastolytic activity was determined by treating the culture-free supernatant with allium cepa plant extract at various concentration and untreated supernatant was kept as control. The test was done using the Elastin Congo Red (ECR) method (18). 900 µl of ECR buffer(20mg Himedia Laboratories Pvt Ltd) was mixed with 100 µl of the cell-free supernatant and incubated in shaker incubator for 3hrs at 37°C. Insoluble ECR was removed and the elastolytic activity was measured as a function of the change in OD495 µg−1 of protein in comparison with the control

Determination Rhamnolipids production

Quantification of Rhamnolipids by the organism in presence of *Allium cepa* extract done using the orcinol method as described by (19) . Cell-free culture supernatants treated and untreated extract as control were taken and orcinol reagent was added to it. The mixture was heated at 80°C for 30 min, and the OD was measured at 421 nm and compared with the control. Test organisms (PAO1, RRLP1, and RRLP2) were treated with sub-MIC levels of extract and untreated organisms were kept as control.

All the results were presented as % reduction and calculated using the formula% Inhibition = [(Control OD - Test OD)/× Control OD] × 100

RESULT AND DISCUSSION

Determination of sub-MIC levels and growth curve study

The MIC of the extract was detected to be 900 µg/ml, 1048 µg/ml, and 1100 µg/ml for *P. aeruginosa* PAO1(P1), *P. aeruginosa* RRLP1(P2), and *P. aeruginosa* RRLP2(P3) respectively. All the further anti-QS analysis of the plant material was assessed at sub-MIC levels (0 µg/ml- 900µg/ml). The growth pattern of sub-MIC levels of the plant extract against tested organisms was studied through a dose-response growth curve study (Figure 1). Despite an extended lag phase, all the tested organisms exhibited similar growth patterns with the respective controls.

The plant material at sub-MIC levels exhibited the significant anti-virulence property

Pathogenesis of *Pseudomonas aeruginosa* is determined by virulence factors and quorum regulated factors protease, elastase, Rahmnolipids, Payocyanin production, etc(20). Multidrug resistance Uropathogenic strain of Pseudemys plays a role in biofilm formation due to UTI infections.

The present study showed that the given plant extract *Allium cepa* plays a major role in the inhibition of those virulence factors against the tested organism

Inhibition of Biofilm production by the tested organisms after the plant material treatment was tested (Figure 1). Treatment with the plant material, reduced the biofilm biomass of PAO1 and RRLP1 by 57.98±2.1% and 45.35±2.1% respectively at 500 µg/ml whereas, RRLP2 exhibited minimum biofilm production at 400 µg/ml (% inhibition - 45.72±1.9).

Extra Polysaccharide formation (EPS) by the test organisms, with or without the extract was quantified. The result showed a significant (Figure2) inhibitory action of the extract against EPS production in all the tested organisms. Plant extract of (400 µg/ml) showed 54.21±2.7% reduction in EPS production against PAO1 strain. Among the other isolates, the plant material at 500 µg/ml exhibited maximum EPS reduction by 51.73±2.19% (RRLP1) and 56.12±2.8% (RRLP2)

Allium cepa plant extract showed a considerable reduction in pyocyanin production by PAO1 was 56.21±3.06% with 500 µg/ml of. Pyocyanin production by the isolates exhibited a significantly dropped when treated with *Allium cepa* extract 56.54±2.01% (500 µg/ml) and 51.32±2.9% (500 µg/ml) in RRLP1 and RRLP2, respectively (Figure 3).

Estimation of protease (LasA) (Figure 4), revealed that at 500 µg/ml exhibited maximum protease depletion in PAO1 (50.34±2.1%) whereas, in clinical isolates (RRLP2 and RRLP1), the rate of inhibition was 55.82±2.8% and 58.13±2.45% respectively.

Treatment with the *Allium cepa* exhibited maximum elastase inhibition in PAO1 (58.61±3.02%) and RRLP1 (41.38±3.0%) at 500 µg/ml whereas, with RRLP2 the reduction was 62.19±2.83% at 500 µg/ml (Figure 5).

Rhamnolipid production inhibition assay was performed with various concentrations, the *Allium cepa* extract of at 500 µg/ml, effectively inhibited the rhamnolipid production in the tested organisms, (Figure 6). Rhamnolipid inhibition was maximum observed in PAO1 (60.11±1.09 %), followed by RRLP2 (65.11±2.45%) and RRLP1 (66.10±2.98%).

