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Assessment of antimicrobial properties of a lectin isolated from *Amaranthus cruentus* Linn

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Abstract

Lectins are diverse class of proteins which have the ability to bind carbohydrates with considerable specificity and involved in many biological processes like host pathogen interactions, cell targeting, cell-cell communications, cancer metastasis and differentiation. Plants are rich sources of lectins that have great implications in medicinal and therapeutic applications. The antimicrobial property of a lectin isolated from *Amaranthus cruentus* Linn seeds has been explored in this study. ACL (*Amaranthus cruentus* Linn lectin) was isolated, purified and characterised by hemagglutination assay with human ABO blood groups and the molecular weight was determined by SDS PAGE. ACL was subjected to evaluation for inhibition of microbial growth by disc diffusion against human pathogenic Gram positive and Gram negative bacterial strains and some fungal strains. MIC and MBC were assessed. The effects of ACL on biofilm forming bacterial strains was assessed using crystal violet assay. The isolated and purified ACL have a molecular weight of 60 KDa. The Hemagglutination activity of ACL was inhibited by N-acetyl galactosamine. ACL exhibited both antibacterial and antifungal activities with considerable zones of inhibition. Biofilm mass reduction was observed in ACL treated bacteria. These findings indicate that ACL is having therapeutic applications with great importance in clinical microbiology.

Keywords: *Amaranthus cruentus* Linn, antimicrobial, hemagglutination

1. Introduction

Lectins are heterogeneous group of proteins with different carbohydrate binding specificity and biological activities (Van-damme *et al.*, 1998) [28]. The lectin binding sites within the carbohydrate, called the carbohydrate recognition domain (CRD), promote specific recognition which are in accordance with the key-lock model (Kennedy *et al.*, 1995) [13]. These proteins, which can be detected through hemagglutination assays, interact with different carbohydrates present in erythrocyte cell surface. These interactions can play a role in cellular adhesion and other cellular recognition events (Irlanda *et al.*, 2017) [9]. Lectins play important physiological roles depending on their properties and distribution in tissues. The characteristic property of lectins to recognize other molecules in a distinct way makes it relevant in research involving purification, structural analysis, *in vitro/in vivo* applications of these macromolecules and biotechnological uses in different areas such as molecular and cell biology, immunology, pharmacology, medicine, clinical analysis, nanotechnology as well as in systems for drug release (Santos *et al.*, 2013) [23]. Lectins have been shown to exhibit mitogenic effects (Chan *et al.*, 2011) [5] and antiproliferative activities on cell lines of human cancer (Liu *et al.*, 2009) [15], inhibition of bacterial and fungal growth, action as promoting agents in cell aggregation, immunomodulatory activities and toxic effects. These natural source of proteins are promising drugs for treatment and in diagnosis of human diseases with less side effects over chemical drugs.

Plant lectins are mostly present in the seed cotyledons besides roots, stems and leaves. Lectins have been reported to possess antimicrobial activity. The role of lectins in the antibacterial defense of *Arabidopsis thaliana* is correlated with bacteria mediated pattern triggered immunity and with stomatal innate immunity acting in the bacteria mediated stomatal closure (Singh *et al.*, 2012) [25]. Lectin from *Amaranthus viridis* Linn presents antifungal activity, inhibiting the growth of the phytopathogenic fungi *Botrytis cinerea* and *Fusarium oxysporum* (Kaur *et al.*, 2006) [11]. Ricin is another lectin having a role in plant defense against pathogens and insects besides its antiviral activity. A new lectin isolated from *Calliandra surinamensis* leaf pinnulae with cytotoxicity to cancer cells, antimicrobial activity and was reported to possess antibiofilm effect (Thamara *et al.*, 2017) [26].

