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ANTIMICROBIAL EFFECT OF WALNUT LEAVES (*JUGLANS REGIA*) ON STREPTOCOCCUS MUTANS IN DENTAL CARIES AMONG SCHOOL GOING CHILDREN IN QUETTA

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Abstract

Streptococcus mutans and Streptococcus sobrinus are oral opportunistic pathogens which cause dental caries particularly in children. The objective of study was to determine the status of dental caries in Government school's children age between 10-14 years. Four hundred dental samples were collected among the students, cultured on selective media and biochemically identified. The molecular identification was also done by specific primer for each bacterial species. Out of 400 samples, 111 (27%) were identified as S. mutans and 33 (8.25%) as S. sobrinus. The lowest prevalence of S. mutans was observed in 10-year age group, with 13 positive cases out of 90 dental samples while the highest rate recorded in the 14-year age group, with 32 positive cases out of 50 samples. Similarly, in 13-year age group, 5 out of 78 samples were identified as S. sobrinus whereas in 14-year age group, 9 out of 50 samples tested positive. Another key objective of this research was to evaluate the efficacy of walnut leaves (Juglans regia) against isolates. Different concentrations of Walnut leaves extract (ethanolic) was tested against S. mutans and S. sobrinus, showed significant antibacterial activity in well diffusion assay. The MIC and MBC values were also determined. The MIC of Walnut leave extract was 50µl/ml whereas the MBC value was evaluated at 100µl/ml. Furthermore, the antibiotic sensitivity test was also performed for isolates. The most effective antibiotic against S. mutans was Sulphamethoxazole that formed 22 mm of inhibition zone while Nalidixic acid showed low sensitivity with 10 mm of inhibition zone. The antibiotics which formed 20 mm inhibition zone against S. sobrinus included Oxytetracycline and Sulphamethoxazole. Both isolated bacterial species showed antibiotic resistance against Erythromycin, Penicillin-G, Clindamycin, Bacitracin, Lincomycin and Norfloxacin.

Keywords: *Antimicrobial activity, Walnut leaves (J. regia), S. mutans, S. sobrinus, Dental carries, School children*

INTRODUCTION

Dental caries is a common issue of oral health that presents a serious burden across the world. The children between the ages of 10 to 14 years are more vulnerable to develop dental caries. They consume sugary diet, acidic foods and beverages, which favor the growth of oral bacteria. Children often neglect proper oral hygiene practice, such as regular brushing and flossing, which leads to dental caries and plaque build-up on their teeth. The bacterial species, such as *S. mutans* and *S. sobrinus* are known to play a critical role in dental caries. These bacteria produce acid which causes tooth decay that disintegrates the enamel of teeth, which leads to cavities (1).

The growth of microbes is supported in oral cavity due to biological and physical factors. The oral cavity has normal temperature 37 ℃ and saliva has pH about 6.5 to 7 which favor the growth of several bacterial species. After the gut, oral cavity is thought to be the second largest microbial flora in human body (2). The main causative agent of dental carries is *S. mutans*. It's a Gram positive coccus, facultative anaerobe and mesophilic (18-40 ℃) bacteria. They usually forms chain during their growth and are non-motile. This microbe demineralizes the calcium and phosphate of tooth enamel which results in dental caries (3). Children between the ages of 5 to 14 are susceptible to dental caries because of poor oral hygiene condition. Dental caries is probably prevailed and fourth most expensive disease to treat in Pakistan. Reports of World Health Organization (WHO) showed, 29 thousands individuals in 21 district of Pakistan founded dental caries (4).

Several previous studies have identified the risk factors of dental caries in children and explored the ways to improve their oral hygiene. One such example is the use of *J. regia* leaves extract as a mouth wash to promote dental hygiene. It has antibacterial activity that inhibits the growth of *Streptococcus*, *E. coli* and *Pseudomonas* when used in high concentration. The walnut leaves have antifungal, antibacterial and anti-plaque activity. The extract of walnut shells and leaves, have traditionally been used around the world for oral hygiene as a preventative measures (5).

A study analyzed the status of dental caries in schools children ranging in age from 10 to 14. The Etiological cause of dental caries and plaque formation is *S. mutans* and other *Streptococcal* species, and the activity of other plaque micro-biota in mouth. Children with age of 12 have great interest in developing of dental caries and evaluation of oral health. This knowledge is essential for establishing preventative strategies in children to promote oral health (6).

Another chief aspect of cariogenic bacteria is expending antibiotic resistance. The resistance pattern causes high financial loss and fatalities in developing countries. Besides dental caries *S. mutans* also causes endocarditis therefore, it needs appropriate treatment. A study conducted in 2014, showed complete resistance of *S. mutans* against ampicillin and penicillin (7). It has been observed that dental caries is difficult to treat with conventional treatment. In recent study on *S. mutans* revealed a gradual increase in resistance to Erythromycin, Penicillin, Ciprofloxacin and different antibiotics (8).

