p - ISSN: 2536 – 6866
e - ISSN: 2659 - 1529

ANTHOCYANIN EXTRACTED FROM WALNUT (*JUGLAN REGIA)* AND SOYBEAN (*GLYCINE MAX)* AS ANTI-BIOFILM AGENT AGAINST SPECIES OF *PSEUDOMONAS* AND *KLEBSIELLA.*

[1]*Enaigbe, A. A., [1]Okafor-Elenwo, E. J., [1]Akpoka, A. O. and [2]Idemudia, I. B.

[1]Department of Biological Sciences, College of Applied and Natural Sciences, Igbinedion University, Okada, Nigeria.
[2]Department of Microbiology, Faculty of Life Sciences, University of Benin, Benin City, Nigeria.

***Corresponding author:** andrewenaigbe89@gmail.com; +2348131230105

Abstract - Anthocyanins are phytochemicals whose extracts contained active principles that are antibiofilm that could potentially be administered to alleviate the risk of biofilm related-diseases and considered as a factor contributing to drug resistance in micro-organisms. The aim of this study was to determine the activity of anthocyanin extract as natural effective alternate antibiofilm agent. The pulp of the fruits was extracted using 75% methanol, prior to sub-fractionation and lyophilization to obtain the anthocyanin component. The antibiofilm activity was performed using micro-dilution assay on iron and polystyrene (hydrocarbon) surfaces. Microbial growth was determined through optical density (OD_{650}) while adhesion and biofilm formation was conducted using standard crystal violet staining methods. The results revealed that, both extracts of walnut and soybean reduced the microorganisms (*Pseudomonas* and *Klebiella* species) surveyed but the extract from soybean (*Glycine max*) was more effective (0.10 - 0475 mg/ml). In addition, increased extract concentration resulted in less biofilm formation and demonstrated a considerable impact as natural inhibiting agent against the preliminary stages of biofilm formation with the capacity to disrupt the attachment to surfaces and with *Klebsiella* species, shown as the more susceptible organism.

Key words: Anthocyanin, bacteria, biofilm inhibition, extraction, adhesion assay.

INTRODUCTION

Biofilms have been associated with wide range of infectious diseases and caused damages to drinking water distribution systems materials (Gilbert, 2002). Consequently, various methods had unsuccessfully been attempted to eradicate biofilm formation. This however led to this new approach of the use of phytochemicals as anti-biofilms. The species of *Pseudomonas* and *Klebsiella* are widespread in finished waters, pipe biofilms and are opportunistic pathogens that caused nosocomial infections in susceptible hosts. The *Pseudomonas* is very difficult to eradicate because of their high intrinsic resistance to a variety of antibiotics and has been linked with possible life threatening illnesses such as cystic fibrosis, low blood pressure and ear infection. The *Klebsiella* is implicated in gastroenteritis, osteomyelitis and cholecystitis (Gilber, 2002).

Anthocyanin is a water-soluble, vacuolar pigment that imparts colour to the fruits of plants and the chemical composition consists of phenylbenzopyrilium linking the hydroxyl group and sugars and the various types include pelagonidin, cyanidin and malvidin. Anthocyanins have been reported in avocado

(*Persea americana*), walnut (*Juglan regia*), soybean (*Glycine max*), coffee (*Coffee arabica*) and berries to contain active chemical principles such as polyphenols, flavonoids, catechins and proanthocyanidins (Badawy *et al*., 2013; Kavita and Yong, 2017). Anthocyanin acts as an antioxidant that may inhibit inflammation and tumor growth, boasts production of detoxifying enzymes in the body, prevents arteriosclerosis and inhibits aggregation of bacteria from building up biofilms (Esenbona *et al*., 2004; Silva *et al,* 2016).

Conventional methods of killing bacteria (physical and chemicals) are often ineffective with biofilm bacteria. The huge doses of antimicrobials required to rid systems of biofilm bacterial may result to drug abuse, environmentally undesirable and medically impracticable as what would kill the micro-organisms would also kill the patient. So, lack of proper biofilm management, drive this research in finding new strategies based on the use of anti-biofilm agents from plant sources of no deleterious public health impact.