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Amaranthus cruentus Linn collectively known as amaranth or pigweed is an annual flowering plant in the family Amaranthaceae that yield staple amaranth grain. It is a tall annual herb with clusters of dark pink flowers. The plant can grow up to 2m in height, and blooms in summer to fall (Flora of Tamil Nadu, VOL. II, 1987). The present study was undertaken to assess antimicrobial property of *Amaranthus cruentus* Linn seed lectins.

2. Materials and Methods

2.1 Chemicals

Mueller Hinton agar, Nutrient broth, Luria Bertani broth, Gentamicin, Sabouraud's Dextrose Agar, DMEM, Streptomycin, Dimethyl sulphoxide (DMSO), Crystal Violet, were of analysis grade commercially available from Sigma Chemical Co., St. Louis, MO, USA.

2.2 Collection, Extraction & purification of ACL

Plant sample was collected from Vallakadavu, Thiruvananthapuram and identified by Dr G Valsaladevi, curator (Retd.), Department of Botany, University of Kerala. A voucher specimen was put into Kerala University Botanical Herbarium with voucher No. KUBH 7501. *Amaranthus cruentus* Linn seeds were weighed and homogenised using mortar and pestle in 5mM PBS (PH 7.4); centrifuged, filtered and the supernatant was collected. The supernatant was subjected for ammonium sulphate (0-60%) precipitation and kept for half an hour at 4°C. After the period, the centrifuged precipitate so obtained was dissolved in minimum volume of buffer (1mL PBS in each tube). Dialysis was done against 1mM PBS at 4°C with a three times buffer change with constant stirring in a magnetic stirrer. The dialysed sample was subjected to gel filtration using Sephadex G-75 1.5 x 75 cm column equilibrated with 5mM PBS. Loaded the column with 1mL sample and allowed to pass into the gel matrix. Elution was done with PBS and 2mL fractions were collected. Absorbance of the eluted samples were measured at 280nm with PBS as blank. An elution profile is plotted with OD at 280nm against number of fractions. Each fraction was tested for heamagglutination.

2.3 Heamagglutination assay

The heamagglutinating activity was assayed on U bottomed microtiter plates according to the protocol of Debray. Human ABO blood groups were used in the native form. A volume of 100µL of the two fold serially diluted lectin solution and 100µL of erythrocytes were added to each well and incubated for 30 min at room temperature. Heamagglutination Units were defined as the reciprocal of the titre that corresponds to the highest dilution giving visible heamagglutination.

2.4 Protein determination

Total protein content of crude sample and purified sample was determined by Folin-Lowry's method and yield of isolated lectin was assayed using UV visible spectrophotometer with an absorbance of 620nm.

2.5 Determination of total carbohydrate

Total carbohydrate content of crude sample and purified sample was determined by Phenol sulphuric acid method and the percentage of total carbohydrate present was calculated.

2.6 Determination of molecular mass

The molecular size of the protein was determined by polyacrylamide Gel Electrophoresis. SDS-PAGE was done

according to the procedure of Laemmli (1970). Partially purified lectin was mixed with an equal amount of sample buffer (1X loading buffer) and then boiled at 95°C for 2 minutes. The tubes were placed on ice after boiling and 50µl aliquots were applied to the wells of a mini slab gel with a protein marker. After SDS-PAGE, the gel was stained with staining solution followed by discoloration by excessive staining with destaining solution.

2.7 Carbohydrate binding specificity

The study was performed analogous to heamagglutination assay using different sugars. 100µL of 100mM sugar solutions were added to a 96 well round bottomed plate and to this equal volume of lectin was added and serially diluted. The plates were incubated for 1 hour at 37°C. Then added RBC suspension and the mixture was allowed to stand for 1 hour at room temperature. The degree of heamagglutination inhibition was recorded as the titre defined as highest dilution giving positive heamagglutination (Ravindranath *et al.* 1985) ^[19].

