Effect of traditional medicinal plants on the pathogenic potential of the versatile pathogen *Staphylococcus aureus*

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Abstract

This study explores the effects of Neem (*Azadirachta indica*) and Papaya (*Carica papaya*) leaf extracts on the pathogenicity of *Staphylococcus aureus*. Through *in vitro* assays, we investigated the impact of these medicinal plant extracts on bacterial metabolic activity and pathogenic factors. Results indicate that the growth of *Staphylococcus aureus* was differentially decreased by the plant extracts, with Neem demonstrating a greater reduction compared to Papaya. Moreover, both Neem and Papaya extracts, either alone or in combination, significantly inhibited bacterial biofilm formation, with reductions of 39%, 48%, and 7%, respectively. Both aqueous and ethanolic extracts exhibited inhibitory effects on bacterial growth, with ethanolic extract showing stronger potency. However, the extracts did not influence the bacteria's haemolytic characteristics. Overall, these findings suggest that traditional medicinal plant extracts possess potential in mitigating *S. aureus* pathogenicity, however, further mechanistic investigations are warranted to elucidate underlying mechanisms.

INTRODUCTION

*Staphylococcus aureus* stands as an essential example of microbial versatility, showcasing its ability to adapt, persist, and pose significant health threats in diverse environments. This bacterium, commonly found on the human skin and mucous membranes, has gained attention not only for its pathogenic potential but also for its resilience against conventional antimicrobial treatments (Lee *et al.*, 2018; Tang and Stratton, 2010). The organism is a versatile pathogen which is transitory colonizer of skin and bodily entrance sites including ears, nasal passages, eyes, etc. and resides on the upper respiratory tract including nares, nasopharynx, and mucus membrane of the 20-30% of the human population (Mousavi *et al.*, 2017). This bacterium can asymptomatically colonize healthy individuals but it can cause disease if it successfully penetrates the body through a variety of routes, such as a cut or scrapes on the skin, an injection, or the implantation of a medical device. This is true not only for immunocompromised patients who are suffering from diabetes or AIDS and defects in neutrophil function but also for everyone with an altered microbiota (Mousavi *et al.*, 2017; Reygaert, 2013).

*S. aureus* is a prominent causative agent across a spectrum of conditions, from superficial lesions like skin inflammations and ulcer infections, to more severe conditions such as endocarditis, pneumonia, osteomyelitis, and bacteremia (Reygaert, 2013). Additionally, it is associated with toxemic syndromes like toxic shock syndrome (TSS) and staphylococcal scarlet fever, both linked to toxic shock syndrome toxin-1 (TSST-1) and

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staphylococcal enterotoxins (SEs). Furthermore, it is responsible for staphylococcal scalded-skin syndrome (SSSS) due to exfoliations, and staphylococcal food poisoning (Reygaert, 2013). Though the primary focus of microbiologists has been understanding the mechanisms of infection processes, our knowledge is still expanding on the effect of traditional medicinal plant extract on the pathogenicity of such versatile pathogens and searching remedies.

Endogenous pathogens, such as members of the ESKAPE (Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, and Enterobacter species), are becoming a threat to human life because their increasing rate of resistance to available antibiotics raises alarms about a forthcoming disaster (De Oliveira et al., 2020). As a consequence, it has become increasingly important to continue attempts to produce an inhibitory molecule derived from naturally occurring plant elements. Various research studies have pointed to phytocompounds as the most promising potential treatment option for multidrug-resistant bacterial infections and supported as the best alternative (Garo et al., 2007; Coutinho et al., 2009).

Throughout ancient times, people looked to plants as a way to heal illness and alleviate human suffering. Since the dawn of time, millions of people around the world have relied on plants for their main health care (Jain, 2010). Paleoanthropological assessments significantly contributed to divulging the first natural products approximately more than 6000 years ago (Solecki, 1975). Traditionally, medicinal plant extracts are frequently taken to help disease alleviation. Intaking medicinal plant leaf juice to enhance immunity and boost its function has been a longstanding practice. Besides, some medicinal plants are externally applied as juice or boiled in water to get rid of diverse problems and/or as part of rituals.

