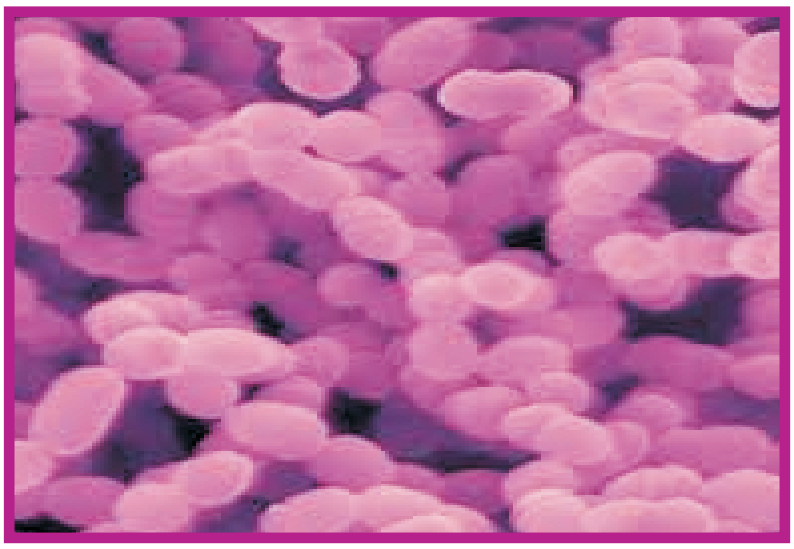


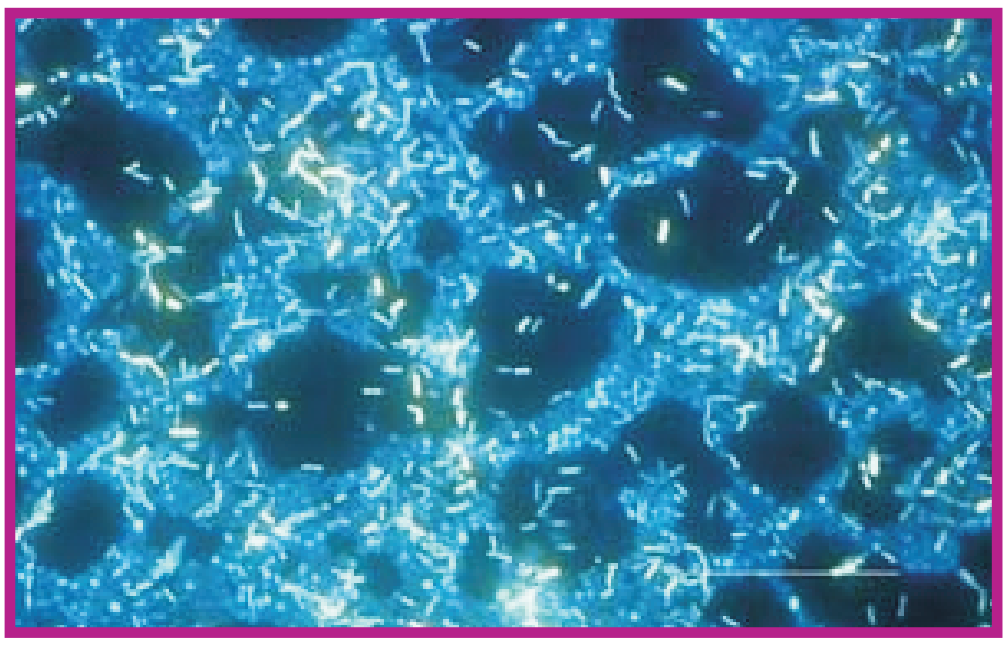
Abstract:-

Most of the pathogens have the ability to form biofilms which makes them resistant to the antimicrobial agents. The emergence of new multi drug resistant strains is a big concern. Hence there is a growing need to find alternative antimicrobial agents. Plants have been used for ancient years to control many diseases. In the present study we evaluated antibiofilm activity of Salix alba bark extract against the Streptococcus mutans and Staphylococcus aureus which are the main cause of dental plaque formation. This is the first study of its kind where bark extract of Slaix alba was used for antibiofilm activity.In the present study it was found that the Salix alba bark extract has a good effect on the microbial biofilm formation. Hence the use of Salix alba can be considered for controlling the biofilm formation. This is the primary study and more research is needed in this regard.