2.8 Strains of tested organisms

The bacterial and fungal strains used in the study were obtained from the Microbiology Laboratory, Department of Biotechnology, University of Kerala. Gram positive bacterial strains were *Staphylococcus aureus* and *Streptococcus pyogenes* and Gram negative strains were *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumonia*, *Salmonella typhi*, *Proteus vulgaris*, *Serratia marcescens* and *Vibrio cholera*. The fungal pathogens were *Aspergillus flavus*, *Candida albicans* (yeast) and *Penicillium chrysogenum*.

2.9 Antibacterial activity screening

The partially purified lectin were screened for their antibacterial activity by the standard Disc Diffusion method. Inoculated the dried surface of a Mueller-Hinton agar plate by streaking the swab over the entire sterile agar surface. Disc were prepared by impregnating plant lectin and antibiotic solution of about 20-25µL per disc. Incubated at 37°C for 24 hours. Examined each plate and measure the diameters of the zones of complete inhibition, including the diameter of the disk (Alfred Bauer *et al.*, 1959) ^[4].

2.10 Determination of MIC and MBC

100µL of plant lectin was serially diluted with 100µL nutrient broth in the dilutions 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, and 1:256 in a 96 well plate. 1×10^6 cells from culture plate was transferred to 1mL normal saline. It was diluted to 1: 20 ratio with normal saline. 5µL of this culture is transferred to each well and the plate was incubated for 37°C for 18-24 hours (Tripathi K.D. *et al.*, 2013) ^[27]. To determine MBC, 100µL of the sample from the MIC plates are plated on agar plates and incubated at 37°C for 18-24 hours and number of colony forming units were counted (Amyes S *et al.*, 1996) ^[2].

2.11 Antifungal activity screening

Antifungal activity was assessed by disc diffusion in Sabouraud's Dextrose agar. Inoculated the dried surface of a Sabouraud's Dextrose agar plate by streaking the swab over the entire sterile agar surface. Disc were prepared by impregnating plant lectin and antifungal solution of about 20-25µL per disc. Place the appropriate disks evenly on the surface of the agar plate either by using a sterile forceps or the dispensing apparatus. Incubate the plates at 25°C for 24-48 hours. Examine each plate and measure the diameters of the

zones of complete inhibition, including the diameter of the disk (Barry A L, Brown S D *et al*, 1996).

2.12 Antibiofilm activity

100µL of overnight bacterial broth cultures and 48 hours incubated fungal strain *Candida albicans* were seeded as triplicates in micro titre plates. The plates were incubated at 37°C for 48-72 hours. 100µL ACL was added to seeded wells except control and incubated at 37°C for 24 hours. The wells were washed with PBS and 100µL of crystal violet solution was added and incubated for 10 minutes. Pipette out the dye, and 100µL of DMSO/Methanol is added and OD was measured at 600nm. Biofilm mass reduction was compared with that of control wells (Stepanovic *et al*, 2007).

3. Results

3.1 Purification of ACL

The supernatant of homogenized ACL was found to have strong agglutinating activity and was purified with gel filtration chromatography. The active fractions (10 and 11) were pooled together and showed strong hemagglutinating activity against human blood erythrocytes. ACL showed a single protein band with an apparent molecular mass of 60KDa by SDS PAGE with and without 10 mM 2-mercaptoethanol in the sample buffer.

3.2 Hemagglutinating activity

ACL agglutinated native human ABO blood groups and among them, A⁺ group showed maximum hemagglutination with a titre value of 16.

3.3 Carbohydrate binding Specificity

One of the most important biochemical properties of lectin is their carbohydrate binding specificity. *Amaranthus cruentus* Linn lectin showed affinity to N-acetyl galactosamine with characteristic inhibition with a titre value of 8 among twelve other sugars at a concentration of 100mM.

3.4 Antibacterial Activity

ACL exhibited a strong antibacterial effect on Gram positive *Streptococcus pyogenes* with a zone of inhibition of 8 mm with the addition of ACL. However, the lectin showed less inhibitory effect on *Staphylococcus aureus*. ACL showed a strong antibacterial activity on Gram negative *Serratia marcescens* than the other strains with an antibacterial zone of 9 mm. ACL did not inhibit well against Gram negative *Pseudomonas aeruginosa*.

