ORIGINAL ARTICLE

Antibacterial effect of Juglans Regia against dental caries Streptococcus mutans

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ABSTRACT

Background: Dental caries is one of the most common and costly diseases in the world and, *Streptococcus mutans* have been commonly associated as major cariogenic bacteria.
Aim: To evaluate the efficiency of some drugs in Kurdistan against S. mutans and study antimicrobial activity of ethanolic and aqueous extract of Juglans *regia* leave and bark of the walnut on the same isolates.
Method: The dental carries samples were collected from 250 students during October 2017 to April 2018, of both sexes with the mean age of 20 years. Biochemical and molecular identification were used for bacterial identification then antibiotic susceptibility assessed by disc diffusion method by using twenty antibiotics. Aqueous and alcoholic extracts of *Juglans regia* leaves and barks were used for inhibition the growth of isolated bacteria
Results: 80 isolates of *Streptococcus mutans* were identified, there was a wide range of resistant *S. mutans* against antibiotics, the resistance started from 15% as low percentage of all isolates against each of MY, VA, N and NA antibiotics while, the high percentage of resistance was 95% toward ME and DO. The minimum inhibitory concentration (MIC) for alcoholic and aqueous extracts of *Juglans regia* was 1400, and 1800 µg/ml respectively, ethyl and methyl alcoholic extracts of both parts most effective against *S. mutans* than aqueous extract.
Conclusion: *Juglans regia* is considered one of the most likely preferable alternative medical plant that used to treat dental caries to avoid the upcoming bacterial resistance to the antibiotic.
Keywords: *S. mutans*, J. *regia*. antibiotics

INTRODUCTION

Oral infections related to dental biofilm carrying on affecting the majority of the world's human population. Among them, dental caries remains to be the single most prevalent and costly oral infectious disease. A biofilm coats the tooth surface – a slime coating composed of millions of bacterial cells, salivary polymers and food particles. Uncontrolled, this biofilm can easily reach hundreds of cells on the teeth's surfaces. The developed biofilm, also known as plaque, provides an excellent adhesion site for many bacterial species to colonize and grow[1].

Streptococcus mutans gives its name to a group of seven closely related species collectively referred to as the *mutans streptococci*. The primary habitats for *S. mutans* are mouth, pharynx, and intestine, around fifty years ago, it was proved that *S. mutans* played a vital role in cariogenesis[2]. *S. mutans* is a gram-positive coccus, non-motile facultative anaerobic microorganism which can metabolize carbohydrates and is considered to be the essential etiological agent of dental caries[1]. Caries is a consequence of fermentation and breaking down of carbohydrate by bacteria in the plaque, to provide energy requirements and produce end products, including lactic acid, it allows the pH of the plaque to drop from 4.0 to 4.5 in a matter of minutes and thus overcomes the saturation of the saliva and plaque with calcium and phosphate and demineralization[3].

In a wide range of plant extracts and natural products, antimicrobial properties were recorded during the last few years, attempting to determine new chemical classes of antibiotics that could resolve the problems such as the appearance of undesirable side effects and the development of earlier uncommon infections[4].

The presence of phenolic compounds, terpenoids, alkaloids, flavonoids and steroids in the plant substantial may be the causes of the antibacterial properties of these materials. Therefore, antimicrobial activity study of the shell and leave extracts of *J. regia* L. were tested against the microbes present in the saliva samples of patients suffering from dental caries[5].

The *Juglans* (family Juglandaceae) contains several species and is broadly spread throughout the world. The pharmaceutical and cosmetic industries use Green walnuts, shells, kernels and seeds, bark[6]. Walnut leaves have been used intensively in traditional medicine to treat venous insufficiency, hemorrhoids, hypoglycemia, diarrhea, and fungal or microbial infections and considered to be a source of healthcare compounds[7]. Phytochemically *J. regia* L contains naphthaquinones, monoterpenes, sesquiterpenes, juglone, ketones like, regiolone, sterol and flavonoid, flavonoids, tannic acids and gallic acid[6,8] . Oil is the main constituent, ranging from 61.32 to 69.35% followed by carbohydrates (9.05-18.92%) and protein (10.58-18.19%)[9].

