Anti-Biofilm Activity of Asiatic Acid Against Cariogenic Bacteria

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Abstract

The accumulation of cariogenic microorganisms within a biofilm is one of the causes of dental caries. Streptococcus mutans and Lactobacillus casei bacteria are cariogenic bacteria because they have acidogenic and aciduric (acid-resistant) properties. The Asiatic acid isolate from Centella asiatica contains a number of anti-biofilm-active components. The objective of this investigation is to determine the efficacy of the anti-biofilm isolate Asiatic acid against Streptococcus mutans and Lactobacillus casei.

In culture media, S. mutans ATCC 25175 and L. casei ATCC 393 were grown. The MIC test was then conducted with Asiatic acid isolates ranging from 0.1% to 2.5%, followed by a time kill assay. A nucleotide leakage assay was conducted to confirm bacterial membrane instability. Using a biofilm assay, the anti-biofilm activity of the Asiatic acid isolate was evaluated.

The minimum inhibitory concentration of Asiatic acid isolate for S.mutans and L.casei is between 2% and 2.5%. The time-kill assay revealed that Asiatic acid has bacteriostatic activity. The concentration of 2MIC isolate Asiatic acid is efficacious in nucleotide leakage assays and biofilm formation assays.

These results imply that Asiatic acid may be a potent antibacterial agent that inhibits the formation of biofilm by cariogenic bacteria.

Experimental article (J Int Dent Med Res 2023; 16(3): 1104-1110)

Keywords: Antibiofilm, Asiatic acid, Strepcoccus mutans, Lactobacillus casei.

Received date: 05 June 2023 **Accept date:** 18 July 2023

Introduction

There are 700 - 1000 species of microorganisms in the oral cavity that affect the health of human teeth and mouth [1]. These microorganisms are closely linked to oral diseases like dental caries, periodontal disease, and oral cancer [2]. Dental caries requires the most attention because it is the most prevalent disease in society and impacts the body's systemic health. The accumulation of pathogenic biofilms is one of the primary causes of dental caries. [3].

Bacterial biofilms are communities of communicating microorganisms that settle on specific surfaces, adhere to one another, and are surrounded by layers of organic and inorganic substances they produce [4]. Biofilms are aggregations of bacterial cells encased in extracellular polymeric substance (EPS) that cover biotic and abiotic surfaces [5]. The proliferation of aciduric microorganisms in the biofilm, such as Streptococcus, Lactobacillus, Bifidobacterium, Actinomyces, Veillonella, etc., causes the pH of the mouth to drop below the critical point, resulting in the demineralization of tooth tissue [6].

Streptococcus mutans bacteria are primarily responsible for the initiation of caries, whereas Lactobacillus bacteria are responsible for the development of caries lesions [7,8]. Streptococcus mutans is virulent because it can produce acid from carbohydrate metabolism, persist in an environment with a low pH, and form adhesive biofilms [3,9]. Lactobacilli and dental caries have been linked for over a century. Lactobacillus casei is a strain of the Lactobacillus genus [10]. In a healthy oral cavity, there is typically

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no habitat for Lactobacillus casei or the number of this bacteria is minimal [11]. However, the proliferation of this bacteria will increase at active caries sites, particularly in dentinal caries [12]. By inhibiting the formation of biofilms resulting from the emergence of microbial colonization, caries development can be inhibited [13]. Consequently, anti-biofilm materials are required to prevent dental caries.

Currently, herbal ingredients are commonly used as a substitute ingredient and has antibacterial properties. Asiatic acid is a pentacyclic triterpenoid derived from the traditional Asian medicinal plant Centella Asiatica (Gotu Kola) [14]. Asiatic acid has been demonstrated to have beneficial effects not only for antiinflammation and neuroprotective activity, but also antimicrobial activity against pathogenic microorganisms [15,16]. The effect of Asiatic acid's antibiofim activity on S. Mutans and L. Casei, however, has not been reported.

Materials and methods

The protocol of this study has registered and approved by Komisi Etik Penelitian Kesehatan, Fakultas Kedokteran Gigi Universitas Islam Sultan Agung, with registration number 330/B.1-KEPK/SA-FKG/XI/2021 (Approval date: 25 November 2021). All methods were performed in accordance with relevant guidelines and regulations.