The minimum inhibitory concentration of ACL was found to be 0.289 mg/mL which inhibit the microbial growth effectively. Colony forming units are counted from MBC plates of different lectin concentration. Visible bacterial colonies are countable from MBC plates. This suggest that lower lectin concentration may be bacteriostatic in nature.

3.5 Antifungal Activity

In vitro antifungal susceptibility by ACL was determined against three pathogenic fungi with Amphotericin B as positive control. ACL showed significant inhibition of fungal growth against *Candida albicans* and *Penicillium chrysogenum* whereas *Aspergillus flavus* did not produced characteristic zone of inhibition and is resistant to ACL. The inhibition of fungal growth can occur through lectin binding to hyphas resulting in poor absorption of nutrients as well as by interference on spore germination process.

3.6 Antibiofilm Activity

The effect of ACL on biofilm formation under *in vitro* conditions was monitored by the binding of crystal violet to adherent cells, which directly reflects the ability against the biofilm formation of different Gram negative and Gram positive bacteria. 0.572mg/mL of ACL is the dosage taken for biofilm assay. The data pointed out that, ACL effectively inhibits the biofilm formation. *Streptococcus pyogenes* showed a large biofilm mass reduction than the other strains indicating that Gram positive organisms are more susceptible to the lectin as compared to Gram negative bacteria. Biofilm mass reduction was very low in *Staphylococcus aureus*, *Klebsiella pneumonia* and *Vibrio cholera*. Biofilm mass reduction was observed in *Candida* biofilms on ACL action with a significant decrease of about 75.29% biofilm mass reduction with a dosage of 0.572 mg/mL of ACL.

4. Discussion

The present study reported the purification and antimicrobial characteristics of a lectin from ACL. ACL was isolated and partially purified from *Amaranthus cruentus* Linn successfully with good agglutination in human ABO blood groups. Most investigations have detected lectins by hemagglutination using a freshly drawn human erythrocyte suspension (Lis *et al.*, 1973).

Amaranthus cruentus Linn Lectin (ACL) was isolated and purified by ammonium sulphate precipitation with a concentration range of 0-60% which is followed by dialysis against phosphate buffer saline. Sugar binding specificity indicate that ACL showed specificity to N-acetyl galactosamine. Gel filtration chromatography on Sephadex G-75 was the method of partial purification of ACL. The second and third fractions of elution showed maximum absorbance and possess maximal hemagglutination activity. SDS PAGE of ACL gave a single band in the presence of β mercaptoethanol. SDS PAGE pattern of ACL showed a molecular weight of about 60 KDa. The result of the purification part of the study was supported by similar work on *Amaranthus cruentus* lectin by Koepe *et al.* (1988) ^[21] that study showed a molecular weight of 66KDa. The yield of lectin was assessed by Folin Lowry's method. The yield was 57.2mg/100g of seed.

Partially purified ACL when subjected to antibacterial screening with different strains of bacteria with Gentamicin as positive control. ACL against *Serratia marcescens*, *Streptococcus pyogenes*, *Klebsiella pneumoniae*, *Salmonella typhi*, and *Proteus vulgaris* showed more antibacterial activity than other bacteria. Antibacterial effect of lectins are mediated through the interaction of lectins with components of the bacterial cell wall including teichoic acid, peptidoglycan and lipopolysaccharide. It is well established that some plants contain lectins that inhibit microbial growth (Cowan, 1999). MIC was performed with various concentrations of ACL on *Serratia marcescens*. The activity confirms the dose depended inhibition by ACL. These results are comparable with that of the *in vitro* antihelminthic activity of *Amaranthus cruentus* by Torane *et al.* (2017) ^[26]. Lectin from chick pea (*Cicer arietinum* L.) was reported to possess antibacterial activity against *P. aeruginosa* and *S. marcescens* (Ajay Kumar *et al.*, 2018).