Not only in ancient times but also in the present day, the WHO described nearly 80% of human illnesses, such as cancer, infection caused by bacteria, immunological disorder, etc. being treated with plant-derived natural products and their derivatives, and one-fourth of all prescribed drugs worldwide come from plants (Karunamoorthi et al., 2013; Rates, 2001). In developing countries, like Bangladesh, there exist an esteemed tradition of using herbal medicines and people are heavily dependent on plant-based therapeutics. It is estimated that half of the thousand species containing medicinal components exist in Bangladesh, of which approximately 250 are being used to prepare traditional remedies (Uddin et al., 2011).

These medicinal plants either naturally produce or store secondary metabolites such as volatile oils, sterols, alkaloids, resins, lactones, flavonoids, tannins, terpenes, and so on and these secondary metabolites aid in human defense against numerous diseases and provide the body with protection (Kar et al., 2014). Moreover, the compounds bind to the cell wall synthesizing protein and form a complex, which is responsible for inactivating or disrupting the cell wall of the organism (Subramani et al., 2017). Many studies have been conducted on the use of phytogetic immune boosters, with an emphasis on their antibacterial capabilities, Azadirachta indica or Neem is one of them (Sahoo & Sharma, 2022). Neem leaves, offer a wide range of beneficial qualities, including immunological
modulation, anti-inflammation, anti-hyperglycemic, anti-malarial, antifungal, antibacterial, antiviral, antioxidant, antimitogenic, and anticarcinogenic effects (reviewed by Latif et al., 2020). Besides, leaf extract of Neem showed significant antimicrobial activity against Gram-positive bacteria, such as *Staphylococcus aureus* (Akinduti et al., 2022). In addition, the efficacy of Neem has been demonstrated in dentistry (Lakshmi et al., 2015; Latif et al., 2020; Pai et al., 2004) and in poultry gut (Ali et al., 2021; Sujatha et al., 2017).

Other than Neem, Papaya is another important fruit and vegetable yielding, as well as medicinal plant in Bangladesh (Saleh & Kamisah, 2020). They are of huge importance on their own accords; literature demonstrates the significance of these medicinal plants in curing many diseases (Reddy et al., 2021; Singh et al., 2020). Papaya exhibits antimicrobial activity, fights against inflammation, and acts as an antioxidant (Dawkins et al., 2003; Gupta et al., 2000). Due to its antibacterial properties, it could be beneficial in the treatment of persistent skin ulcers in order to facilitate the healing process (Dawkins et al., 2003). Moreover, it is a traditional remedy for numerous skin disorders, such as wounds. In developing countries, it is a readily accessible and efficacious remedy for a variety of wounds, especially burns (Nayak et al., 2012). Furthermore, Papaya captured enormous attention as fruit, but leaves remained quite unexplored until recently when dengue and Covid-19 have hit the world (Ochani et al., 2023). The efficacy of Papaya leaf juice was mentioned as a gross remedy, however, the mechanisms of curation remained veiled.

Till to date, *Staphylococcus aureus*, a versatile human pathogen known for its significant role in severe hospital-acquired infections, its tendency to create biofilms, and its ability to develop antibiotic resistance (Lee et al., 2018), continues to pose a formidable challenge for scientists seeking effective treatments, especially targeting its biofilm formations (Tang and Stratton, 2010). So, this research study *S. aureus* and its relationship with traditional Bangladeshi medicinal plants, *Azadirachta indica* and *Carica papaya*, it is blending ancient wisdom with modern science. The study aimed to find new ways to fight stubborn bacteria by exploring the natural remedies passed down through generations.

**MATERIALS AND METHODS**

**Bacterial strain:** The bacterial strain, *Staphylococcus aureus* ATCC 25923, was obtained from Microbiology Laboratory, BRAC University, Bangladesh as a gift. *S. aureus* ATCC 25923 was initially maintained on Mannitol Salt Agar (MSA) and grown on Nutrient broth (NB), Nutrient Agar (NA), Tryptic Soy Agar and Broth (TS-A/B).