The majority of nosocomial UTI are related to bacterial colonization and biofilm formation in urethral catheterization [21] Biofilm inhibition is one of the major ways to prevent these device-associated nosocomial infections. Reports of earlier workers showed that phytochemicals are found to have prominent anti-biofilm properties [22]. The present study also proved that the anti-biofilm activity of sub-MIC levels of the *Allium cepa* against UTI causing *P. aeruginosa* reference strains as well as clinical isolates. Antibiofilm and anti-virulence properties due to the bioactive properties of plant extract preventing the EPS production leading to failure in biofilm establishment. In biofilm-forming *P. aeruginosa* antibiotics show reduced efficacy due to the impermeable polysaccharide matrix and active efflux pumps in the cells. Thus biofilm act as a physical and/or cellular barriers do not allow antibiotics to reach their desired target(s) [23]. Evidence from the earlier works showed that combination drug therapies with both anti-virulence agent(s) and antibiotics may result in reduced biofilm formation that makes microbes susceptible to a reduced dose of antibiotics [24-26].

Many natural or synthetic agents are proved to have anti-virulence properties and the Quantification of virulence phenotypes its anti-virulence properties [23]. This study also proved that virulence factors that are significant for the pathogenesis of CAUTIs can be utilized to evaluate the anti-virulence property of sub-MIC levels any bioactive compounds. Quorum sensing-controlled expression of an array of virulence determinants (EPS, pyocyanin, protease, elastase, and rhamnolipid) in ***P. aeruginosa*** and marked suppression of these VFs by the plant material indicated that it is an active and potent anti- virulence candidate.

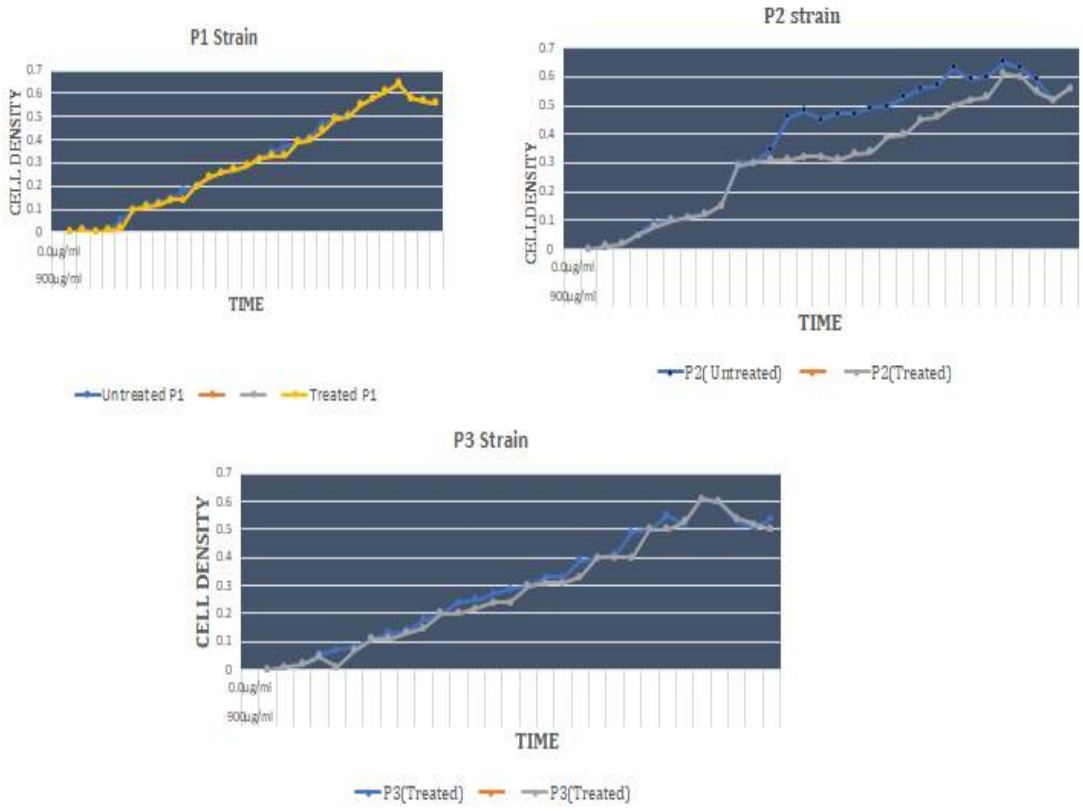

Figure 1 Cell density compared with treated and untreated Allium cepa extract with *Pseudomonas aeruginosa* (PI P2 and P3)

Figure 2 Biofilm biomass reduction Assay of Plant extract against Pseudomonas strains