Antifungal activity was screened in *Aspergillus flavus*, *Candida albicans* and *Penicillium chrysogenum*. *Aspergillus flavus* was found to be resistant where as large zone of inhibition was seen in the other two species. The inhibition of fungal growth can occur through lectin binding to hyphas

resulting in poor absorption of nutrients as well as by interference on spore germination process. This is similar to the antifungal work in leaf extracts from *Amaranthus spinosus* by Biradar *et al.* (2016) [20]. Antifungal activity of *Pongamia glabra* seed lectins against *Candida albicans* agrees with the results obtained from the current work and from other plant legume lectins (Yan *et al.*, 2005; Ye *et al.*, 2001). The activity may be related to the lectin carbohydrate binding property (Damico *et al.*, 2003) that might endow lectin molecules with binding activity towards certain carbohydrate components in the fungal cell wall affecting its activity and viability as most lectins recognize either N-acetylneuraminic acid, N-acetylglucosamine, N-acetylgalactosamine, galactose, mannose, or fucose (Lis *et al.*, 1998).

The work investigated the effect of ACL on growth and biofilm formation in bacteria and a yeast *Candida albicans*. Biofilm mass reduction was observed in all strains. ACL on *Streptococcus pyogenes* showed a large biofilm mass reduction (%) among the bacterial strains. *Candida albicans* also resulted in effective biofilm mass reduction. These results are in reference with studies of a lectin in potato by Kabir *et al.* (2014). The antibacterial activity of lectins occur due to the specific recognition of components of the bacterial surface. Some studies reported that lectins could inhibit biofilm formation by interaction with bacterial cells constituents of the biofilm and alter the expression of genes associated with virulence and biofilm formation (Marques *et al.*, 2017). Research has shown that gram-negative bacteria are less susceptible to lectins than gram-positive bacteria

because of their outer membrane structure, the cell membrane which restricts diffusion of hydrophobic compounds through its lipopolysaccharide covering (Sartoratto *et al.*, 2004). This explains why the gram-positive bacteria *Streptococcus pyogenes* and fungus *Candida albicans* were more susceptible than the gram-negative bacteria. The antibacterial and antifungal activities of ACL is further supported by the work of Ahmed *et al.* (2013) in their study of phytochemical profiling with antioxidant and antimicrobial screening of *Amaranthus viridis* leaf and seed extracts. The emergence and clinical significance of drug resistant bacterial infection has created an urgent need for the rapid and continued development of new classes of antibiotics. Antibacterial activity is well established from the study of Sharma *et al.* (2009); in which a novel plant lectin inhibit *Streptococcus mutans* biofilms on saliva coated surfaces. These plant lectins have different structures and different action when compared with antimicrobials conventionally used to control microbial growth and survival (Nascimento *et al.*, 2000).

These findings indicate that ACL tested in this study may be natural alternative antimicrobial agents; however further studies are required to better elucidate the functional use of these lectins.

Table 1: Purification table of ACL

Sample	Total protein (mg/ml)	Relative yield of protein
CRUDE	0.968	100
ACL	0.572	59.09

Table 2: Antibacterial activity of ACL against gram positive and gram negative bacteria

Serial No.	Microorganism	Zone of inhibition ACL (mm) (114.4 µg/disc)	Zone of inhibition of Gentamicin* (mm) (400 µg/disc)
1.	<i>Escherichia coli</i>	7±1	16±1
2.	<i>Pseudomonas aeruginosa</i>	6±1	18±1
3.	<i>Staphylococcus aureus</i>	6±1	17±1
4.	<i>Streptococcus pyogenes</i>	8±1	9±1
5.	<i>Klebsiella pneumonia</i>	8±1	14±1
6.	<i>Salmonella typhi</i>	8±1	17±1
7.	<i>Proteus vulgaris</i>	8±1	17±1
8.	<i>Serratia marcescens</i>	9±1	20±1
9.	<i>Vibrio cholera</i>	7±1	22±1