The research investigated the possible antibacterial activities of the walnut leaves extract in relation to the cariogenic bacteria. Several chemicals found in walnut leaves, such as the phenolic compounds and essential oils which have the potential to inhibit the growth of cariogenic bacteria (1).

The antimicrobial effect of *J. regia* extract in dental caries can be useful for oral hygiene. The ethanolic extract of walnut leaves and its tree's bark inhibits the growth of oral bacteria. The antimicrobial activity of leaves is due to the presence of phenolic compounds like terpenoids, flavonoids, alkaloids and steroids. The extract has showed antimicrobial activity against cariogenic bacteria reported in studies, such as *S. mutans*, *S. salivarius* and *Actinomyces viscosus* (9, 10).

In order to determine antibacterial properties of *J. regia* leaves, clinical strains of *Streptococcal* species acquired from dental caries sample and cultured in the laboratory. To check the efficacy of the walnut leave extract as an inhibitor against cariogenic bacteria, the study used both the agar well diffusion method and the broth micro-dilution technique (6).

Dental caries is an oral health problem in children and its prevalence has been reported 48% to 95% in 8-13 years while 53% in group of 15 years old children. The walnut leaves have oral health care compounds and used in different traditional medicine (10).

METHODOLOGY

STUDY DESIGN

A cross-sectional study was conducted in Quetta from August to October 2024 to determine the occurrence of dental caries among school-aged children (10-14). A total 400 dental caries samples were collected from three boy's schools. The samples were analyzed in-vitro to identify the bacterial species. After isolating of pure culture of *S. mutans* and *S. sobrinus,* biochemical tests were performed. The isolated microbes were further confirmed using PCR. Walnut leaves extract was prepared and its antimicrobial activity against isolates was evaluated using standard methods. Moreover, the antibiotic sensitivity test was conducted and Statistics was applied to compile the results.

SAMPLE COLLECTION

The samples were collected from students aged 10 to 14 years. One hundred eighty samples from Comprehensive high school, one hundred from Sandeman higher secondary school and one hundred twenty samples from Killi Ismail high school, Quetta. Dental caries samples were collected with sterile cotton swab. The samples were processed to isolate *S. mutans* and *S. sobrinus* in vitro identification at Center for Advanced Studies in Vaccinology and Biotechnology (CASVAB).

CULTURE MEDIA

The selective media, Mitis Salivarius Agar with 1% Potassium Tellurite was used for the growth of *S. mutans* and *S. sobrinus*. Initially, oral samples were inoculated in Tryptone soya broth (TSB) and incubated for 24 hours. After incubation, the positive samples were then inoculated on MSA plates and incubated for 24 hours at 37°C. The appeared morphological characteristic of desired microbes were observed and noted.

GRAM STAINING AND MICROSCOPY

The procedure of Gram staining was performed to differentiate between Gram positive and Gram negative bacteria. The bacterial species which involved in dental caries are *S. mutans* and *S. sobrinus* were identified by colony morphology and staining results.

BIOCHEMICAL TEST

The biochemical test including catalase, bacterial growth in 6.5% NaCl saline, and blood haemolysis on blood agar were performed. Finally, Voges Proskauer test was performed to confirm the isolates and results were recorded (16).

PCR CONFIRMATION

DNA EXTRACTION

The boiling method was used for the extraction of bacterial DNA. The bacterial culture was grown in BHI broth. 1 ml from overnight culture was taken into eppendrof tube and centrifuged 12000^{x} **g** for 5 minutes. The pellet formed was boiled with 1 ml of Tris HCL (10 mM) at 100 °C for 10 minutes. Again the tubes were centrifuged at 12000^{x} g for 10 minutes. 5 µl was taken from supernatant for PCR reaction (15).

PCR IDENTIFICATION (S. MUTANS)

The PCR technique was used to detect the virulence *gtf-B* genes. The pair of primers used that targeted the *gtf-B* gene. The sequence of primer was 5'-ACTACACTTTCGGGTGGCTTGG-3' (Forward) and 5'-CAGTATAAGCGCCASTTTCATC-3' (Reverse). The size expected for PCR amplicon product was 517 bp. The condition for amplication consisted on 95°C for 4 minutes; 30 cycles of 95°C for 30 second, 59°C for 30 seconds, and 72 °C for 1 minute and 72°C for seven minutes (11).

PCR IDENTIFICATION (S. SOBRINUS)

The primers used to identify the *S. sobrinus* was *gtf-I* gene 5'-GAAACCAACCCAACTTTAGCTTGGAT-3' (forward) and 5'-ATGGATGATTTTCCATCGGTACTTG-3' (Reverse). The expected size for PCR amplicon product was 319 bp. The PCR system was set on forty cycles. Each cycle consisted on 95°C for ten minutes; denaturation occurs at 95 °C for fifteen seconds, annealing and extension at 60°C for one minute (12).