MATERIALS AND METHODS

Sample Collection

Walnut (*Juglan regia*) and soybean (*Glycine max*) fruits were purchased from a major trading centre in Benin metropolis (New Benin Market). The fruits containing soluble solids were measured with an automated refractometer. The three bacterial species each of *Pseudomonas* and *Klebsiella* were obtained from the Nigerian Institute for Oil-palm Research (NIFOR) Culture Collection Centre, Edo State, Nigeria (*Pseudomonas aeruginosa, Pseudomonas minosa* and *Pseudomonas maltophilia; Klebsiella pneumoniae, Klebsiella oxytoca* and *Klebsiella granulomatis).*

Extraction of plant materials

The pulp of Walnut (*Juglan regia*) and soybean (*Glycine max*) fruits were extracted with 75 % methanol at a substrate to solvent ratio (1: 4) using a blender, centrifuged at 4000 rpm for 5 min and the supernatant was filtered. The filtrates were evaporated at 30 ^{o}C and freeze-dried at - 60 ^{o}C. The extracts were divided into sections and dissolved in 200 ml of water, followed by extraction with 100 ml ethyl ethanoate for two times. The aqueous medium was concentrated at 35 ^{o}C in an evaporator, lyophilized

to produce anthocyanin portion and eventually kept at - 60 ^{o}C for use (Silva *et al*., 2016 and Sahra, 2018).

Anti-biofilm testing

The potentials of the phytochemical (anthocyanin) portions from walnut and soybean were screened against three species of each test organisms (*Pseudomonas* and *Klebsiella)*. The biofilm inhibitory activity was assessed by ascertaining the minimum biofilm inhibitory concentration (MBIC) using a microlitre plate assay as enunciated by Nadef, (2018) with some modifications. MBIC is the least concentration of an agent to stop biofilm formation. The bacteria were grown in 5% Dextrose in Water (5DW) infusion broth (10 ml) at 37 ^{o}C for 24 hr. The bacterial suspension was regulated to 0.5 Table top spectrophotometer standard (0.5 – 0.8 absorbance at 650 nm). The bacterial suspension was diluted in 5DW broth (1:10) and sterilized with a Miltex PTFE Millipore filter (0.25µm). The sample was serially diluted in 40-well microlitre plates, following inoculation with 100 µm of the bacterial suspension, incubated at 37 ^{o}C for 24 hr. The broths in the wells were removed and washed with water twice to keep the biofilms that were aggregated to the bottom of each well. Thereafter, 100 µl of 0.1 % crystal violet was applied to stain the biofilm. 75 % methanol was used to dissolve the colour to determine the absorbance index at 650 nm. The negative control was bacterial suspension with distilled water, while the positive control was bacterial suspension with the inclusion of anthocyanin. The strains were sub-divided into the following sections: $OD_{650} < 2$ (weak biofilm former), $OD_{650} < 4$ (moderate biofilm former) and $OD_{650} > 4$ (strong biofilm former) (Hui and Manach *et al*., 2004; Nadef, 2018).

Adhesion assay

This was performed with slight modifications where polystyrene petri dishes and new iron rods were cleaned with distilled water and sterilized by autoclaving. Bacterial cells were centrifuged at 5000 rpm for 10 min and the resultant pellets were rinsed with phosphate buffered saline (PBS). The anthocyanin portion from walnut and soybean and bacterial cells were transferred to a petri dish containing the hydrocarbon pellets and iron rods and incubated at 35 ^{o}C for 2 hr. Thereafter, the hydrocarbon pellets and iron rods were cleaned with PBS to eliminate any loosely adhered bacterial cells and then stained with 0.1 % crystal violet stain. The attached cells were viewed under light microscope with 1000x magnification and colonies counted and expressed as log CFU/ cm^2

***p* - ISSN: 2536 – 6866**
***e* - ISSN: 2659 - 1529**

Statistical analysis

The investigations were independently conducted in duplicates to verify claims. The data obtained were analyzed applying the one-way analysis of variance (ANOVA) followed by Spearman's test and level of significance was determined at $P < 0.05$ applying a HSPsp 2 data file template.