*Standard antibacterial antibiotic

Table 3: Antifungal Activity of ACL against fungi

Serial No.	Microorganism	Zone of inhibition of ACL lectin (mm) (114.4µg/disc)	Zone of inhibition of Amphotericin B* (mm) (4µg/disc)
1.	<i>Candida albicans</i>	33±1	8±1
2.	<i>Penicillium chrysogenum</i>	30±1	10±1
3.	<i>Aspergillus flavus</i>	NIL	10±1

*Standard antifungal antibiotic, growth measured- radial growth in mm.

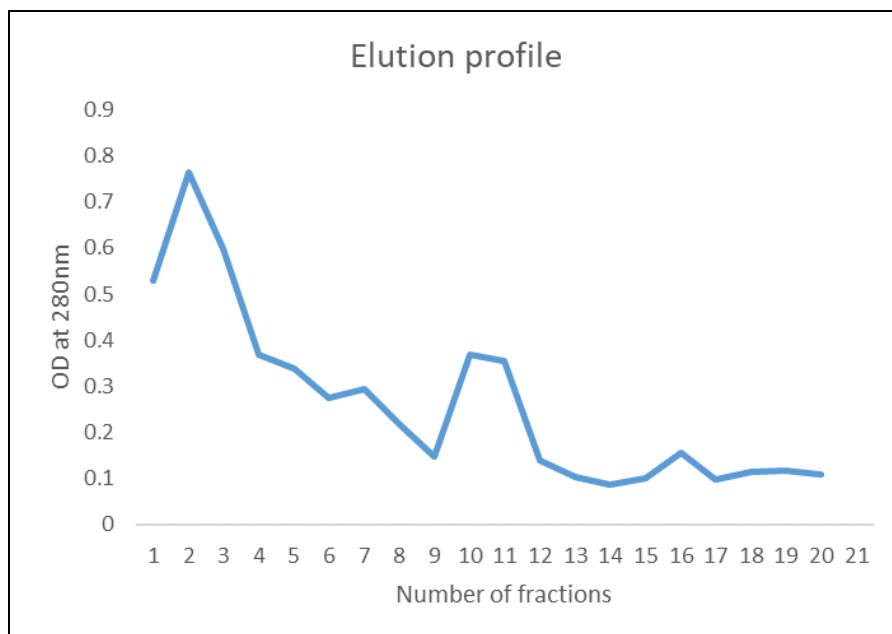


Fig 1: GFC of ACL. Dialysed sample was subjected to gel filtration chromatography with Sephadex G 75 column equilibrated with 5mM PBS and eluted. The second fraction showed maximum absorbance at 280nm.

