AJBCPS African Journal of Biological, Chemical and Physical Sciences

Vol 3, Issue 1, 2024

ISSN: 2790-9522 (Print) | 2790-9530 (Online)
Website: https://journals.jozacpublishers.com/ajbcps/index

OPEN ACCESS

Inhibition of biofilm production by lactobacillus SPP from dental caries using Azadirachta indica

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Received: June 28, 2024 | **Accepted:** July 18, 2024 | **Published:** July 26, 2024

Abstract

Biofilm is a matrix formed by microorganisms due to the secretion of exopolymeric substances. Lactobacillus is a gram positive rod shape bacteria, it causes dental caries and has the ability of forming biofilms. Azadiratcha indica (neem plant) is a fast growing plants used in traditional medicines and has antimicrobial activities. This study aimed at the determination of inhibitory activities of Azadirachta indica on biofilm production by Lactobacillus spp. The phytochemical extraction was done using cold maceration method. The Minimum Inhibitory Concentration (MIC) and Sub-Minimum Inhibitory Concentration (Sub-MIC) were evaluated using micro broth dilution method. The biofilm and antibiofilm determination were done using the crystal violet assay. Results from the study showed that neem Leafs and stem contain tannins, saponins, phenols, flavonoids, alkaloids and cardenoloids. Lactobacillus spp is a positive biofilm former (Moderate biofilm) with 0.201 mean optical density (OD) value at 620 nm. Sub-MIC for Leaf ethanolic extract (LAE), Leaf aqueous extract (LEE), Stem ethanolic extract (SAE), and stem aqueous extract (SEE) against Lactobacillus spp was at 0.78 mg/mL, 0.20 mg/mL, 0.78 mg/mL, and 0.39 mg/mL respectively. The LEE, SAE, and SEE inhibited biofilm production from moderate biofilm former at 0.201 to a weak biofilm former at 0.121, 0.140, and 0.093 mean OD value at 620nm respectively. A. indica inhibited biofilm production of Lactobacillus spp this might be due to phytochemical compounds present. Neem plants may be combined with antibiotics to improve effectiveness in treatment of dental caries and plaques.

Keywords: Biofilm, Bioactive, Dental Caries, Lactobacillus, Inhibition

1. Introduction

Lactobacillus is historically among microorganisms causing dental plaque (Badet & Thebaud, 2008). Lactobacillus is considered the second most cariogenic microorganism, it plays important role in

dental plaque progression (Ahirwar et al., 2017). Biofilm is a matrix formed as a result of exopolymeric substance production, which serve as a defense mechanisms (Peeters et al., 2008). Exoploymeric substance production by microorganisms causing dental carries has made treatment of dental infections difficult. Studies have shown that microorganisms with abilities of biofilm production reduces the effectiveness of antibiotics. (Attaran et al., 2017; Bae & Jeon, 2013; Vestby et al., 2020; Yonezawa et al., 2019). According to a report by WHO about 60-90% of pupil in school and 100% of adults have dental plaques due to poor oral hygiene and unhealthy food (Jurgensen and Petersen, 2013; Kassebaum et al., 2015; WHO, 2016). *Azadirachta indica* (Neem plant) is used worldwide in the treatment of diverse diseases (Mohammad, 2016). Neem plants contains bioactive compounds such as Azidiratcin, polyphenol among others with high antimicrobial activities (Efferth and Koch, 2011). The broad spectrum activity of neem plant has been explored in dentistry and bacteriology (Marina & Marrell, 2022). Neem stalk, tree, leaf has been used locally in brushing teeth (Mohammad, 2016). The increasing rise in antimicrobial resistance due to defense mechanisms such as gene modification and biofilm formation has led to an increase in the demand for new antimicrobial agents that can be readily available, body-friendly, and with cost effectiveness. Natural medicines have low toxicity than synthetic drugs, phytomedicine is important to oral health (Brahmachari, 2004). This study therefore aim at inhibition of biofilm production by *Lactobacillus* spp using neem plants.