GEL ELECTROPHORESIS

The agarose gel (1.2%) was used to analyze the amplified DNA. To run the electrophoresis 7 µl of PCR product was placed into the well of agarose gel. The electrophoresis was run on 400 mA current with voltage of 100 volts for 30 minutes (11).

GEL DOCUMENTATION

The gel documentation system was used for PCR product, to visualize the DNA bands. The results were analyzed for *S. mutans and S. sobrinus* in 10-14 years age group in children (11).

WALNUT LEAVES EXTRACT PREPARATION (*JUGLANS REGIA*)

Walnut leaves were collected from district Ziarat. The method used for the extraction of walnut leaves extract has different steps. Firstly, the plant leaves were washed by tap water, cut into small pieces then air dried in shade at 22°C for 72 hours. Dried leaves were grinded into fine powder by Homogenizer.

Secondly, powder was used to prepare alcoholic extract from leaves with ratio of 1 gm of walnut leaves into 10 ml of ethanol and shaken for 48 hours. The leaves extract was filtered aseptically and evaporated the solvent by rotary evaporator. Finally, the concentrated extract crude made in 200 mg/ml of DMSO and stored in dark place at 4 °C (10).

EVALUATION OF ANTIMICROBIAL EFFECT OF WALNUT LEAVES EXTRACT

The evaluation of the antimicrobial activity including minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were determined by well diffusion method and broth dilution method.

WELL DIFFUSION METHOD

The inoculum was prepared according to clinical laboratory standard. Colonies of 24 hours culture of bacterial species were suspended in Muller-Hiton broth and adjusted to 0.5 McFarland standards. The bacterial strain was streaked by using a sterile swab to Muller-Hiton agar and Nutrient agar plates and left to dry for 15 minutes to perform sensitivity test. The wells were made by cork borer with 6-8 mm in diameter on agar plates (13). The antibiotic disc placed at the center of agar plates was used as the positive control. The leaves extract was poured in wells as 25, 50, 75 and 100 micro-liters. The plates were incubated for 24 hours at 37 °C. The antibacterial activity was measured in inhibition zone (14).

BROTH DILUTION METHOD (MIC AND MBC)

The walnut leaves extract (ethanolic) was used to demonstrate the MIC and MBC. Muller Hinton broth was prepared and poured into test tubes. The colony of each testing bacteria was inoculated separately in 2ml MH broth and incubated for 24 hours at 37 °C. A serial dilution of walnut leaves extract was performed to reach the concentration of 100 to 1.56 mg/ml. 100 µl from each prepared bacterial strain transferred to MH broth (1 ml broth) tubes. 1 ml MH broth in test tube was left un-inoculated for negative control. 100 µl of each bacterial strain were inoculated in MH broth tubes without leaves extract for the positive control and incubated at 37 °C for 24 hours. The lowest concentration that inhibits the microbial growth was considered as MIC value for tested bacteria. To evaluate MBC value, MH agar was poured into culture plates and allowed to solidify. The cultures tubes that showed no visible growth in tubes were streaked on agar plates and incubated for 24 hours at 37 °C. The lowest concentration at which no bacterial growth was observed on the agar plates was considered the MBC (13).

ANTIBIOTIC SENSITIVITY TEST

The antibiotic sensitivity test was performed for isolates by using disc diffusion method. The microbial inoculum was prepared and adjusted with 0.5 McFarland standards. The lawn was made on BHI agar by swabbing the inoculum and allowed to dry for 15 minutes. The antibiotics used were: Erythromycin E-30 (30-µg), Penicillin-G (P) 10 units, Sulphamethoxazole (SXT) 25µg, Cloxacillin (OB) 5µg, Clindamycin (DA) 2µg, Lincomycin (MY) 10 µg, Polymyxin-B (PB) 300 units, Colistin Sulphate (CT) 25 µg, Rifampacin (RD) 5 µg, Norfloxacin (NOR) 10 µg, Bacitracin (BC) 0.04 units, Nalidixic acid (NA) 30 µg, Enrofloxacin (ENR) 5 µg, Tobramycin (TOB) 10 µg, Furazolidone (FR) 15 µg, Oxytetracycline (OT) 30 µg. The inhibition zone was measured after 24 hours of incubation at 37 °C. The experiment was performed in duplicate and results were recorded.

RESULTS

MICROSCOPIC EXAMINATION OF ISOLATES

One hundred and eleven samples were positive on the basis of colony morphology and Gram staining. The colonies of *S. mutans* appeared roughly in blue colour on MS agar. The bacterial species appeared in coccus (round) shaped, purple in colour and formed chain (Fig. 1). The data in Table I show the positive result of samples according to age.