MATERIALS AND METHODS

Specimen's collection: 250 swabs were taken from dental caries of teeth during the periods from October 2017 to September 2018, of both sexes with the mean age of 20 years. Samples were taken with sterile cotton swab and cultured on nutrient agar for 24 hours at 37°C, then transfer single colonies to Blood, MacConkey and Mannitol Salt agar directly and incubated for 24 hours. A total of 80 isolates of *S. mutans* were isolated and identified according to morphology, cultural characteristics, and biochemical

tests was performed such as VP test, catalase test, urea hydrolysis and salt tolerance test.

Culture media: Different culture media such as nutrient agar, MacConkey agar and Blood agar Mannitol salt agar was used to isolate and identify *S. mutans*; these media were prepared as described1[0,11].

PCR for differentiation of *Streptococcus mutans:* Bacterial DNA for PCR amplification was extracted using Presto™ Mini gDNA Bacteria Kit following the manufacturer's instructions (Geneaid, Taiwan). Because the biochemical identification of several isolates was not accurate, amplification and sequencing of the 16S rRNA for 80 isolates was performed to confirm bacterial identification. Universal primer for 16S rRNA Eubac 27F (5' AGA GTT TGA TCC TGG CTC AG 3') and 1492R (5' GGT TAC CTT GTT ACG ACT T) and specific primer Sm479F (5' TC G CGA AAA AGA TAA ACA AAC A 3') and Sm479R (5' GCC CCT TCA CAG TTG GTT AG 3') were used. The extracted DNA, primers, and PCR master were thawed at 4°C. PCR mixture was set up in a total volume of 25 µL (0.2 mL sterile PCR tubes) include 12.5 µL of 2x HotStart Taq Master Mix (Amplicon, Denmark), 1.5 µL of each forward and reverse primers, different µL of template DNA depending upon the extracted DNA concentration in one microliter, and the rest volume was completed with free nuclease water, then spin down to mix and bring the contents to the bottom of the tubes, in addition to another negative control, contained all material except template DNA was used to the comparison. PCR reaction tubes had been placed in the PCR machine ($Alpha_{max}$, UK), DNA of 16S rRNA and Sm479 genes were amplified under the following PCR conditions, initial denaturation at 96°C for 2 min, followed by 30 cycles of denaturation at 95°C for 30 sec, annealing at 59°C for 30 sec and extension at 72°C for 1 min, with the amplicon size was 1500 bps[12] and initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 60 sec, annealing at 56°C for 60 sec and extension at 72°C for 60 sec, with the amplicon size was 433 bps[13] . PCR products were separated on 1.2% agarose gel, and then DNA bands were visualized with Red safe dye.

Susceptibility screening: The antibiotic sensitivity test was performed using twenty types of antibiotics against 80 isolates of *S. mutans* which included; Trimethoprim–Sulfamethoxazole (SXT 1.25+23.75 µg), Oxacillin (OX 1 µg), Methicillin (ME 5 µg), Lincomycin MY 10 µg), Ofloxacin (OF 5 µg), Tetracycline (TE 30 µg), Ampicillin (AMP 10 µg), Clindamycin (DA 2 µg), Vancomycin (VA 30 µg), Gentamicin (G 10 µg), Erythromycin (E 15 µg), Tobramycin (TO 10 µg), Doxycycline (DO 15 µg), Neomycin (N 10 µg), Rifampicin (RA 5 µg), Cefoxitin (FOX 30 µg), Carbenicillin (CAR 100 µg), Cephalothin (KF 30 µg), Nalidixic acid (NA 30 µg), and Amikacin (AK 30 µg), as clarified in Table (1) which is determined according to CLSI (Clinical and Laboratory Standards Institute) standards. 20 ml of Muller Hinton agar melted and cooled at 45 °C was poured into sterile petri dishes and allowed to solidify completely. A lawn of test pathogen was prepared by evenly spreading 100 µl inoculums (1.5×10^8 CFU/ml) (according to 0.5 McFarland standard solution[14] using a sterilized swab onto the entire surface of Mueller Hinton Agar plate. The plates were allowed to dry before applying antibiotic disc. The disc was applied firmly to the agar plate surface within 15 minutes of inoculation[15,16]..