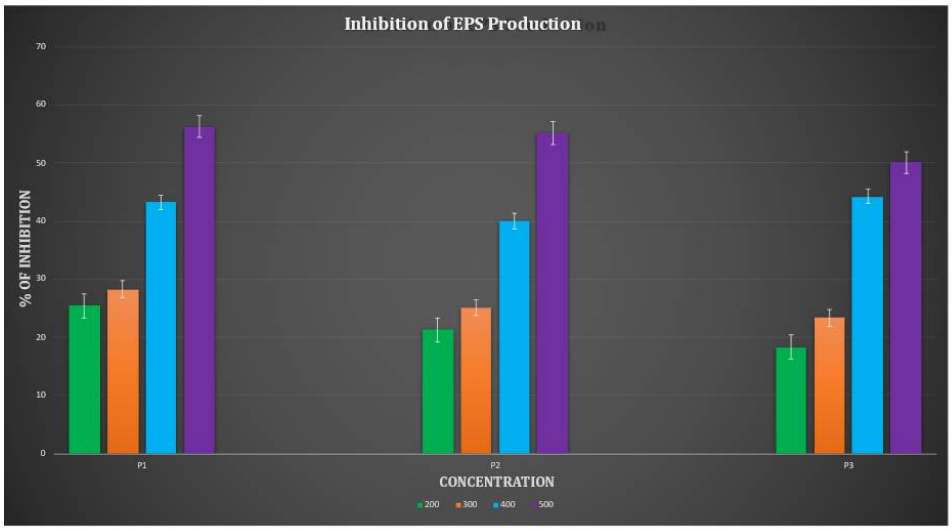

Figure 3 EPS Inhibition Assay of *Allium cepa* extract against Pseudomonas strains