Materials

Asiatic acid (Asiatic acid 95%, catalogue number 546712-500MG, Sigma Aldrich, St. Louis, Missouri, United States); Dimethyl sulfoxide (DMSO) (Merck, Germany); The antimicrobial test strain corresponded to the American Type Culture Collection (ATCC), an international reference of S. mutans ATCC 25175 (MBRIO, Indonesia) and L.casei ATCC 393 (Dipa Puspa Labsains, Indonesia). Before cultivation, the stored at -80°C strain was reactivated by a reactivation phase 48 hours prior to the experiment and then maintained at 37°C.

Asiatic acid

Asiatic acid was stored 2°- 8° C. Asiatic acid was dissolved in DMSO to create concentrations. Asiatic acid concentrations ranged from 0.5% - 2.5% (v/w).

Preparation of bacterial culture

Streptococcus mutans and Lactobacillus casei bacteria were incubated in 10 ml of brain-heart infusion broth at 37 °C with 5% CO2 for 24 to 48 hours. Examine the turbidity of the medium and perform a gram stain to demonstrate that no other bacteria are contaminating the culture.

Saliva collection

Saliva samples were collected from three healthy volunteers aged 24 to 28. Samples were centrifuged at 10,000 x g for 10 minutes at 4 C, sterilized using a 0.22-m Millex-GP Filter Unit (Merck Millipore, Darmstadt, Germany), and deposited on the wells of the plate (Sumitomo Bakelite, Tokyo, Japan) for biofilm formation assays.

Minimum inhibitory concentration (MIC)

According to the group, 100µl of bacterial culture containing 10^6 CFU/ml was inoculated into 5 ml of Mueller-Hinton broth (MHB), and 100µl of Asiatic acid concentration ranging from 0.1% to 2.5% was added to each tube. All containers were incubated for 24 to 48 hours at 37°C in a CO_2 incubator. The minimum inhibitory concentration (MIC) is recorded at the lowest concentration of Asiatic acid that inhibits bacterial growth; there is no turbidity in the region. The turbidity was determined by measuring the optical density (OD) at 600 nm using a UV spectrophotometer; the experiment was repeated three times.

Time-kill assay

In vitro time-kill of Asiatic acid at 0.5MIC, 1MIC, and 2MIC against the test strain of bacteria was observed in 5 ml of MHB at 37°C after inoculation with 10^6 CFU of the bacterial culture. At 0, 3, 6, 9, and 12 hours, 100 µl of bacterial suspension was cultured on MHB plates for CFU/ml determination. Plates were incubated at 37°Celsius for 24 hours, and colonies were enumerated.

Nucleotide leakage

Nucleotide release is measured utilizing a spectrophotometer. Each vial contained 1 ml of MHB. A 100µl bacterial suspension was treated for 2 hours at 37°C with 100µl Asiatic acid at concentrations of 0.5MIC, 1MIC, and 2MIC. The absorbance at 260 nm of the supernatant was determined after 10 minutes of centrifugation at 10,000x g.

Biofilm formation

The biofilm formation was cultivated for 1 hour at 4˚C in 96-well polystyrene microtiter plates (Sumitomo Bake-lite, Tokyo, Japan) coated with human saliva. After coating, the wells were cleansed twice with phosphate-buffered

saline (PBS). S. mutans and L. casei were inoculated into wells containing 180 µl of TSB with 0.25% sucrose at an optical density (OD600) of 0.4. Plates were incubated with 20 µl of Asiatic acid at concentrations of 0.5MIC, 1MIC, and 2MIC for 16 hours at 37˚C with 5% CO_2. The well containing TSB was used as a negative control. Following incubation, adherent cells were stained for 15 minutes with 0.25% safranin and planktonic cells were eliminated by flushing with distilled water. After two washes with distilled water, safranin was extracted from the biofilm using 70% (v/v) ethanol and measured at 492 nm absorbance.