Keywords:

Biofilm, Alkaloids, Antimicrobial, Salix Alba, Antibiotics, Antimicrobial Resistance.

ANTIBIOFILM ACTIVITY OF SALIX ALBA PLANT EXTRACT AGAINST STREPTOCOCCUS MUTANS AND STREPTOCOCCUS AUREUS ISOLATED FROM DENTAL PLAQUE SAMPLES



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INTRODUCTION

Dental caries is an infectious microbial disease that results in localized dissolution and destruction of the calcified tissues of the teeth [1]. *Streptococcus mutans* is known as the causative bacteria in the formation of dental plaque and dental caries. The acid producing *S. mutans* inhabiting the mouth causes damage by dissolving tooth structures in the presence of fermentable carbohydrates such as sucrose, fructose, and glucose [2]. Persistent dental disease is painful, and most importantly, it has also been suggestively linked to diabetes, high blood pressure, heart disease, and multiple sclerosis later in life. The pain can be worsened by heat, cold or sweet foods and drinks [3, 4]. Dental plaque is a type of microbial biofilm,

Biofilms - adherent communities of bacteria surrounded by a matrix of extracellular polymeric substance (EPS) - are the prevailing microbial lifestyle in the environment [5]. The role of the biofilm is to attach to abiotic surfaces, the epithelia of multicellular organisms, and interfaces such as that between air and water [6]. Surface adhesion of bacteria is an essential step and is required for the bacteria to arrange themselves favorably in their environment [7].

Now the biofilm is considered as major target for the pharmacological development of drugs. A biofilm serves to promote bacteria persistence by resisting antibiotic treatment and host immune responses [8]. Antibiotics are rendered ineffective when biofilms form due to their relative impermeability, the variable physiological status of microorganisms, subpopulations of persistent strains, and variations of phenotypes present [9]. Biofilms have been reported to show increased resistance to antimicrobial agents including antibiotics compared to free-floating cells [10].

Plant extracts and other biologically active compounds isolated from plants have gained widespread interest in this regard as they have been known to cure diseases and illnesses since ancient times [11].

The search for natural-product antifoulants has been greatly encouraged by the fact that these compounds are less or non-toxic to the environment. The majority of natural product antifoulants identified so far are terpenoids, steroids, carotenoids, phenolics, furanones, alkaloids, peptides and lactones [12]. They have been isolated from a wide range of organisms such as sponges, corals, seaweeds and microbes [13,14]. As biofouling causes huge loss to the marine domain, intensive studies towards the development of eco-friendly antifouling agents are progressing worldwide [15,16,17]. Most of the studies on natural-product antifoulants are concentrated mainly on marine invertebrates, seaweeds, tunicates and microbes. There is a lack of information on antifouling agents from terrestrial sources.

Salix alba (white willow) White willow bark has been used throughout the world as an antipyretic and analgesic. Since the development of synthetic acetylsalicylic acid in the 1890's, willow bark has fallen into disuse and has not undergone rigorous scientific evaluation. Ancient Egyptians used the bark of the white willow to treat pain and inflammation [18].

Hippocrates and Dioscorides recommended willow bark as a remedy for gout and rheumatic joint diseases, but it fell into disuse in Europe during the Middle Ages. Kuan-Yin, the bodhisattva of compassion, is often depicted holding a willow branch as a symbol of healing.

Native American healers also relied on willow for its analgesic properties [19]. Knowing the fact that no literature is available on the anticariogenic property of *Salix alba* the study is focused on assessing the plant extracts with different solvents.