**Preparation of standard culture inoculum of the bacteria:** Before each experiment, single colony from overnight incubated culture (streaked from stock) was inoculated into 10 ml of Nutrient broth and incubated for 24 hours at 37 °C. The bacterial concentration at the working cultures was adjusted to obtain an optical density of 0.5 at 600 nm (OD$_{600}$ = 0.5). Later, the bacteria were grown on NA and TSB. The bacteria were stocked in 50% v/v glycerol in TSB and stored in -20 °C for future use.
Traditional medicinal plant extracts: Neem (*Azadirachta indica*) and Papaya (*Carica papaya*) plants that were already identified, leaf samples were collected from the botanical garden of Jahangirnagar University and were verified according to relevant literature. Leaves of the plants were washed, chopped, and dried. Grinded leaves were soaked into ethanol, crude ethanolic extracts were prepared using Soxhlet device (Heidolph, Germany), followed by evaporation using rotary evaporator according to the procedure previously mentioned (Zaidan *et al.*, 2005). The plant extracts were analysed for the presence of metabolites using spectrophotometry (Analytik jena, Specord) at a range of 190 to 800 nm wavelengths and peaks were determined. Similarly, aqueous extract was also prepared.

Effect of plant extracts on the bacterial growth: *S. aureus* were exposed to 125 µg/ml concentration of plant extracts in TSB and growth was measured as representative of bacterial metabolism. A single colony from an overnight culture plate was inoculated into 10 ml of the liquid TSB medium at planktonic culture condition and was incubated at 37 °C for 24 hours at aerobic condition. Growth was measured as absorbance at 450 nm using spectrophotometry.

Biofilm formation assay: The *in vitro* biofilm assay was performed to evaluate the effect of the medicinal plants on the ability of the bacteria to form a biofilm on glass plates (5 cm) in planktonic culture condition. Bacteria from the stock was grown on NA, followed by growing in TSB at 37 °C for overnight. The culture was inoculated into fresh liquid media with/without plant extracts at a concentration of 1:200 and incubated for 48 hours continuously at 37 °C. The Crystal Violet- Acetic Acid biofilm formation assay was performed according to the protocol previously described (Shil & Chichger, 2021). Amount of biofilm formed was measured as absorbance of the retained Crystal Violet at 450 nm using spectrophotometry and data was normalised with control.

Haemolysis assay: Blood Agar Base containing 7% Sheep Blood was used to investigate the haemolytic properties of the bacteria after exposure to the plant extracts (125 µg/ml) or vehicle.

Inhibitory effect of the plant extracts on the bacteria: Both ethanolic and aqueous extracts of the Neem and Papaya leaves were employed on the *S. aureus* using disc diffusion assay on Mueller Hinton Agar (MHA) plates. Briefly, blotting papers were hewed into 6 mm round pieces and were impregnated with 20 µl of the plant extracts of desired concentrations (125 µg/ml and 250 µg/ml). *S. aureus* lawn was prepared on the MHA plates using a single colony of fresh sub-culture. The discs, soaked with either Neem or Papaya plant extracts, were placed on the surface of the plates. Doxycycline was used as a positive control whilst ethanol and water were used as vehicle controls, respectively (Cunha *et al.*, 1982). The plates were incubated at 37 °C for 48 hours with an observation at 6, 8, 12, 24 and 48 hours. Inhibition zone was measured at 24 hours with a scale and three independent sets of experiments were performed.

Statistical analysis: Experiments were performed as independent sets (*n = 3*), data were gathered on Excel (Microsoft Office) files, and analysed using GraphPad Prism (v.9.5.0). A one-way ANOVA with uncorrected Dunn’s test was performed to statistically analyse the variation, significance was reached when *p < 0.05*. 
RESULTS AND DISCUSSION

The plant extracts contained considerable phytocompounds: *Azadirachta indica* (Neem) has been extensively exploited at all possible forms from the ancient times as medication and home remedies of various diseases (Bandyopadhyay & Bindu, 2011). *Carica papaya* (Papaya) also is well-known as vegetable and fruit, however, the possibilities of leaf extracts attracted huge attention recently (Saleh & Kamisah, 2020). Both Neem and Papaya ethanolic extracts showed peaks in between 190 nm and 703 nm, indicating the presence of phenolic compounds, terpenoids, and glycoside compounds (Figure 1) (Karpagasundari & Kulothungan, 2014).