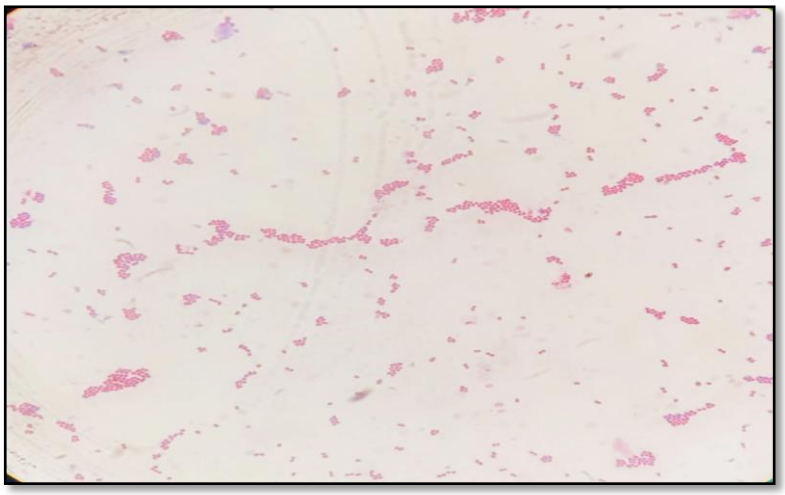

Fig. 1. Microscopic view of *S. mutans*

Table I. Age wise positive results of *S. mutans*

Age	Total students	Percentage	No. of Students (positive)	% of positive students	Over all %
10	90	22.5	13	11.72	3.25
11	97	24.4	18	16.21	4.5
12	85	21.25	21	18.93	5.25
13	78	19.5	27	24.34	6.75
14	50	12.25	32	28.82	8
Total	400	100	111	100	27.75

The *S. sobrinus* exhibited smooth light blue colonies on Mitis Salivarius agar. After Gram stain the species appeared as coccus, gram positive, blue colour, coccus (round) shaped, formed chains during growth (Fig. 2). Thirty three positive samples out of four hundred showed characteristics of *S. sobrinus*. The data in Table II show the positive results of samples according to age.

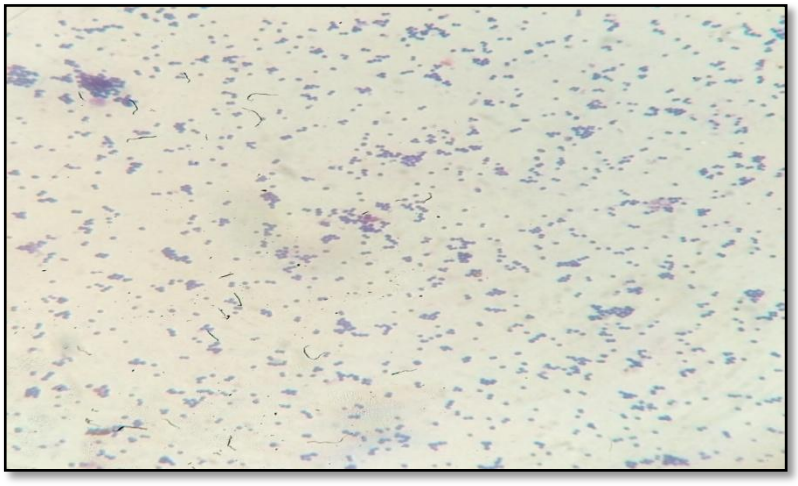

Fig. 2. Microscopic view of *S. sobrinus*

Table II. Age wise positive results of *S. sobrinus*

Age	Total students	Percentage	No. of Students (positive)	% of positive Students	Over all %
10	90	22.5	6	18.18	1.5
11	97	24.4	7	21.22	1.75
12	85	21.25	6	18.18	1.5
13	78	19.5	5	15.15	1.25
14	50	12.25	9	27.27	2.25
Total	400	100	33	100	8.25

BIOCHEMICAL TESTS

The isolated *S. mutans* and *S. sobrinus* caused Alpha haemolysis on blood agar. The microorganisms did not grow in 6.5% of NaCl. Both microbial species tested negative for catalase activity with hydrogen

peroxide, while both bacterial isolates showed a positive Voges-Proskauer (VP) reaction (Fig. 3). Table III shows the results of biochemical tests.

Table III. Biochemical test of isolates

S. No	Biochemical test	Result
1	Alpha-haemolysis	Positive
2	Growth in 6.5% NaCl	Negative
3	Catalase	Negative
4	VP	Positive

Fig. 3. Voges Proskauer test with positive and negative control

PCR IDNTIFICATION OF ISOLATES

A total of 111 positive dental samples identified as *S. mutans* through colony morphology and staining, were further subjected to molecular identification. Out of these, only 90 samples were confirmed as *S. mutans* by targeting the *gtf-B* gene of 517 base pairs (Fig. 4). Table IV shows the positive results of PCR.