Data analysis

The data is displayed as the mean standard error. Utilizing SPSS software (IBM SPSS, Armonk, New York, United States), statistical analyses were conducted. The significance level was set at p □ 0.05, after which the normality of the data was examined, followed by an analysis of variance (ANOVA), then a post-hoc (Tukey test)

Results

Asiatic acid inhibits the growth of S.mutans and L.casei

Using a UV OD 600nm spectrophotometer, the Minimum Inhibitory Concentration (MIC) of Asiatic acid was determined. The minimum inhibitory concentration (MIC) is the lowest Asiatic acid concentration at which growth is inhibited. In this experiment, Asiatic acid inhibited the S.mutans strain with a MIC of 2% and the L.casei strain with a MIC of 2.5% significantly (Table. 1).

Strain		MIC
S. Mutans	ATCC 25175	2%
L.Casei	ATCC 393	2.5%

Table 1. Minimum Inhibitory Concentration (MIC) of Asiatic acid against cariogenic bacteria.

Using a time-kill assay, the effect of Asiatic acid on the growth of S. mutans and L. casei was determined. Figure 1 displays time-kill graphs for Asiatic acid at 0.5 MIC, 1 MIC, and 2 MIC against S. Mutans and L. Casei strains. The time-kill curves of Asiatic acid extracts against S. mutans and L. casei indicate a reduction in the number of cells at nine hours. In comparison to the control, the growth cycle curves of S. mutans and L. casei were inhibited at all concentrations tested. The most effective concentration of acetic acid to reduce the number of bacteria is 2 MIC.

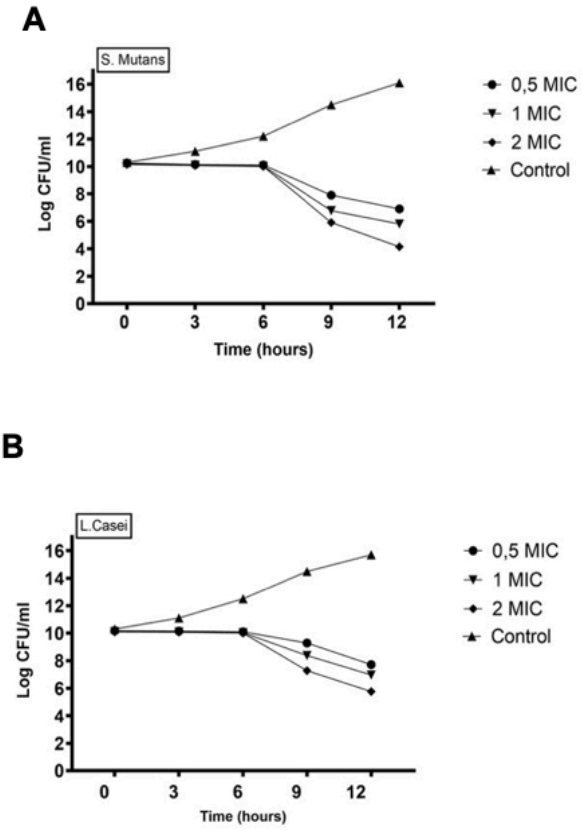

Figure 1. Time kill curve of S. Mutans and L. Casei treated with Asiatic acid. S. Mutans and L. Casei were cultivated in BHI broth medium containing varying concentrations of Asiatic acid and DMSO as a control. The growth kinetics were followed for 12 hours at 3-hour intervals.

Asiatic acid disrupt S.mutans dan L.casei membrane permeability

After exposure to Asiatic acid concentrations of 0.5MIC, 1MIC, and 2MIC, nucleotide leakage indicates bacterial cell lysis. Higher absorbance values indicate more efficacy since they indicate greater numbers of lysed bacterial cells at the concentration of exposure. Asiatic acid administration resulted in a significant increase in OD260 in S. Mutans and L. Casei, significant differences were found between all groups with p<0.0000, and the same significance was found when comparing 0.5MIC, 1MIC, 2MIC, and the control group (Figure. 2)