![Absorbance](image)

\textbf{Fig. 1. Ultraviolet visualisation of the plant extracts in ethanol.}
Crude ethanolic extract was prepared from dried grinded leaves using Soxhlet device and concentrated using rotary evaporator. Absorbance was measured using spectrophotometry (Analytik jena, Specord) at a range of 190 to 790 nm wavelengths against ethanol as vehicle.

Both Neem and Papaya plant extracts inhibited the growth of the *S. aureus*:

The bacterial growth upon exposure to the neem and papaya plant extracts were measured as absorbance at 450 nm, culture without plant extracts and vehicle was kept as control (Figure 2). Whilst *S. aureus* organism demonstrated 100% growth without the presence of plant extracts, the growth however drastically dropped due to the presence of antibiotic (approximately 8%), as expected. Neem extract decreased *S. aureus* growth by approximately 22% compared to the control, whilst Papaya extract demonstrated the opposite by increasing growth to 143%, although the change was statistically insignificant. Nevertheless, when *S. aureus* was exposed to both the plant extracts (Neem and Papaya), the organism showed slight decreased growth (93%) a little bit less than the uninfluenced one.
Fig. 2. Effect of Neem and Papaya leaf extracts on *S. aureus* growth. A single colony from overnight culture was inoculated into 10 ml liquid medium and incubated for 24 hours at 37 °C. Growth was measured as absorbance at 450 nm using spectrophotometer. Data are presented as the mean ± S.E.M., n=3.

The medicinal plant extracts did not change the haemolysis pattern of the bacteria: The haemolysis assay was performed on blood agar plates under *in vitro* conditions to evaluate the effect of the plant extracts on haemolytic ability of the bacteria. Overnight exposure to Neem or Papaya leaf extracts did not affect the haemolysis activity (Figure 3).

The plant extracts decreased the biofilm formation ability of the *S. aureus*: Analysing *S. aureus* biofilm data showed decreased biofilm development for exposure at the tested plant extracts. Neem reduced *S. aureus* biofilm formation by 39% whilst Papaya inhibited even more (48%). Application of both neem and papaya extracts inhibited *S. aureus* biofilm formation lower (7%) than the tested antibiotic, Doxycycline (14%). Both plant extracts reduced *S. aureus* biofilm formation (Figure 4) upon exposure to the extracts for short term at 37 °C. Papaya leaf extract (52.4%) was more effective to inhibit the biofilm formation of *S. aureus* than Neem (61.9%).
Effect of traditional medicinal plants

Fig. 3. Effect of Neem and Papaya plant extracts on haemolysin production of *S. aureus*.

A single colony of the organism was exposed to 125 µg/ml concentration of either Neem or Papaya or vehicle or antibiotic for 24 hours in Tryptic Soy Broth and the culture was streaked on blood agar plates using cotton swab. Growth was observed for haemolysis at 24 hours. The plant extracts did not affect the haemolytic property of the organism.

Inhibitory effect of the plants extracts on *S. aureus*: Both Neem and Papaya at all concentrations of ethanolic extract created a zone of opaque region indicating inhibition, however, a clear zone alike the antibiotic was absent in all cases (Figure 5). At 250 µg/ml
concentration, dispersion of a brown colour was more prominent, and the growth of the *S. aureus* was absent whilst at 125 µg/ml concentration, a light brown colour dispersion was visible, and growth was absent alike 250 µg/ml. The clear inhibition zone created by Doxycycline and no inhibition by the vehicle justifies the authenticity of the experiment.

The aqueous extracts at 250 µg/ml concentration of both plant extracts created greater opaque zones than that made by 125 µg/ml concentrations (Figure 6). Alike the ethanolic extracts, the aqueous extracts did not create clear inhibition zone but a decreased growth around the discs. The outcomes imply that although a clear zone of inhibition was absent, however, both the plant extracts have antibacterial effect on the tested *S. aureus*.