RESULTS AND DISCUSSION

The results of the minimum biofilm inhibitory concentration (MBIC) of anthocyanin on biofilm formation revealed that, MBIC of anthocyanin fraction from walnut was between 0.183 to 1.10 mg/ml against the *Pseudomonas* species, and ranged from 0.183 to 0.475 mg/ml against *Klebsiella* species (Table 1). The MBIC of the anthocyanin component from soybean against *Pseudomonas* species was recorded to range between 0.183 to 0.285 mg/ml, while extract from soybean against *Klebsiella* was between 0.105 to 0.474 mg/ml. The lowest MBIC (0.183 mg/ml) was recorded from the extracts of walnut on *P. maltophilia* and *K. oxytoca,* while the highest MBIC was obtained from anthocyanin fraction of soybean on *P. mimosa* (0.285 mg/ml). At 2.0 mg/ml, the anthocyanin portion from walnut inhibited 16.0 to 27.0 % of *Pseudomonas* species and 20.4 to 41.0 % of *Klebsiella* species (Table 1). At the standard concentration (2.0 mg/ml), extract from soybean cleared 16.0 to 28.0 % of *Pseudomonas* species and between 20.7 to 40.9 % of *Klebsiella* species.

Table 1: Minimum biofilm inhibitory concentration (MBIC) of anthocyanin portions from walnut (WN) and soybean (SB) (2.0 mg/ml) against species of *Pseudomonas* and *Klebsiella*

Organisms	Biofilm formation status	MBIC (mg/ml		Percentage of biofilm inhibition at 2.0 mg/ml	
		WN	SB	WN	SB
P. aeruginosa	Strong	1.10	0.183	26.3 ± 1.4	28.0 ± 2.1
P. minosa	Strong	1.10	0.285	16.1 ± 0.6	16.1 ± 0.3
P. maltophila	moderate	0.183	0.183	22.0 ± 1.7	22.0 ± 1.3
K. pneumoniae	Weak	0.475	0.475	41.0 ± 0.3	41.0 ± 3.0
K. oxytoca	moderate	0.183	0.10	31.5 ± 0.4	32.4 ± 0.4
K. granulomatis	Weak	0.475	0.10	20.4 ± 0.5	21.2 ± 0.4

MBIC values were means of two biological replicates.
Values for percentage biofilm inhibition were expressed as means ± standard deviation.
Weak: $OD_{650} < 2$; moderate: OD < 4 and strong: > 4 (Nadef, 2018).
Significant difference within the bacterial species at $P < 0.05$.

***p* - ISSN: 2536 – 6866**
***e* - ISSN: 2659 - 1529**

Activity of anthocyanin extracts on cell-surface hydrophobicity

The anthocyanin component from walnut and soybean significantly reduced the hydrophobicity of the two organisms used ($P < 0.05$). The *Pseudomonas* showed hydrophobicity level from 08.2 to 16.5 mg/ml and *Klebsiella* species hydrophobicity level recorded for negative control, ranged from 02.0 to 12.6 mg ml. (Table 2). This is different from the hydrophobicity index as reported by other studies that indicated higher levels (56 – 80 mg/ml) of environmental isolates of *Pseudomonas aeruginosa* attachment to surfaces. The type of hydrocarbon and environmental conditions may be responsible for differences recorded in hydrophobicity determination.

Table 2: Activity of anthocyanin extracts from walnut (WN) and soybean (SB) (2.0 mg/ml) of cell-surface hydrophobicity

Organisms	WN	SB	NC
P. aeruginosa	37.0 ± 0.3	36.6 ± 03	12.6 ± 0.1
P. mimosa	9.84 ± 0.3	42.9 ± 0.4	08.2 ± 0.3
P. maltophila	8.07 ± 0.2	35.2 ± 0.5	16.5 ± 0.1
K. pneumoniae	40.5 ± 0.1	20.0 ± 0.4	02 ± 0.2
K. oxytoca	46.3 ± 0.4	40.7 ± 0.2	10.1 ± 0.3
K. granulomatis	52.3 ± 0.2	35.2 ± 5	05.0 ± 01

Significant difference within same bacterial species at $P < 0.05$;
NC: negative control.