2. MATERIALS AND METHODS

2.1 Plant materials:

Healthy and fresh bark of *Salix alba* was collected from the respective plants grown in Tral Kashmir, the material was authenticated at Department of Botany, Annamalai University .Collected materials were washed in tap water followed by successive washing in distilled water. Washed materials were shade dried. Dried material was homogenized in domestic mixture into fine powder, stored in plastic container at room temperature used for further studies.

2.2 Dental Plaque samples:

The dental plaque sample was taken from the patients attending the Outdoor patients department of the Raja Muthiah Dental College and Hospital, Annamalai University, Chidambaram TN. The plaque samples were then transferred to VMGA III transport media.

2.3 Isolation of Microorganisms used in the study:

The dental plaque samples were serially diluted and 0.1 ml of 10-6 dilution was plated on the petriplates containing the growth media. MS Agar media was used for the isolation of *Streptococcus mutans*. For isolation of *staphylococcus aurues* MSA agar medium was used. The organisms were identified by Gram staining and biochemical tests.

2.4 BIOCHEMICAL REACTIONS

2.4.1 Gram's Reaction

Gram staining was carried out as per the Hucker's modification (Rangaswami, 1975) [20].

2.4.2 Motility Test

Semisolid agar medium was prepared and the bacterial isolates were stabbed into the semisolid agar medium. These inoculated stabs were incubated at 30°C for 72 h and observed for motility. The distance of growth from the point of stab showed motility.

2.4.3 Catalase test

The tryptosone soy agar was prepared and dispersed in the test tubes and sterilized in an autoclave. The bacterial isolates were incubated at 37°C for 24 to 48 h. suitable inoculated controls were maintained. Hydrogen peroxidase was added to culture tubes including the un-inoculated control through the sides of the test tube so as to flow gently. The bubbling of oxygen within a minute after addition of hydrogen peroxide indicated the positive character of the isolates tested.

2.4.4 Oxidase test

A piece of whatman No. 42 paper was smeared with oxidase test reagent and loopfull of organism was smeared over a small area of the paper and the changes were observed.

2.4.5 Hydrogen Sulfide (H2S) production test

Sulfide indole motility agar, medium was prepared, poured into tubes and sterilized at 25 lbs for 15 min. the agar tubes were stabbed with sterile needle containing bacterial culture and kept at 32°C for 48 h. the tubes were examined for presence or absence of black precipitate along the line of inoculation and observations were noted.

2.4.6 Utilization of citrate

Simmon's citrate medium was prepared and sterilized. The isolates were inoculated on the slants by streak method and incubated at 37°c for 48 h. change in the colour of medium from green to blue indicated citrate positive, while as the green colour (no Change in colour) indicated the citrate negative.

2.4.7 Nitrate reductase activity

The nitrate reductase activity of various bacterial isolated were tested based on the method described by Yordy and Rudoff (1981) [21]. Bacterial isolated were inoculated in 10 ml of malate broth supplemented with 10 mM of sodium nitrate and incubated at room temperature for five days on a rotary shaker.

The broth was centrifuged at 800 rpm for 10 min and the supernatant was collected. To 10 ml of the supernatant, 0.3 ml of one percent sulphanilmide in 1.5 N hydrochloric acid and 0.2 ml of 0.002 per cent N (1-napthyl) ethylene diamine dihydro chloride were added. The appearance of pink colour indicated the presence of nitrate reductase activity.

2.4.8 Voges-Proskauer test

The Voges – Proskauer test was carried out by following the method described by Aneja (1993) [22]. The change in the colour is indication of positive VP test while no change in colour is regarded as negative test.

2.4.9 Indole test

Tryptone broth was prepared poured into test tubes and sterilized. The medium was inoculated with a loopful of culture and incubated at 32°c for 48 h mixed with 3 ml of Kovac's reagent, shaken well and allowed to stand for 5 min. the observations were recorded.