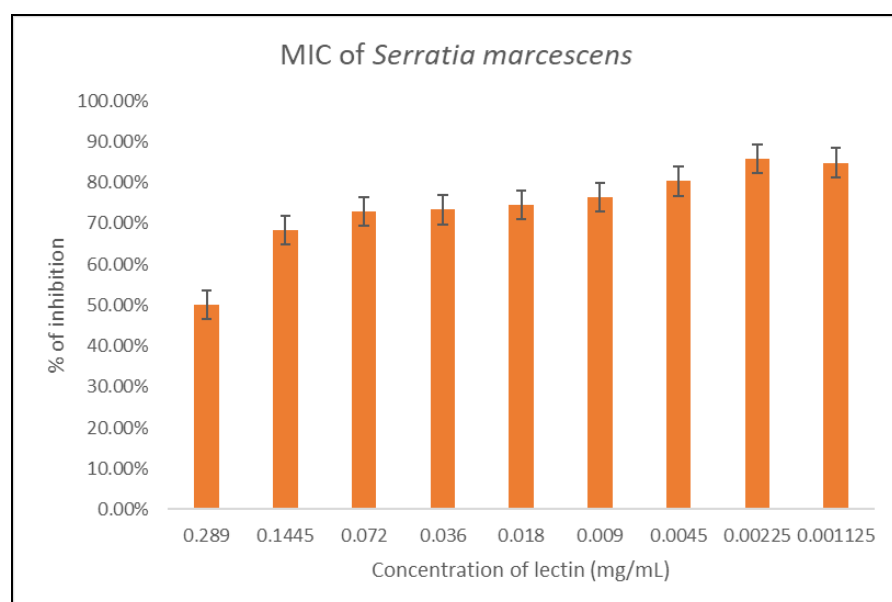


Fig 2: MIC of *Serratia marcescens* by broth dilution method

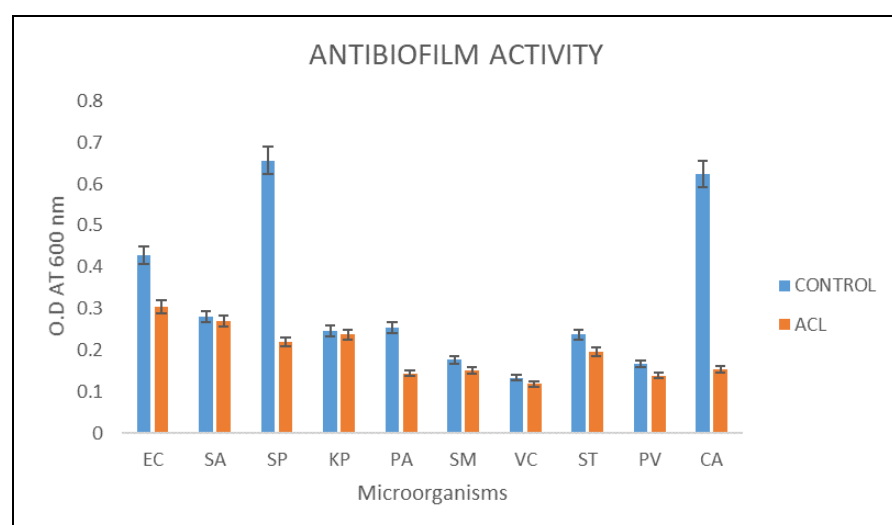


Fig 3: Antibiofilm activity of pathogenic gram positive and gram negative bacteria and the yeast *Candida albicans*

5. Conclusion

We herein present the first report on antimicrobial properties of a lectin from *Amaranthus cruentus* Linn, a commonly available herb. The study has showed that ACL is a potent antimicrobial agent showing antibacterial activity against Gram positive *Streptococcus pyogenes* and Gram negative *Serratia marcescens*. ACL was found to be bacteriostatic in nature which regains its growth capability when sub cultured in a new nutrient rich medium. ACL also possess strong antifungal activity and has profound effect on biofilm mass reduction under *in vitro* conditions. Reduction of biofilm activity can be considered a promising activity, considering that its effect could prevent the emergence of drug resistant strains. In conclusion, the antibacterial activity of ACL could be enhanced if the lectin is completely purified. From all these observations, the isolated plant lectin serves to be a potential source of novel herbal drug for treating infections caused by some clinical pathogens. The isolated lectin also has profound applications in the field of cancer therapeutics.

Appendix

Abbreviations used: ACL- *Amaranthus cruentus* Linn Lectin, KDa- Kilo Dalton, LB- Luria Bertani, MBC- Minimum Bactericidal Concentration, MH- Mueller Hinton, MIC- Minimum Inhibitory Concentration, PAGE- Poly acrylamide gel electrophoresis, PBS- Phosphate buffered saline, SDA- Sabouraud's Dextrose Agar, SDS- Sodium dodecyl sulphate, TEMED- Tetra methyl ethylene diamine

Conflict Of Interest: No conflict of interest

6. Acknowledgements

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