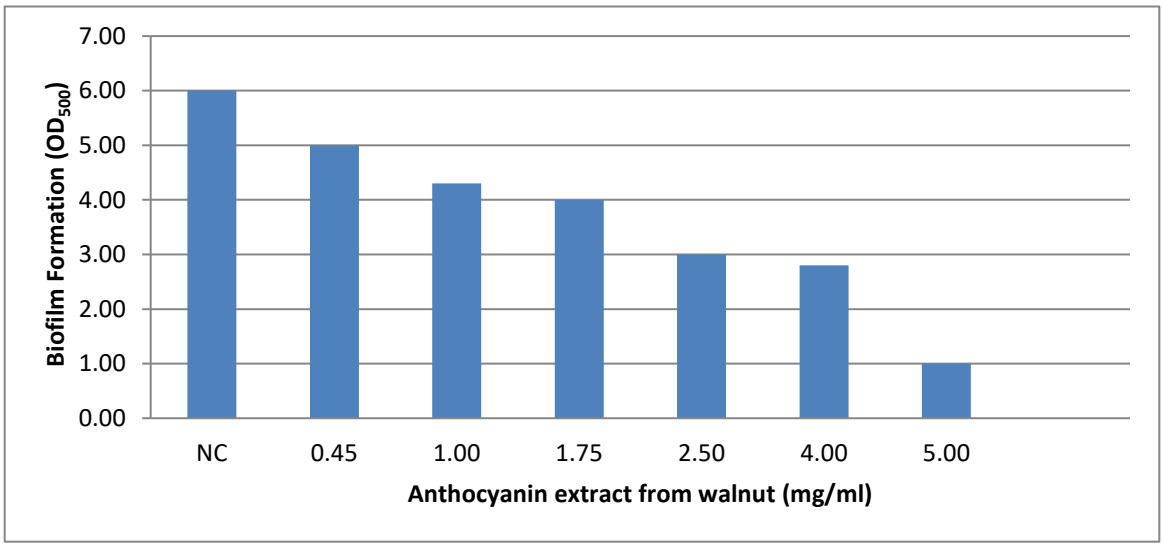
Effect of anthocyanin on bacterial adhesion to Polystyrene (hydrocarbon) surface

Prior to the inclusion of anthocyanin fraction, the level of *Pseudomonas* and *Klebsiella* which adhered to iron rod was 4.3 – 6.6% and 3.2 – 7.4% respectively. The extracts from both walnut and soybean recorded lower levels when compared to the negative control. Relatively, the anthocyanin extract from soybean on *Klebsiella* species ranged from 16.0 to 40.3 mg/ml, the least was recorded for *K. granulomatis* (16.0 mg/ml) and the highest was recorded for *K. pneumonia* (40.3 mg/ml). Conversely, the walnut fraction ranged from 15.0 to 35.5 mg/ml for *Klebsiella* species. The least value was reported from walnut extract on *P. minosa* (11.0 mg/ml) and the highest value of anthocyanin activity was recorded from the walnut extract against *P. maltophila* (30.5 mg/ml).

p - ISSN: 2536 – 6866
e - ISSN: 2659 - 1529

Table 3: Activity of anthocyanin extract from walnut (WN) and soyabean (SB) (2.0 mg/ml) on the adhesion of species of *Pseudomonas* and *Klebsiella* to polystyrene (hydrocarbon)

Organisms	WN	SB	NC
P. aeruginosa	27.6± 0.3	20.5 ± 0.2	4.3 ± 0.1
P. mimosa	11.0 ± 1	18.7 ± 0.2	5.61 ± 0.3
P. maltophila	30.5 ± 0.4	26.1 ± 0.2	6.55 ± 0.2
K. pneumonia	35.5 ± 0.3	40.3 ± 0.1	6.03 ± 0.5
K. oxytoca	15.0 ± 0.2	32.0 ± 0.2	7.35 ± 0.4
K. granulomatis	26.3 ± 0.3	16. *NC* 4 ± 0.1	3.20 ± 0.2



Significant difference within the same bacterial at $P < 0.05$; NC: negative control