2. Literature Review

Biofilm are aggregate matrix formed as a result of microorganisms adhering to living or nonliving walls. The formation of biofilm provides protection to microbes against environmental stress and increases their resistance to antimicrobial agents which enhances infection persistence and toxicity (Peeters et al., 2008). The adherence of bacteria to tooth surfaces has increased dental caries. The existence of Lactobacillus in the oral cavity can be traced to over a century. A researcher known as Lewkowicz in 1901 was the first to isolate Lactobacillus from dental carries (Ollie et al. 2017). Lactobacillus are aciduric bacteria that includes *Lactobacillus acidophilus*, *L. rhamnosus*, *L. casein,* and *L. oris* capable of breaking down sugars to acids. Lactobacillus can attach to enamel, dentin, cementum, gingiva, and oral mucosa to form biofilm (Ahirwar et al., 2017). WHO estimated that about 60-90% of pupil in school and 100% of adults have dental plaques/carries due to poor oral hygiene and unhealthy food (Kassebaum et al., 2015; WHO, 2016).

Azadiractha indica popularly known as neem plants is traced to be a traditional medicine in the treatment of diseases globally (Noorul, 2016). It is 20-23m tall with a diameter of about 4-5 ft. *A. indica* has complex compounds including nimbin, nimbidin, nimbolide, and limonoid with high efficacy in infection treatment. *A. indica* is nontoxic and readily available worldwide (Aumeeruddy, 2021). The various parts of neem is effective as antipyretic, antacid, antiparasitic, antibacterial, antifungal, anticancer, antioxidant and other protective properties (Alzohairy, 2016). Millions of Africans often use neem stem as chew stems for oral health management (Brahmachari, 2004; Gupta et al., 2017). Kanagasanthosh et al. (2015) observed that 200 mg/kg of ethanolic neem leaf extract could not cause death in mice this justifies the less toxicity of neem. The rising antimicrobial resistance of microorganisms has reduced the available antimicrobial agents in the market for infection treatment, therefore there is a need for the discovery of novel antimicrobials with high efficacy and less toxicity.

In a research carried out by Mistry et al. (2015); Nayak (2017); Singh et al. (2020), they reported that neem parts has antimicrobial effect on pathogens causing dental plaques such as streptococcus, Lactobacillus, Staphylococcus, and Candida. In another study by Nimbulkar et al. (2020) and Selvaraj et al. (2020) reported that neem based tooth paste can inhibit formation of dental plaque. Neem plants has antibiofilm production activities through quorum sensing inhibition, efflux pump inhibition and metal chaletors inhibition (Borges et al., 2016). In a study to check the effectiveness of neem based for mouth rising the study confirmed that *A. indica* was also good in minimizing periodontal infection as chlorhexidine (Chatterjee et al., 2011). The joint interaction between many constituent found in plants, has explained the effectiveness of low dose of herbal products than synthetic ones (Aiyegoro & Okoh, 2009).

3. Research methodology

3.1. Plant Collection and Preparation

The Leafs and the stem were rinsed with distilled water and dried in the shade for 12 – 16 days. The stem and leave were grounded into powdered. 250grams of the grounded sample was weighed in to 2 separate conical flasks for each sample and soaked using 1.2 liters of the solvent (water and ethanol) respectively. It was soaked for 24 and 48h for distilled water and ethanol respectively. The soaked sample and was sieved with muslin cloth and then filtered with by Whatman filter paper no.1. The extracts were concentrated respectively according to their boiling point (Gwana *et al*. 2014).

3.2. Microorganism

The *Lactobacillu*s spp used in this studies was collected from the Microbiology Laboratory Department of Microbiology Nasarawa State University Keffi and were re-identified using biochemical tests.

3.3 Phytochemical Screening

Phytochemical analysis was carried out using a procedure as described by Aziz (Aziz, 2015).

Reducing sugar: Exactly 5 mL Fehling solutions A and B was dispensed into 2 mL of the sample in a tube, it was boiled for 2 min. The precipitation of brick-red copper (I) oxide indicates reducing sugar.