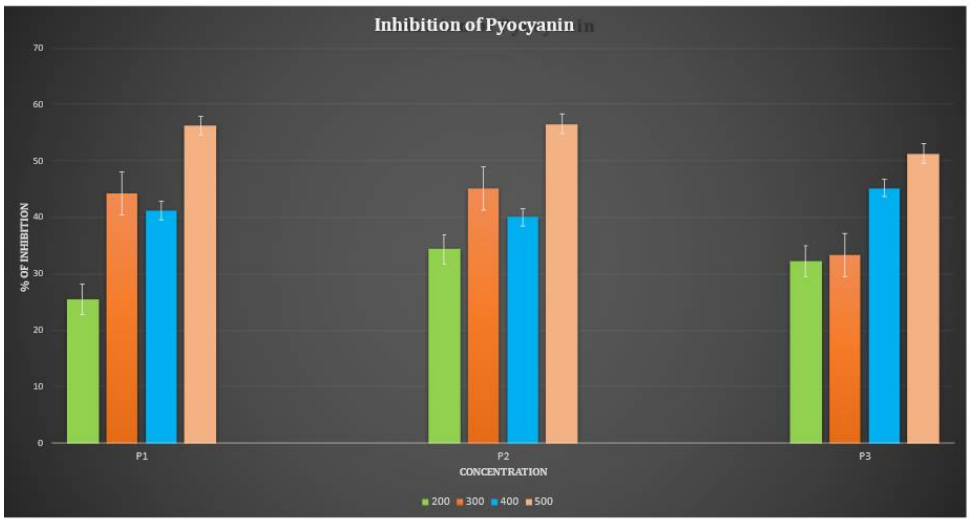

Figure 4 Pyocyanin Inhibition Assay of Pyocyanin against Pseudomonas strains

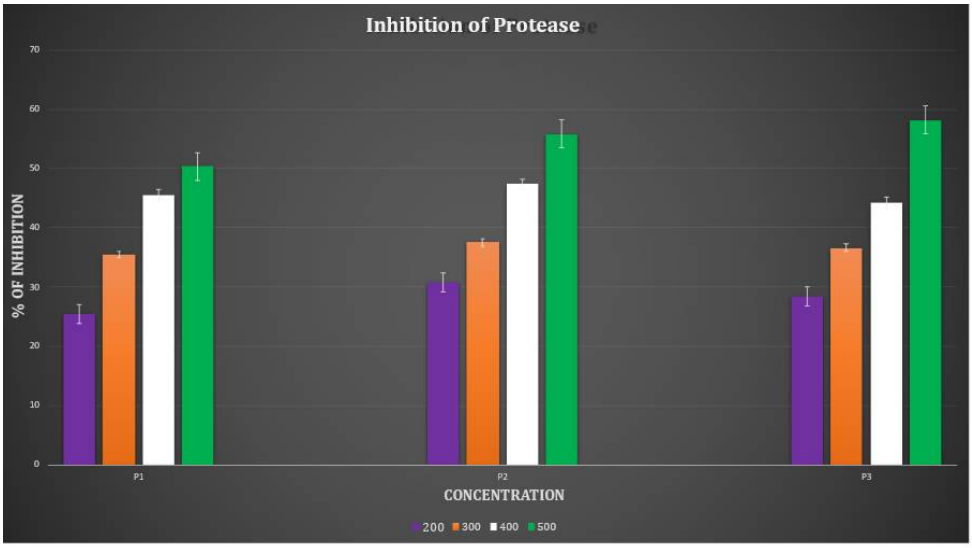

Figure 5 Inhibition Assay of Protease activity by *Allium cepa* extract

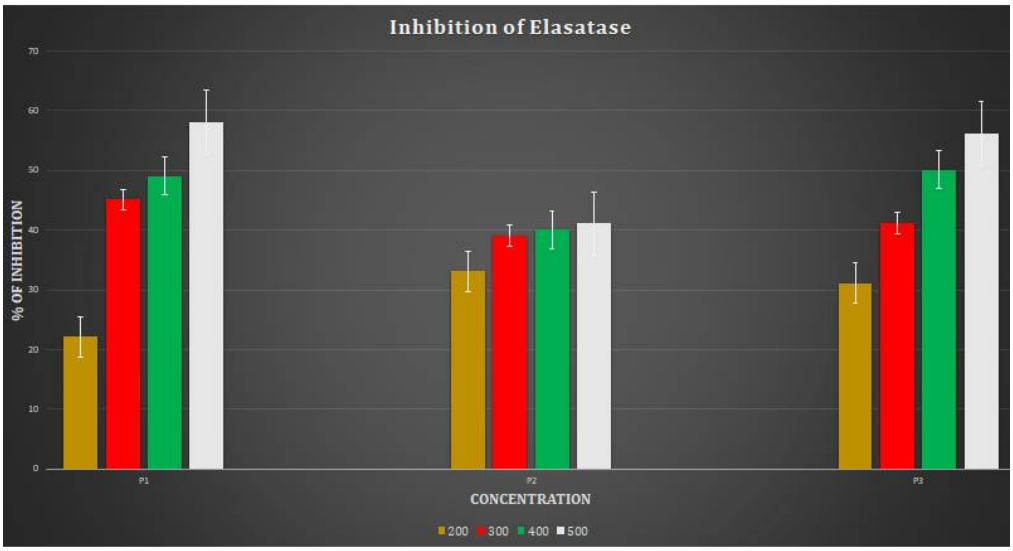

Figure 6 Inhibition of Elastase activity by *Allium cepa* extract

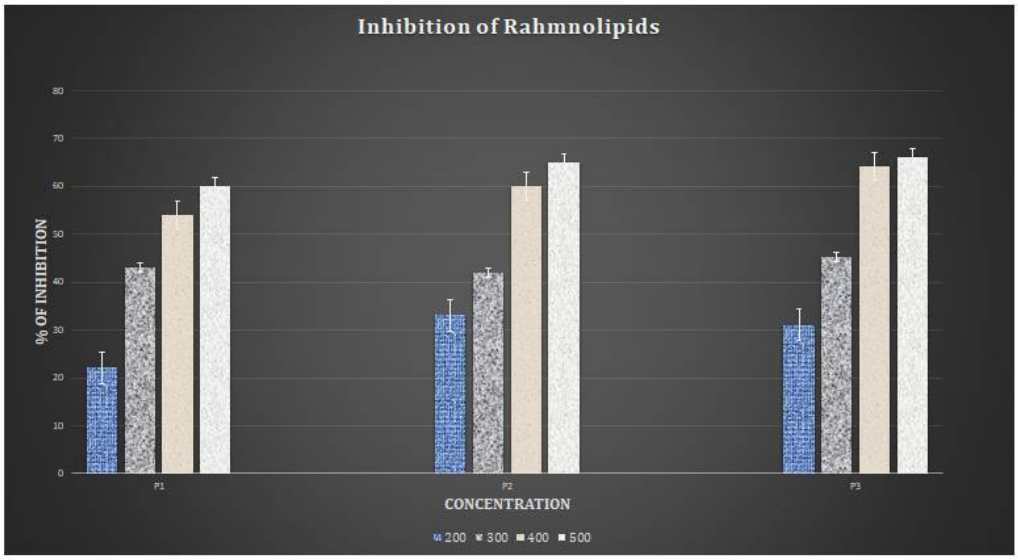

Figure 7 Inhibition of Rahmnolipids activity by *Allium cepa* extract

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