Fig 1: Effect of Anthocyanin extract from walnut on Biofilm formation

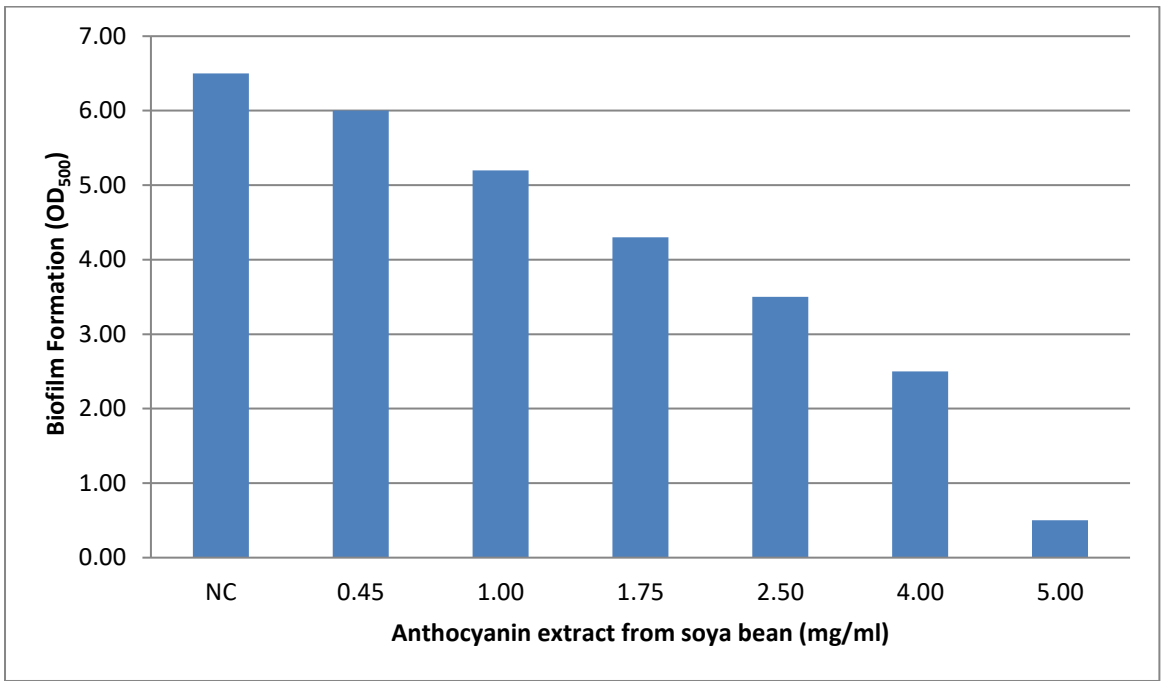


Fig 2: Effect of Anthocyanin extract from soya bean on Biofilm formation

***p* - ISSN: 2536 – 6866**
***e* - ISSN: 2659 - 1529**

The regulation of biofilm formation is intricate to eradicate because of the structural and chemical composition of the extra-cellular polymeric substance (EPS) that is composed of polysaccharides, cross linked with hexose sugars, glycolipids amino acids, organic and inorganic biomolecules and its high level of resistance to antimicrobials (Wilgender and Flemming 1999).

The percentage of biofilm inhibition was determined by dividing the optical density (OD) at 2.0 mg/ml by the OD of the negative control. At 2.0 mg/ml, the anthocyanin moiety from walnut cleared (16.0 to 27.0% and 20.4 to 41.0%) of the *Pseudomonas* and *klebsiella* species respectively (Table I). Overall, the anthocyanin potions from the soybean and walnut showed antibiofilm action against both bacteria, as higher activity was reported from the extracts of soybean on the *klebsiella* species.

Furthermore, evaluation of the biofilm inhibitory activity revealed that when the concentration of anthocyanin extract increased, lesser biofilms were produced by tested bacteria (*Pseudomonas* and *klebsiella*) in the wells or combs and therefore reduced absorbance reading because less biomass of biofilm was being stained by Crystal violet. The MBIC was quantified when there was a significant difference between the surveyed group at the lowest concentration and negative control ($P < 0.05$) (figures 1and 2).