**Fig. 5. Assessing the inhibitory effect of the ethanolic plant extracts on *S. aureus*.**

Paper discs were impregnated with 20 µl of the plant extracts and placed on a bacterial lawn. Plates were incubated for 24 hours and visually observed for inhibition zones in comparison to the antibiotic. The small a, b, and d, e denote Neem 250 & 125 µg/ml, and Papaya 250 & 125 µg/ml, respectively; c and f represent vehicle and the antibiotic control, respectively.

**Fig.6. Inhibitory effect of aqueous extracts of Neem and Papaya plant leaf on *S. aureus* on MHA plate.**
Paper discs were saturated with 20 µl of plant extracts and positioned on a bacterial culture. After 24 hours of incubation, the plates were inspected visually for zones of inhibition, comparing them with the antibiotic. The labels a, b, and d, e correspond to Neem concentrations of 250 & 125 µg/ml, and Papaya concentrations of 250 & 125 µg/ml, respectively; while c and f stand for the vehicle and antibiotic controls, respectively.

The main focus of this study was to examine the therapeutic potential of phytochemicals from *Azadirachta indica* and *Carica papaya* and their impact on the versatile pathogen, *Staphylococcus aureus*. The ultimate goal was to evaluate the traditional use of these plants. This research provided insights into the effects of crude leaf extracts and their potential against the bacteria. The focus was observing the changes in growth and pathogenicity of the bacteria. The Neem plant extract decreased the growth of the bacteria insignificantly (Figure 2), however, neither Neem nor Papaya affected their haemolytic behaviour (Figure 3). In addition, exposure to the medicinal plant extracts reduced bacterial pathogenic feature (biofilm development) in comparison to the control. The study forms a basis for future research to characterize how medicinal plant extracts modulate pathogenic bacterial growth and development.

In the past, Neem oil and extract were employed to combat *S. aureus* both in liquid medium (Sultana *et al.*, 2016) and on Petri plates (Sarmiento *et al.*, 2011). Similarly, the antibacterial efficacy of phytochemicals presents in the crude ethanolic and aqueous extract of Neem and Papaya against pathogenic bacteria was evaluated using Petri plates containing MHA media, and assessments included analyzing the zones of inhibition and determining the effect of concentration on bacterial metabolism.

To elucidate the potential mechanisms underlying the effects of the extracts and their components on *S. aureus*, prior investigations suggest that the antimicrobial efficacy of natural compounds against this bacterium may encompass diverse pathways, such as hindering cell wall synthesis and disrupting vital metabolic processes (Nappi & Singh, 2023; Subramani *et al.*, 2017). Additionally, research has proposed that specific bacteriocins produced by staphylococci could impede the proliferation of *S. aureus*, presenting promising avenues for further exploration (Newstead *et al.*, 2020). Furthermore, the antibacterial properties of plant extracts against the bacteria may stem from their capacity to intervene in cellular mechanisms and disturb bacterial cell architecture. For instance, a study demonstrated that two concentrations of *Dracocephalum moldavica* L. ethanolic extract not only suppressed the growth of *S. aureus* isolates by up to 90% but also disrupted 24-hour biofilm formation by inducing damage to the cell membrane and altering cell morphology. Moreover, the ethanolic extract exerted differential regulation on *S. aureus* protein expression, particularly proteins involved in membrane integrity, biofilm inhibition, and alterations in energy metabolism (Yu *et al.*, 2019). Incorporating these findings fosters a comprehensive comprehension of the underlying mechanisms governing the efficacy of the extract and its constituents against the bacterial species.

The antimicrobial properties of *A. indica* and *C. papaya* have been attributed to their phytochemical richness, particularly in antioxidants and antimicrobial agents. Previous
studies have reported the antimicrobial and antioxidant properties of *A. indica* (Yu et al., 2019; Quelemes et al., 2015) and *C. papaya* (Karo-Karo et al., 2023; Dagne et al., 2021; Ajiboye & Olawoyin, 2020). Additionally, ethno-based studies have highlighted the effectiveness of *A. indica* and *C. papaya* phytochemicals against *S. aureus* (Singh et al., 2023).