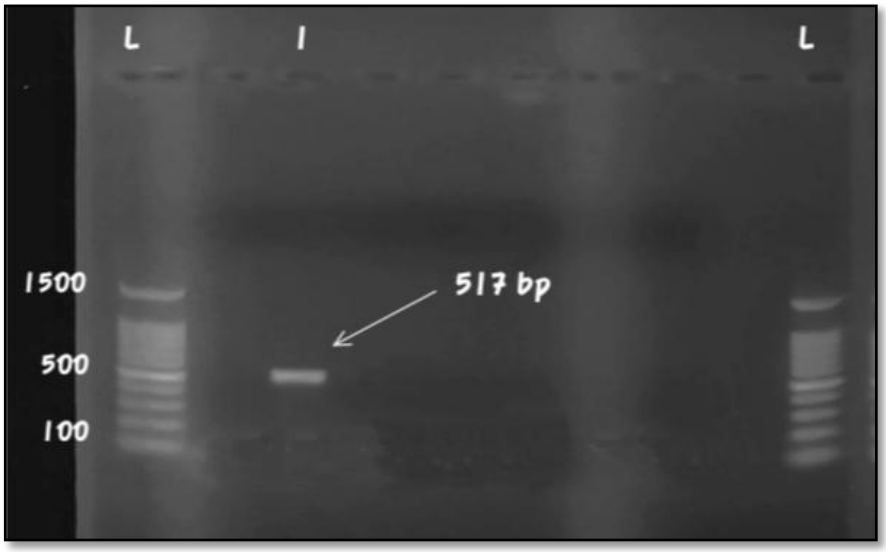

Fig. 4. Agarose gel electrophoresis of PCR amplification of *gtf-B* gene of *S. mutans*. DNA 100 bps ladder was used, 517 bps was positive for *S. mutans* (*gtf-B* gene)

Table IV. Positive PCR results for *gtf-B* gene

Age of Students	*gtf-B* gene	Total %	Over all %
10	11	12.22	2.75
11	10	11.11	2.5
12	18	20	4.5
13	22	24.45	5.5
14	29	32.22	7.25
Total	90	100	22.5

Twenty seven dental caries samples were identified as *S. sobrinus* based on colony morphology and staining, and were further subjected to molecular identification. Only 20 isolates were confirmed as *S. sobrinus* by the targeting *gtf-I* gene of 319 base pairs (Fig. 5). Table V shows the positive results of PCR.

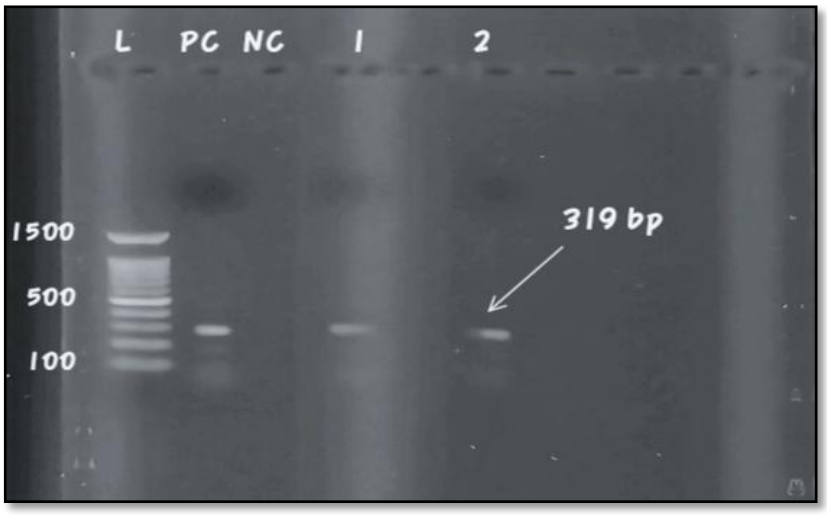

Fig. 5. Agarose gel electrophoresis of PCR amplification of *gtf-I* gene of *S. sobrinus*. DNA 100 bps ladder used, NC is negative control, PC is positive control. The sample 1 and 2 show positive result at 319 bps for *S. sobrinus* (*gtf-I* gene)

Table V. Positive PCR results of *gtf-I* gene.

Age of the students	*gtf-I* gene	Total %	Over all %
10	4	20	1
11	3	15	0.75
12	5	25	1.25
13	3	15	0.75
14	5	25	1.25
Total	20	100	5

EVALUATION OF ANTIMICROBIAL ACTIVITY OF WALNUT LEAVES

WELL DIFFUSION METHOD

Walnut leaves extract, tested at different concentration, and showed strong antimicrobial activity against the isolates on agar plates. At 100 µl, it produces a 27 mm (Fig. 6a) inhibition zone against *S. mutans*, while the same concentration formed a 24 mm (Fig. 6b) inhibition zone against *S. sobrinus*. The result of well diffusion method of ethanolic extract of walnut leaves are shown in Table VI.

Table VI. Antimicrobial activity of walnut leaves extract (ethanolic) used for isolates

Microorganism	Concentration in µl	Inhibition zone (Diameter)
S. mutans	25µl	14mm
	50µl	19mm
	75µl	24mm
	100µl	27mm
S. sobrinus	25µl	15mm
	50µl	17mm
	75µl	20mm
	100µl	24mm

MIC AND MBC

The walnut leaves extract (DMSO solution) was used in two fold dilution method from 100 to 1.563µl/ml. The MIC for *S. mutans* and *S. sobrinus* was determined in tube containing 50µl/ml, the concentration at which the bacterial growth inhibited. The MBC value was determined at 100µl/ml, as confirmed by the absence of the growth when samples were inoculated onto culture plates. The experiment was performed in duplicate.