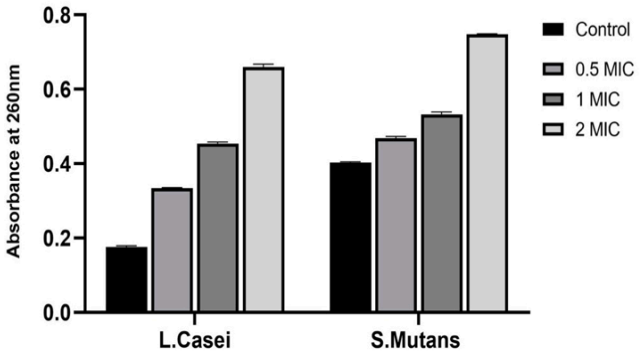

Figure 2. Effects of asiatic acid on bacterial nucleotide leakage. Asiatic acid at 0.5MIC, 1MIC, 2 MIC and control (DMSO) was added into a Mueller Hinton broth containing S. mutans and L.Casei. Bar graphs represent the relative nucleic acid leakage as quantified by measuring absorbance at 260 nm.

Effects of Asiatic acid on Biofilms

Asiatic acid's ability to disrupt preformed biofilms and effect the adhesion ability of S.mutans and L.casei at 0.5, 1, and 2 MIC was evaluated using the biofilm assay. According to the analysis, there are statistically significant differences between the concentrations of 2 MICs and S. Mutans ($p<0.0000$) and L. Casei ($p<0.0001$) biofilms. These results indicate that a concentration of 2 MIC inhibits S Mutans and L.casei biofilm formation (Figure.3).

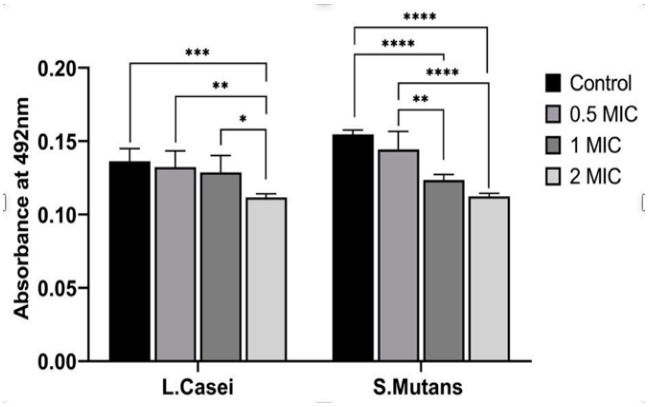

Figure 3. Effects of asiatic acid on Biofilm S.mutans and L.casei.After 16 hours, biofilms were stained with safranin and the absorbance at 492 nm was measured. Using ANOVA and the post hoc test, the differences between parameters were examined. * $p<0.05$; ** $p<0.001$; *** $p<0.0001$; **** $p<0.0000$; ns = not significant (not shown). The group replicated 5 times.

Discussion

The most prevalent use of biodiversity by humans is the production of pharmaceuticals from medicinal plants. According to their biogenetic origins, antibacterial natural products can be categorized as terpenoids, alkaloids, flavonoids, or simple phenols [17]. Triterpenoids, which consist of numerous compounds that can be subdivided into more essential chemical structural groups, are one of the most active compounds. Triterpenoids are primarily represented by tetracyclic and pentacyclic derivatives [18]. Asiatic acid is a pentacyclic triterpenoid that is the primary bioactive component of the Centella Asiatica (L.) Urban [19]. Due to its capacity to inhibit the growth of both gram-positive and gram-negative bacteria, Asiatic acid isolate is used as an antibacterial agent [20,21].

In this investigation, Asiatic acid demonstrated a broad spectrum of antibacterial activity against all bacterial strains. The results of the study revealed that exposure to Asiatic acid concentrations of 0.5% - 2.5% resulted in MIC values of 2% for S. mutans and 2.5% for L. casei. This is because Asiatic acid's acidic conditions can disrupt the Acid Tolerance Response (ATR) mechanism in S. mutans [22]. Acid Tolerance Response (ATR) in S. mutans modifies the composition of the plasma membrane by altering the proton bonds in the bacterial cell membrane under acidic conditions [7,23]. These protons establish bonds with a larger proportion of fatty acids in the cell membrane, protecting S. mutans from harm, and can raise the pH by 0.5 to 1 units [24]. Asiatic acid has bactericidal effectiveness by causing the fatty layer on the bacterial cell membrane structure to thin out until it is finally damaged [25]. The lipid layer on the bacterial cell wall is an essential protective barrier [26]. Lactobacillus Casei bacteria are more resistant to Asiatic acid than S. mutans bacteria. This is because lactobacilli possess multiple mechanisms to cope with acid stresses, such as passive acid efflux via the cell membrane, active proton pump via F1F0-ATPase, sodium-proton antiporters, and various alkaline production pathways, such as glutaminase and arginine deiminase, whose ammonia product can neutralize protons so that proteins and DNA in damaged bacterial cells can be repaired [27,28]. The application of Asiatic acid significantly inhibited