However, the bacterial cell- surface characteristic such as hydrophobicity, has an important aspect in bacterium cell interactions (Hui and Dykes 2012). It has been reported that the introduction of plant extracts with the cell–surface hydrophobicity of Gram negative bacteria such as *klebsiella pneumoniae* and *Streptococcus mutans* (Gram positive) greatly inhibited biofilm accumulation (Esenbona *et al*., 2004). Anthocyanin in *Juglan regia* possibly can affect the hydrophobicity of *P. aeruginosa* by disrupting the bacteria cell–surface. This is enhanced by the hydrophilic tendency of *P. aeruginosa* and the chemical component of phenylbenzopyrilium contained in the plant extract. This result also revealed that, the anthocyanin extract from both plants inhibited biofilm production by *Klebsiella* species on the polystyrene and iron rod through reduction in cell-surface hydrophobicity and surface charge

Table 3 showed that, when compared to the negative control, both anthocyanin moieties from the plants significantly reduced the attachment of bacteria to the hydrocarbon surface ($P < 0.05$). However, the possible mechanism of anthocyanin action may be due to the ability to attach on hydrophobic and

***p* - ISSN: 2536 – 6866**
***e* - ISSN: 2659 - 1529**

hydrophilic non-biotic surfaces, leading to decreased bacteria adhesion and the probability that, hydrophilic fraction of the extract can neutralize bacterial cell-surface hydrophobicity and lower the affinity between the cells and the surface during aggregation to a substratum (Kavita and Yong, 2017; Nadef, 2018). The anthocyanin fraction that significantly reduced adhesion to polystyrene compared to iron surface shown that, hydrophobic modification is one of the pathways of anthocyanin in manifesting the biofilm inhibitory activity.

Nevertheless, though the anthocyanin fraction from soybean recorded the maximum inhibitory effect and decreasing aggregation of cells to both surfaces, the capacity to induce inhibition in walnut cannot be underestimated. This is collaborated by the fact that, the mechanism of action in the plants is slightly different due to relative differences in chemical structure of the plants (Badawy *et al*., 2013; Silva *et al*., 2016).

CONCLUSION

Anthocyanin moieties from both plants recorded significant biofilm inhibitory prowess and most remarkably, their capacity to lower the accumulative potentialities of *Pseudomonas* and *Klebsiella* species to surfaces. Therefore, anthocyanin possesses the innate anti-biofilm agent to militate against the initial stages of biofilm production. In this current study, anthocyanin obtained from walnut (*juglan regia*) and soybean (*Glycine max*) have been highlighted to exhibit this unique potentiality at concentration less than 2.0 mg/ml.

ACKNOWLEDGEMENT

I express my profound gratitude to the staff of Department of Biological Sciences, Igbinedion University, Okada and Prof. Frederick Ekhaise of the Department of Microbiology, University of Benin, Nigeria for their support and encouragement through the period of this study.

***p* - ISSN: 2536 – 6866**
***e* - ISSN: 2659 - 1529**

REFERENCES

Badawy, H.T., Passetto, P., Mouget, J.L. and Milsted, A. (2013). Bacterial adhesion and growth reduction by novel rubber-derived oligomers. *Biochemistry and Biophysics Research Community* 438: 691-696.

Esen bona, M.T., Santos, C. and Rivas- Gonzalo, J.C. (2004). Anthocymin in cereals. *Journal of chemotography* 1054: 129 – 141.

Gilber, P (2002). The physiology and collective recalcitrance of microbial biofilm communities. *Advance microbial physiology* 14: 101-110.

Hui, Y.W. and Dykes, G.A. (2012). Modulation of cell surface hydrophobicity and attachment of bacteria to abiotic surfaces and shrimp by Malaysian herb extracts. *Journals of food protein* 55: 501- 511.

Kavita, S and Yong, R.L. (2017). Systematic expression of active component antibacterial and antibiofilm agent in onion. Journal of food and drug analysis 26: 518 – 528.

Nadef, N.H. (2018). Biofilm inhibition mechanism from extract of *Hymonocallis littoralis* leaves. *Journal of ethnophamacology* 8: 46 – 53.

Sahra, K. (2018). The Methods for Detection of Biofilm and Screening of Antibiofilm agent (2nd Edition). In-tech Open limited, UK. 140pp.

Silva, S. Coata, E.M and Menodes, M. (2016). Antimicrobial antiadhensive and antibiofilm activity of anthocyanin–rich blue berry extract pumped by solid phase extraction. *Journal of applied microbiology* 121 (3): 21 – 29.

Wilgender, J. and Flemming, H. C. (1999). Microbial Extracellular polymeric substance: Characterization, structure and function (Wilgender and Flemming edition). Springer Science Media, UK. 266pp.