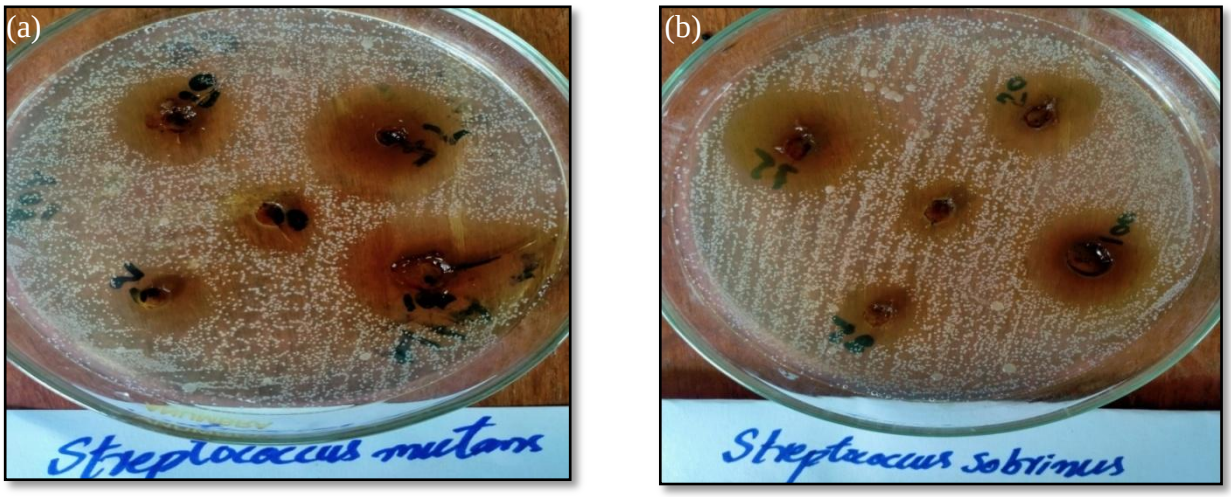

Fig. 6 (a). Walnut leaves extract activity (25µl, 50µl, 75µl, 100µl) against *S. mutans;* **(b).** Walnut leaves extract activity (25µl, 50µl, 75µl, 100µl) against *S. sobrinus*

ANTIBIOTIC SENSITIVITY TEST

The antibiotics discs employed in experiment are commonly prescribed in treatment of dental diseases. The sensitivity test was performed for both bacterial isolates with sixteen different types of antibiotics. The most sensitive antibiotic against *S. mutans* was Sulphamethoxazole which produced a 22 mm inhibition zone while Enrofloxacin and Oxytetracycline produced 15 mm and 12 mm inhibition zone respectively (Fig. 7-a). Likewise, Colistin Sulphate and Furazolidone formed 14 mm and 20 mm inhibition zone respectively (Fig. 7-b). In *S. sobrinus*, most effective antibiotics were Sulphamethoxazole and Oxytetracycline, each produced an inhibition zone of 22 mm while Nalidixic acid produced 11 mm inhibition zone (Fig. 7-c). Both bacterial species showed resistance to Erythromycin, Penicillin-G, Cloxacillin, Clindamycin, Lincomycin, Rifampacin, Norfloxacin, and Bacitracin. Tobramycin was only antibiotic that showed low sensitivity against *S. sobrinus* but had no effect against *S. mutans*. The results have been shown in Table VII.

Table VII. Antibiotics name, abbreviations and zone of inhibition (R-Resistant)

S. No	Antibiotics	Abbreviation	Concentration	Diameter of zone	
				S. mutans	*S. sobrinus*
1	Erythromycin	E	30-µg	R	R
2	Penicillin-G	P	10-units	R	R
3	Sulphamethoxazole	SXT	25-µg	22mm	20mm
4	Cloxacillin	OB	5-µg	R	R
5	Clindamycin	DA	2-µg	R	R
6	Lincomycin	MY	10-µg	R	R
7	Polymyxin-B	PB	300-units	13mm	12mm
8	Colistin Sulphate	CT	25-µg	14mm	15mm
9	Rifampacin	RD	5-µg	R	R
10	Norfloxacin	NOR	10-µg	R	R
11	Bacitracin	BC	0.04-units	R	R
12	Nalidixic acid	NA	10-µg	10mm	11mm
13	Enrofloxacin	ENR	5-µg	15mm	13mm
14	Tobramycin	TOB	10-µg	R	10mm
15	Furazolidone	FR	15-µg	20mm	17mm
16	Oxytetracycline	OT	30-µg	12mm	20mm

DISCUSSION

Maintaining oral health is universal aspect of human of human well-being, and every individual has the fundamental right to prioritize oral hygiene in their daily life. Naturally, a dental caries is ubiquitous in world and causes disease in communities (8). Different factors which contribute in dental caries including over-growth of cariogenic bacteria, desirable bacterial substrates and children with poor oral hygiene are more endangered to Periodontitis and dental cavities (6).