Anthraquinone (Borntrager's test): 0.5g of the sample was added to 10 mL of Chloroform and Ammonia. Presence of bright pink color confirms anthraquinone.

Saponins: 5 mL of distilled water was added to 0.5 mL of the sample and vortexed. Appearance of foam indicates that saponin is present.

Cardiac glycosides (Keller-killani test): 1g of the sample was dissolved in chloroform, containing about 1 mL of glacial acetic acid (with small amount of Ferric chloride) and 1 mL of teraoxosulphate (vi) acid. A Brownish red colour at the top confirm that cardiac glycosides is present.

Terpenes (Liebermann-Burchard): 0.5 mL of the crude extracts was combined with 2 mL of chloroform and 3 mL of sulphuric acid. A reddish brown indicates that Terpenes is present.

Steroids: 1 mL of the sample and 10 mL of chloroform and sulfuric acid were mixed together. The appearance of bilayer of red up layer and greenish down layer indicates that steroids is present.

Alkaloids: 100 mL of distill water was used to dissolve the sample, the solution was filtered and steamed. Few drops of 1% HCL was added. About 1 mL of the heated solution was added to 6 mL of

mayer-wagner reagent. The presences of brown color indicates alkaloids.

Flavonoids (Ferric chlorides Test): Acetone was used to dissolve 0.5g of the sample, and was left to evaporate and then dissolved in warm water. Little drops of 10% ferric chloride was added. A green or blue solution indicates that flavonoids is present.

Tannins: 10 mL distil water was added to 200 mg of the sample to dissolved and then boiled. Ferric chloride (0.1%) was dispensed in to the solution, blue black color reveals the presence of tannins.

Phlobatannins: 2 drops of 1% HCl was mixed with 1 mL of sample and boiled. Appearance of reddish precipitation reveal it positive.

Resins: 2 mL of crude extract was added to 2 mL acetic anhydride solution, drops of concentrated H_2SO_4. An appearance of violet coloration confirms the presence of resins.

Phenols: About 0.4g of the extract was dispensed in 1:1 volume ration of 1 % ferric (III) chloride/water. Precipitation of dirty green color indicates the presence of phenol.

3.4. Biofilm assay

The method described by Musleh and Jebur (2014), was used. A colony from tryptic soy agar was cultured into nutrient broth (10 mL) and then kept at 37°C for 18-24h. Using a sterile pipette 1 mL of the cultured broth was added 9 mL of sterile nutrient broth. 200 µL of isolates suspension was dispensed using a sterile pipette into three wells (triplicate). Nutrient broth containing no isolate was dispensed into three wells as control and incubated at 37°C for 48 h. After incubation the isolate suspension in the microtitre wells was poured off and then washed in three container containing clean water. The plate was blotted on a clean towel. The microtitre plate was allowed to air dry. 200 µL of 0.1% crystal violet was dispensed into each wells using a pipette, and was allowed to atay for 30minute. The stain was rinsed in three containers containing clean water. The plate was blotted using a clean towel and was allowed to dry. 200 µL of 80% of ethanol and 20% Acetone was dispensed into the wells to detained each well.100 µL of the solution was transferred in to another microtitre plate for each isolate, the plate was read using spectrophotometer at 620nm.

3.5. Preparation of Stock Solution

250 mg/mL concentration was prepared by dissolving 0.5g of the extract in 2 mL of 10% Dimethyle Sulphoxide.

3.6. Evaluation of Minimum Inhibitory Concentration (MIC), and Sub Minimum Inhibitory Concentration (Sub MIC) in mg/mL

In the first row hundred microliter of double strength Nutrient Broth was dispensed into the wells, and then single strength of nutrient broth was dispensed into the remaining wells. 100 µL of the stock solution (25 mg/mL) of every extract was dispensed into the first wells separately and then double fold serial dilution was carried out down the column respectively to get a concentration of 12.5 mg/mL, 6.25 mg/mL, 3.13 mg/mL, 1.56 mg/mL, 0.78 mg/mL, 0.39 mg/mL, 0.20 mg/mL, and 0.90 mg/mL. The isolates were standardized to 0.5 Mcfarland standard.100 µL of the isolate was dispensed in to the wells. Hundred microliter of nutrient broth was dispensed in an empty well (negative control) and 100 µL of nutrient broth containing the plant extract was dispensed into another well (positive control). The microtitre pates was kept in the incubator at 37°C for 48h.