the proliferation of S. mutans and L. casei bacteria, as demonstrated by these results. These findings suggest that Asiatic acid is an antibacterial agent with a broad spectrum that is efficacious against two cariogenic bacteria.

Time-kill assays can monitor the growth and death of bacteria treated with antimicrobial compounds and are frequently used to assess the antimicrobial effects over time[29,30]. Time-dependent bactericidal effects occur when the antibacterial drug concentration is greater than the microorganism's minimum inhibitory concentration (MIC), whereas concentration-dependent bactericidal effects occur when an antimicrobial agent has a sufficient concentration at the microorganism's binding site to eradicate it [31]. Our analyses of time-kill kinetics revealed that Asiatic acid exhibited a rapid time-dependent kinetics of bacterial killing against all tested bacteria, mainly at 2MIC value. In the first 9 hours, Asiatic acid displayed a bactericidal effect against S. mutans and L. casei. A time-dependent increase in the number of dead cells following Asiatic acid exposure further supports Asiatic acid's function as a bactericide.

Bacterial membrane integrity is essential not only for self-defense but also for the functions of membrane-associated enzymes involved in energy production, respiration, and redox balance [32,33]. Consequently, bacterial membrane rupture readily compromises these essential functions, affecting the bacteria's survival and growth [34]. In this research, Asiatic acid destroyed the integrity of the test bacteria's membranes. High nucleotide leakage can be observed in S. mutans and L. casei when 2MIC concentrations of Asiatic acid are present. Additionally, nucleotide leakage occurs because Asiatic acid disrupts the homeostasis of potassium action cation (K^+) from the bacterial cytoplasm, causing more (K^+) to be forced out of the bacterial cell. Potassium cation (K^+), which is required for the growth, defense, and virulence of bacteria, is drastically reduced, resulting in the death of bacterial cells [35]. Asiatic acid inhibits bacterial growth by damaging the membrane and altering the structure of the bacterial cell, causing the intracellular substance to seep out. [36]. Due to their strong UV absorption at 260 nm, it is possible to quantify the release of nucleotides and their derivatives, including DNA and RNA, from bacteria by measuring their absorbance at this wavelength [37]

Bacterial biofilm is an aggregated form of bacterial community in which each cell is compacted and interconnected by extracellular polymeric substances [38]. The biofilm assay reveals that the higher the proportion of bacteria that absorb crystal violet, the higher the number of bacteria that die and the better the efficacy of Asiatic acid. The percentage of S. mutans and L. casei biofilm formation that was inhibited by Asiatic acid 2MIC was found to be greater in this study. Asiatic acid is capable of inhibiting the attachment of S.mutans and L.casei to biotic and epithelial surfaces [39,40]. Asiatic acid, which diffuses through the permeability of the cell wall, interferes with genetic proteins and the formation of bacterial organs, alters the morphology of bacterial fimbriae, and causes them to become hydrophobic. The bacteria lose their ability to adhere, thereby harming the biofilm's formation [41]. The reduction of biofilm must be regarded as the initial phase for its control by an antimicrobial agent.

Conclusions

This study reveals that Asiatic acid isolate is a triterpenoid saponin derivative derived from the Centella Asiatica plant that possesses antibacterial activity in S.mutans, and L.casei biofilms. This result was confirmed as Asiatic acid can inhibit the membrane permeability of cariogenic bacteria. To establish the therapeutic applicability of this isolate, it will be necessary to execute animal toxicity studies in future research.

Acknowledgements

The authors would like to thank the Faculty of Dentistry, Universitas Islam Sultan Agung.

Declaration of Interest

The authors report no conflict of interest.

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