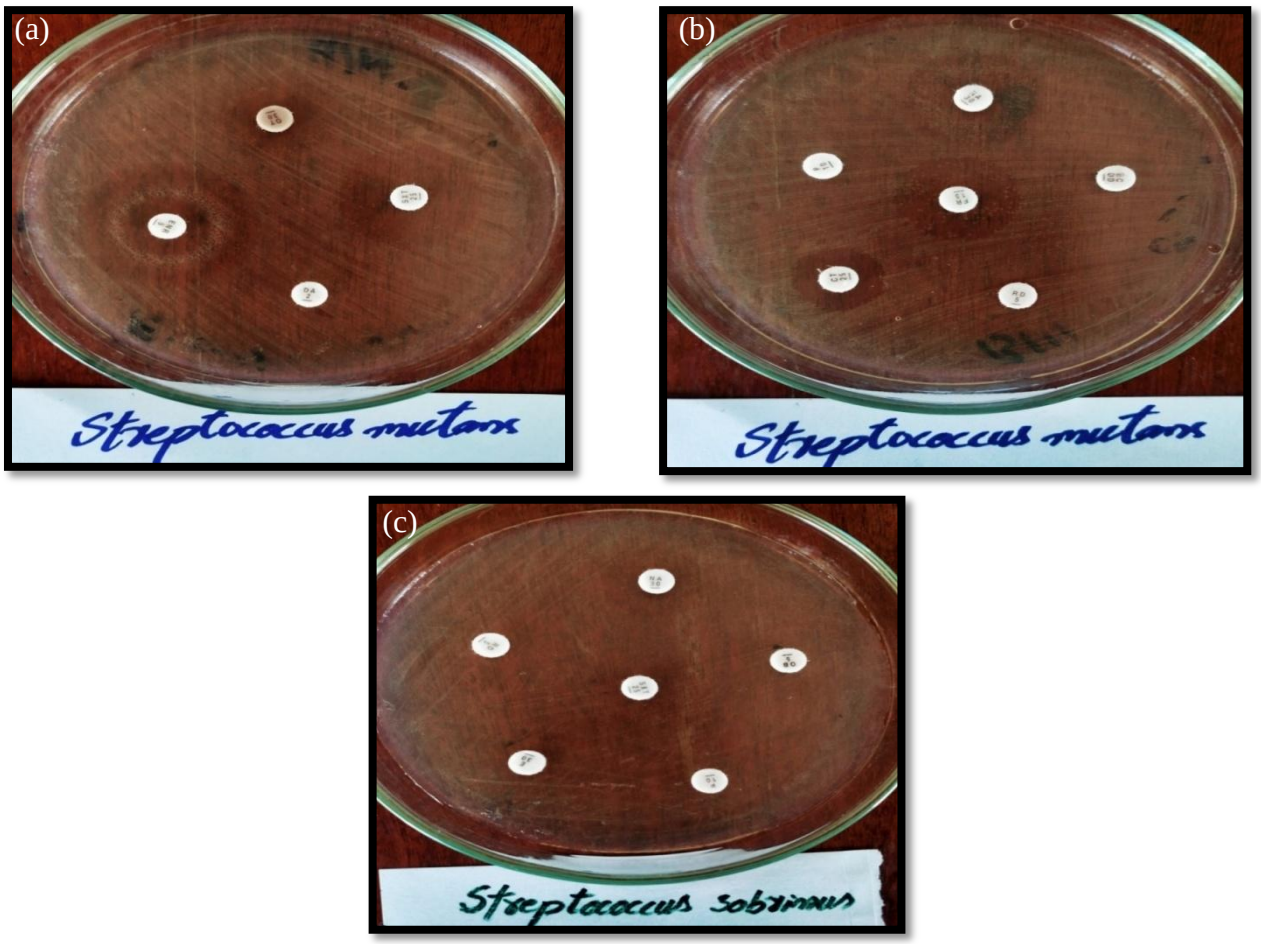

Fig. 7 (a). ENR, OT and SXT discs showing inhibition against isolates on MHA; **(b).** CT and FR discs showing the zone of inhibition against isolates; **(c).** NA and SXT discs showing zone of inhibition on MHA

S. mutans possess many virulence factors that contribute to the development of dental caries. One of the virulence factors is an enzyme known as *glucosyltransferase* encoded by *gtf* gene. This enzyme catalysed the sucrose into water soluble glucans and water insoluble glucans. Amongst, three types of *gtf* enzymes, *gtf-B* synthesized the polymers of water-insoluble glucans (WIG). The glucans polymers provide the attachment of *S. mutans* for plaque formations (3). *S. sobrinus* also has the ability to produce extracellular enzymes known as *glucosyltransferase* that forms WIG. The *gtf-I* enzymes with the help of WIG participates in formation of cariogenic dental biofilm (17). Therefore, in molecular identification, specific gene primers *(gtf-B* & *gtf-I)* were used in PCR to confirm the bacterial species.