Development of Staphylococcal biofilm on implanted biomaterials is a significant virulence factor in the etiology of infections associated with medical devices. The current environmental conditions and exposure to various chemicals, including phytochemicals, antibiotics, etc., have a significant impact on biofilm growth. One of the most used techniques in the lab for assessing an isolate's capacity to create biofilms is the glass plate test. The formation of biofilm, in which the planktonic cells present cling to solid surfaces and multiply and accumulate in multilayer cell clusters embedded on a polymer matrix, is thought to be a typical aspect of *S. aureus'*s life cycle in the environment. Unlike exposed and susceptible planktonic cells, this structure shields the bacterial population from environmental challenges, the host immune system, and antibiotic attacks.

Crude plant extract contains a diverse array of compounds that may remain in different interactions. In the present study, the plant extracts were prepared in water and ethanol to get compounds as much as possible. However, other than an insignificant inhibition effect, both plant extracts established similar outcomes. Previous study mentioned similar i.e., no anti-staphylococcal activity of aqueous Neem leaf extracts in plates (Sarmiento et al., 2011). The UV spectrophotometric data revealed the presence of chemical groups relevant to the targeted phytocompounds (Figure 1), and the *in vitro* inhibition assay reflected a zone of unclear inhibitions (Figure 5 and 6). The phytocompounds may remain in a bond state and therefore interacted differently, a higher concentration of phytocompounds in the extracts may leave an improved effect, or other biological solvents, such as Acetone, Dimethyl sulfoxide, might create clear zones and so on. Nevertheless, the phytocompound concentration in the extracts was low (data not shown) which may be the sole reason for unclear inhibition. Therefore, further evaluation of the phytocompounds is necessary to assess the effect of the plants on *S. aureus* inhibition.

Pathogenic *S. aureus* from clinical samples were found to be β-haemolytic (Shil & Chichger, 2021). As a remedy, medicinal plant extracts might have influence on bacterial conversion from haemolytic to non-haemolytic state. Although *S. aureus* has the capacity to incorporate external gene related to various features (Kim et al., 2018), the bacteria were not haemolytic in the study conditions, however, it may behave differently in a different environmental condition.

The host's epithelial defence mechanisms provide protection, but controlling inflammatory reactivity is crucial (Nagai et al., 2016). Commensals have an impact on the host's immune system even when they do not initiate an epithelial defence response (Petersson et al., 2010). When pathogenic bacteria colonize a host, this damage to the host's cells prompts them to fight against the antigen, which results in an inflammatory reaction (Vijay-Kumar et al., 2010). Colonization is enhanced and protected in a biofilm, however, biofilm formation was reduced after being exposed to the plant extracts (Figure
4), which coincides with previous study showing a significant biofilm adherence inhibition from 62.5 µg/mL Neem ethanolic extract for a sensitive *S. aureus* and 125 µg/mL for two MRSA strain (Quelemes *et al.*, 2015). The evaluation of the antibacterial properties of *C. papaya* leaf crude acetone and aqueous extracts exhibited a zone of inhibition reaching a diameter of 17.90±0.10mm and 15.50±0.50mm, respectively at a concentration of 500 mg/ml against *S. aureus* (Ajiboye & Olawoyin, 2020). Also, the extracts reduced the growth of *S. aureus*, and displayed inhibitory effect in the disc diffusion assay, previous studies evidenced the effectiveness of the subjected plant extracts against *S. aureus* (Mehrotra *et al.*, 2010). In addition, use of appropriate controls (Doxycycline antibiotic as a positive control and a disc with equivalent amount of ethanol/water as vehicle control) represent the standards for the inhibition assay.

To conclude, the current study uses *Staphylococcus aureus* to imply their responses after exposure to the tested plant extracts. The findings indicate that the plant extracts exhibited inhibitory effect on the metabolism and pathogenic feature of the organism. The study adds information on the effect of traditional medicinal plant extracts on the growth and development of pathogenic bacteria. However, further research is necessary to investigate how traditional plants can be exploited in disease prevention and remedy by controlling disease causing bacteria.

REFERENCES