3.7. Antibiofilm Activity Assay

Crystal violet assay as described by Peeters et al. (2008) was used in carrying out antibiofilm assay. 100 µL of standardized inoculum (1 mL of overnight broth containing *Lactobacillus* spp into 9 mL of a sterile nutrient broth) was dispensed in triplicate, 100 µL of sub inhibitory concentration of plant extract was added. 100 µL of nutrient broth was dispensed in an empty well (negative control) and ten 100 µL of nutrient broth containing the plant extract was dispensed into another well (positive control) and incubated at 37°C for 48h. Immediately after 48 h the isolate suspension in the microtitre wells was poured off and then washed in three containers containing clean water. The plate was blotted on a clean towel. The microtitre plates was allowed to dry. 200 µL of 0.1% crystal violet was dispensed into each well using a pipette, and was allowed to stain for 30minute. The stain was poured off gently and then rinsed in three containers containing clean water. The plates was blotted using a clean towel and was allowed to dry. 200 µL of 80% of ethanol and 20% Acetone was dispensed into the wells to distained each well. 100 µL of each was transferred in to another microtitre plate for each isolates, the plates was read using spectrophotometer at 620nm.

4. Data Analysis

The research for each parameter was run in triplicate, the data are presented as average/mean of the result.

5. Results and Discussion

Phytochemical Analysis: Qualitative analysis result of neem extract is shown in Table 1. The ethanolic extracts contained more phytochemicals than the aqueous extracts. The highest number of bioactive compounds was observed in Leaf ethanolic extract.

Biofilm Production: The degree of biofilm formation by *Lactobacillus* spp, it is shown in table 3. The biofilm formation measured at 620 nm was observed to be 0.201.

Antibacterial Activity: Antibacterial activity of neem extract is shown in Table 2. The Leaf ethanolic extract was observed to have more activity at lower concentration with MIC and Sub MIC at 0.39 mg/mL and 0.20 mg/mL respectively.

Antibiofilm Assay: Antibiofilm activity of neem extacts is shown in Table 3. The stem ethanolic had more activity in biofilm production inhibition from 0.201 to 0.090 at 620nm. The Leaf aqueous extract had no activity.

Table 1: Phytochemicals found in Leaf and Stem (Ethanol and Aqueous Extracts) Of *Azadirachta indica*

Parameters	Leaf Extract		Stem Extract	
	Ethanol	Aqueous	Ethanol	Aqueous
Saponins	+	-	+	+
Taninns	+	+	+	+
Anthraquinones	+	+	-	-
Phenols	+	-	+	-
Trapeniods	-	+	-	-

Cardenolids	+	-	+	-
Flavonoids	+	+	+	-
Cardiac Glycoside	+	+	-	-
Glycoside	+	+	-	-
Resins	-	-	-	-
Steroids	+	+	-	-
Alkaloids	+	+	+	+
Phlobotanins	-	-	-	+
Redusing Sugar	+	+	+	+

Key: +: Shows the presence of the phytochemical, -: Shows the absence of phytochemical.

Table 2: Determination of MIC and Sub MIC (mg/mL) of *Azadirachta Indica* Extracts against *Lactobacillus* spp Associated with Dental Caries

Extracts	**Concentrations (mg/mL)**	
	MIC	**Sub MIC**
Leaf Aqueous Extract	1.56	0.78
Leaf Ethanolic Extract	0.39	0.20
Stem Aqueous Extract	1.56	0.78
Stem Ethanolic Extract	0.78	0.39

Table 3: Determination of biofilm production and Antibiofilm Activities of *Azadirachta indica* Extracts against *Lactobacillus* spp Associated with Dental Caries at 620nm