S. mutans is considered a primary contributor, while *S. sobrinus* is often regarded as secondary contributor that plays a critical role in development of dental caries. They are among the most frequently isolated bacterial species from dental cavities. These bacteria demineralize the enamel of teeth, causes disease known as dental caries (11). The research conducted in 2015, reported the presences of cariogenic bacterial species from dental plaque samples. Thirty six (55%) isolates were *S. mutans* while 5 (7%) were identified as *S. sobrinus* out of 65 clinical samples (7). This study has evaluated the status of cariogenic bacteria in school going children between the aged of 10-14 years. Out of 400 dental caries samples 111 (27%) were *S. mutans* while only 33 (8%) were identified as *S. sobrinus*. The research correlates with above studied bacterial species of dental caries. It has been observed that presence of *S. mutans* is predominant and a major contributor to dental caries. Another bacterial species associated with dental caries is *S. sobrinus.*

Most of the studies have examined the antimicrobial potentials of medicinal plants in inhibiting and killing of bacterial growth. The WHO has reported that approximately 80% of population relies on herbal medicine (14). The use of medicinal plant extracts against cariogenic bacteria can be used as preventive measures. Several plants exhibits anti-bacterial activity, like Cinnamon and Cloves solution extracts have been using in treating of dental diseases and swelling of gums (13). Similarly, walnut leave extract also exhibited antibacterial activity against *S. mutans*, *S. salivarius* and others *cariogenic* species that contribute to dental plaque formation (10).

Antibacterial activity of (methanolic) extracts for Cinnamon and Cloves have been demonstrated in disc diffusion method. The disc was dipped in 10µl of Cinnamon extract which formed 14 mm of inhibition zone while Clove extract exhibited 12.6 mm of inhibition zone against *S. mutans* (13). In present study, the ethanolic extract of walnut leaves was evaluated for its antimicrobial properties. The antimicrobial activity of the extract against *S. mutans* increased with concentration, producing inhibition zones of 14 mm, 19 mm, 24 mm and 27 mm at concentrations of 25 µl, 50 µl, 75 µl, and 100 µl, respectively. In *S. sobrinus*, ethanolic extract producing inhibition zones of varying diameters: 25 µl resulted 15 mm zone, 50 µl in a 17 mm zone, 75 µl in a 20 mm zone, and 100 µl in a 24 mm zone. The antimicrobial compound in walnut leaves exhibits high antibacterial activity as compared to Cinnamon and Cloves extract which have demonstrated in previous study.

Another prominent characteristic of bacterial isolates is antibiotic resistance. Several studies have analyzed and documented the rising resistance pattern of antibiotics in cariogenic bacteria. A research conducted in Baghdad, evaluated the antibiotic sensitivity for *S. mutans* isolated from dental caries patients. The isolates showed complete resistance to Erythromycin and Bacitracin (18). Therefore, in this study, antibiotic sensitivity of isolates was test using 16 types of antibiotics. The most effective antibiotic was Sulphamethoxazole which produced 22 mm of inhibition zone against *S. mutans*. Similarly, Sulphamethoxazole produced 20 mm of inhibition zone against *S. sobrinus*. *S. mutans* have showed complete resistance to the following antibiotics: Erythromycin, Penicillin-G, Cloxacillin, Clindamycin, Lincomycin, Rifampacin, Norfloxacin, Bacitracin and Tobramycin. Among the above mentioned antibiotics, *S. sobrinus* was resistant to all except Tobramycin which produced 10 mm inhibition zone.

CONCLUSION

Dental plaque and dental caries are globally important issues. Poor oral hygiene leads to various dental diseases. Primarily, children are victims of dental caries between 10 to 14 years of age. Several traditional herbal medicines are used since ancient time for maintaining oral health. The leaves and barks of walnut tree (*J. regia*) have been using as a preventive measures for many centuries in the world. Therefore, the antimicrobial potential of walnut leaves extract was evaluated and founded to be effective against cariogenic microbes. The leaves and barks of walnut tree can improve the oral hygiene by reducing the bacterial growth in mouth. Furthermore, this research may help the dentists to select the most appropriate antibiotics for the treating dental infections. Pharmaceutical industries can develop and formulate new herbal therapeutic products using walnut leaves extract. The effectiveness of toothpaste and mouth wash can be enhanced by incorporating the walnut leaves extract in suitable concentration.

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Authors' contributions

IA Conceived, the experimental work, and finalized the manuscript; SAK Supervised, study design and data analysis; ISS, MAK, YH & SA Assisted in laboratory experiments, microbial assays, and statistical analysis; MN, SUR & MS Microbiological testing and result validation.

Conflict of interest:

Authors have no conflict of interest.

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