2. 4.10 Carbohydrate Fermentation Test:

Carbohydrate fermentation test was carried out to demonstrate the ability of organism to ferment

various carbohydrates. Inverted Durham's tubes were placed in test tubes were filled 5-6ml of indicator broth medium just above the Durham's tubes and sterilized at 121^{0}C for 15 mins. 0.3 ml of 20% filter sterilized carbohydrate solution was added aseptically.

Each sugar tube was then inoculated with (5 drops) culture and incubated at 37^{0}c for 24-48 hours. Development of yellow colour and presence of bubbles or drop in the fluid level in Durham's tube indicates fermentation.

2.5 Biofilm inhibition assay

Biofilm inhibition carried out in 96 wall plates adopting modified method of biofilm inhibition spectrophotometric assay [23] .100µl of cell suspension of the organism thus prepared was added into 96 well titre plate and different concentration of free and polymer coated plant extracts as 0.125, 0.250, 0.500, 1.00 mg /ml was added and incubated at 37º C for 3 days. After the incubation, the liquid suspension was removed and 100 µl of 1% w/v aqueous solution of crystal violet was added. Following staining at room temperature for 30 minutes the dye was removed and the wells were washed thoroughly, 95% ethanol was added and incubated for 15 minutes. The reaction mixture was read spectrophotometrically at 570nm. Inhibition mediated reduction of biofilm formation was calculated by the following formula

$$\%\ of\ biofilm\ inhibition = \frac{\text{OD in control} - \text{OD in treatment}}{\text{OD in control}} \times 100$$

3. RESULTS AND DISCUSSION

The colonies from both the selective media were observed for morphological characteristics. Gram staining and other biochemical tests confirmed the isolates as *Streptococcus mutans* and *Staphylococcus aureus*. The colonies of *Streptococcus mutans* were found to be white/grey circular. The isolates were Gram positive spherical/ short rods. They were catalase and oxidase negative. The isolates showed a positive growth at 37^{0}C.

The *Staphylococcus aureus* colonies were circular, raised, glistering. These isolates were found to be Gram positive, spherical cells, catalase positive and oxidase negative. (Table I)

Methanol extract showed a better activity against the tested organisms as compared to the ethanol extract. This can be due the fact that methanol extract has more phytochemicals than the ethanol extract. Methanol extract showed a better activity against the Streptococcus mutans at I mg/ml concentration and inhibited 80.03±0.21of biofilm formation while as ethanol extract at this concentration showed a slightly lesser inhibition of 78.13±0.12 (Table III, IV).

Compared to Streptococcus mutans, Staphylococcus aureus was more susceptible to the Salix alba bark extract at the 1 mg/ml concentration the *Streptococcus aureus* biofilm was inhibited by 83.23±0.21 by methanol extract while as at the same concentration ethanol extract inhibited 82.13±0.12 biofilm formation (IV, V).

Due to the resistance to the conventional antimicrobial agents, researchers have been prompted to identify the alternatives for the control of infections. Plant extracts have gained wide importance in the recent years in this regard as they are used since ancient times to cure illness. [24]. All the plant extracts tested inhibited biofilm as dose dependent manner. Anti biofilm effect of various plant extracts against biofilm of human pathogenic bacteria has been reported by workers [25,26,27]. Ours is the first study which reported the antibiofilm activity of Salix alba.

Table I: Grams reaction and biochemical characteristics of the isolates

Isolate Designation	Cell shape	Motility	Gram reaction	catalase	Oxidase	Colony morphology on blood agar	Growth at 37^{0}C	Hemolysis
Streptococcus mutans	Spherical/ short rods	-ve	+ve	-ve	-ve	White, circular	+ve	α- hemolytic
Staphylococcus aureus	Spherical	-ve	+ve	+ve	-ve	Grey,Smooth, raised, glistering, circular	+ve	β- hemolytic

Table II. Influence of *Salix alba* methanol extract on biofilm formation (%) of *Streptococcus mutans* by well plate assay at time period (h).