Extracts	**Biofilm formed**	**Antibiofilm Activities**
Leaf Aqueous Extract	0.201	0.200
Leaf Ethanolic Extract	0.201	0.121
Stem Aqueous Extract	0.201	0.140
Stem Ethanolic Extract	0.201	0.090

The phytochemicals tests performed were qualitative type and from the phytochemical investigation, the identification of tannins, alkaloids, saponins, flavonoids, phenols and cardenoloids in the Leafs and stem of neem plants in this study is in accordance with previous studies (Gupta et al., 2017; Nandita & Reena, 2020; Nagini et al., 2021; Roy & Saraf, 2006; Saleem et al., 2020). From the results in table 4.1 shows that ethanolic extracts of both stem and leave contains more phytochemical than the aqueous extracts. This is because water may not be a good solvent for extraction. Result obtained showed that Lactobacillus is a biofilm producer through the production of exopolymeic substance. Thus, confirming reports by previous studies Ahirwar et al. (2019), Ollie et al. (2017) and Zijnge et al. (2010) who reported that lactobacillus is a positive biofilm former in dental caries. Result from this study revealed that *A indica* exhibited antibacterial activities on *Lactobacillus* spp this aligns with the study of Ashwin et al. 2024 that neem plants significantly had antibacterial effect against lactobacillus.

Most of the ethanolic extracts had more antibacterial activity than water extracts, which may be due to different kinds and concentration of active compounds in the parts of neem plants and the ability of the solvent to extracts broader spectrum of compounds. Chewing sticks from neem plants has high antibacterial efficacy against bacterial associated with dental plaque than other plants (Marina & Marrell, 2022). The stem aqueous and ethanolic extract reduced biofilm formation from 0.201 to 0.140 and 0.090 respectively. Despite the antibacterial activity of Leaf aqueous extracts it could not inhibit biofilm production, this may be the reason for the use of neem stems as chew sticks in dental carries treatment. The antibiofilm activities obtained in this study is inspiring where it showed a reduction in formed biofilm from moderate to weak biofilm, because of the joint antibacterial activities of the phytochemicals present. This study has proved that neem plants extracts has anti adhesive potentials against Lactobacillus spp. Previous studies reported that bioactive compounds can be combined with synthetic antibiotics to increase their efficacy. (Aiyegoro & Okoh 2009; Ayaz et al., 2019; Braga et al., 2021; Marina & Marrell, 2022). This study has shown that neem plants parts such as stem and leaf may be effective in the treatment of dental caries to avoid tooth decay. It can also be used in the as medication in treating infections related lactobacillus. The effectiveness of neem in the inhibition biofilm production shows that oral infection persistence due to biofilm formation can be reduced with the use of extract from the plants parts. The phytochemicals presents in the neem plants has the ability to inhibit exopolymeric substance production and prevent bacteria from attachment to surfaces, due to this infection may not occur. Thus, use of extract from *A indica* may result in eradication of biofilm produced by Lactobacillus, thereby, enhancing the antibacterial effect of other antibiotic drugs. The persistence of dental infections is due to biofilm formation, the inhibition of this biofilm may reduce the severity of oral infection. Chew sticks from neem plants are readily available and cost effective in the treatment of dental carries. Antimicrobial resistance is very common in dental carries due to efflux pumps, but neem plants bioactive compounds may hinder adherences and as such biofilm cannot be formed and resistance would be aborted. The use of neem as mouth wash may be effective in the maintenance of oral health and should be added to tooth paste to inhibit biofilm formation in dental carrier's development.

6. Conclusion

Lactobacillus produced biofilm. Stem ethanolic extract of *A. indica* is more recommended for biofilm removal as its usage lead to a significant reduction of the biofilm produced and the extract also had remarkable antibacterial activity. Thus, bioactive compounds from *A. indica*, especially from the stem, may be used as ingredients in pharmaceuticals for oral wash.

7. Acknowledgement

The Authors sincerely acknowledge the Department of Microbiology, Faculty of Natural and Applied Sciences, Nasarawa State and Biotechnology Advanced Research Center, SHESTCO Abuja for their endless support for making this research a success.

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