Extract	Biofilm inhibition (%)					
Concentration	12(h)	24(h)	36(h)	48(h)	60(h)	72(h)
0.125(mg/ml)	0.0	0.0	17.30±0.10	21.33±0.06	27.10±0.06	32.33±0.31
0.250(mg/ml)	0.0	0.0	32.47±0.15	37.43±0.40	43.00±0.26	50.20±0.10
0.500(mg/ml)	0.0	0.0	47.27±0.25	54.20±0.26	61.00±0.10	68.40±0.10
1.00(mg/ml)	0.0	21.27±0.12	54.33±0.15	68.37±0.12	73.20±0.15	80.03±0.21
Control	0.0	0.0	0.0	0.0	0.0	0.0

DMSO was taken as control. The value is a mean of three replicates ± SD.

Table III. Influence of *Salix alba* ethanol extract on biofilm formation (%) of *Streptococcus mutans* by well plate assay at time period (h).

Extract	Biofilm inhibition (%)					
Concentration	12(h)	24(h)	36(h)	48(h)	60(h)	72(h)
0.125(mg/ml)	0.0	0.0	18.17±0.15	20.30±0.10	28.27±0.06	36.17±0.06
0.250(mg/ml)	0.0	0.0	33.13±0.12	40.07±0.12	45.13±0.06	46.10±0.10
0.500(mg/ml)	0.0	0.0	45.13±0.06	52.30±0.00	57.17±0.15	59.90±0.17
1.00(mg/ml)	0.0	17.87±0.06	47.73±0.25	62.37±0.12	68.33±0.06	78.13±0.12
Control	0.0	0.0	0.0	0.0	0.0	0.0

DMSO was taken as control. The value is a mean of three replicates ± SD.

Table IV. Influence of *Salix alba* methanol extract on biofilm formation (%) of *Streptococcus aureus* by well plate assay at time period (h).

Extract	Biofilm inhibition (%)					
Concentration	12(h)	24(h)	36(h)	46(h)	60(h)	72(h)
0.125(mg/ml)	0.0	0.0	20.13±0.12	24.37±0.12	30.13±0.06	35.43±0.12
0.250(mg/ml)	0.0	0.0	33.30±0.17	40.27±0.15	45.23±0.12	55.13±0.15
0.500(mg/ml)	0.0	23.60±0.10	50.03±0.06	57.57±0.06	65.27±0.25	70.13±0.12
1.00(mg/ml)	0.0	37.43±0.06	55.83±0.06	63.53±0.15	75.27±0.25	83.23±0.21
Control	0.0	0.0	0.0	0.0	0.0	0.0

DMSO was taken as control. The value is a mean of three replicates ± SD.

Table V. Influence of *Salix alba* ethanol extract on biofilm formation (%) of *Streptococcus aureus* by well plate assay at time period (h).

Extract	Biofilm inhibition (%)					
Concentration	12(h)	24(h)	36(h)	48(h)	60(h)	72(h)
0.125(mg/ml)	0.0	0.0	18.50±0.17	22.37±0.12	30.23±0.15	36.90±0.10
0.250(mg/ml)	0.0	0.0	32.30±0.10	38.23±0.15	46.20±0.10	51.83±0.15
0.500(mg/ml)	0.0	0.0	50.53±0.15	55.13±0.15	61.87±0.15	67.87±0.15
1.00(mg/ml)	0.0	0.0	52.67±0.06	60.10±0.10	72.20±0.17	82.13±0.12
Control	0.0	0.0	0.0	0.0	0.0	0.0

DMSO was taken as control. The value is a mean of three replicates ± SD.

4. CONCLUSION:

Biofilm is one of the major virulent factors of most of the pathogenic microorganisms. Plant extracts with antimicrobial activity has received growing interest as effective and safe medicine. The present study demonstrated the antibiofilm activity of Salix alba bark extract against the *Streptococcus mutans* and *Staphylococcus aureus* isolated from dental plaque salmples. Further study would help to understand the molecular mechanism of antibiofilm activity of *Salix alba* extract.

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