PREPARATION OF *J. REGIA* EXTRACTS

Aqueous extraction: 10 g of air-dried powder was taken in distilled water and boiled on slow heat for 2 hrs. It was then filtered and centrifuged at 5000 rpm for 10 minutes. supernatant was collected pooled and concentrated to make the final volume one–fourth of the original volume. It was then autoclaved at 121 °C under 15 Ibs pressure and stored at 4 °C.

Solvent extraction: 10 g air dried powder was taken in 100 ml of organic solvent (methanol or ethanol) in a conical flask, and then kept on a rotary shaker at 250 rpm for 24 h. After 24 h, it was filtered through cloth and centrifuged at 5000 rpm for 10 minutes. The supernatant was collected and the solvent was evaporated to make the final volume one – fourth of the original volume and stored at 4 °C in airtight bottles[17].

Antibacterial assay of *J. regia* L. extracts through the determination of Minimum Inhibitory concentration (MIC): The antibacterial activity of *J. regia* was determined by using broth dilution (MIC) for *S. mutans*. The **ISO 37**, which represented the most resist isolate among all isolates used in our study, were inoculated in nutrient broth tubes, incubated in shaking incubator for 24 hours at 37 ºC, then 0.1 ml of growth culture was added to sterile nutrient broth containing different concentrations of the plant extracts (100, 200, 400, 600, 800, 1000, 1200, 1400, 1600 and 1800) µg/ml separately, in addition to control samples for each concentration. Later, the cultures were incubated for 24 hours at 37 ºC and the bacterial growth was evaluated on the basis of the turbidity of the suspension and all tubes were read by spectrophotometer at 600

RESULTS

Identification of bacterial isolates: A total of eighty isolates of bacteria *S. mutans* were isolated from 250 swabs were taken from dental caries of teeth (53 females, 27 males),based on difference of colony morphological observation on different culture media, Gram stain and biochemical test, the results of our studies showed that *S. mutans* was small smooth circle colonies, gram positive coccus shaped forming pairs and chains, non–motile and non-spore forming bacteria[18].. The results of biochemical tests for *S. mutans* were negative for catalase and urease tests while positive for Voges–Proskauer test, and do not have ability to grow in 6.5% NaCl of saline, and when cultured on Blood agar, all isolates shown α–hemolysis, as shown in Table (2), and this results were compatible to the results of other study that performed by[19,3].

PCR identification of *Streptococcus mutans:* Initial attempts at confirmation of *S. mutans* identification via PCR utilized the 16S rRNA Eubac 27F and 1492R primer (Figure 1) and Sam479 primer (Figure 2). Isolates (previously identified by conventional methods) from 80 isolates (53 females, 27 males), all with *S. mutans* colonization, were selected to test the primers. An amplification of 16S rRNA and Sam479 from 80 isolates was performed to confirm bacterial identification. Primers for conserved region were designed and used for amplification of DNA of *S. mutans* isolates by PCR then

PCR products were separated on agarose gel. The result demonstrated that 80 isolates (100%) of *S. mutans* had 16S rRNA gene band with 1500 bps and Sam479 with 433 bps band. Identification of *S. mutans* isolates by using 16S rRNA and Sam479 are more accurate than bacteriological and biochemical assays. As[20] demonstrate that 16S rRNA and Sam479 genes were PCR was sensitivity, specific, and used for diagnosis of culture negative bacterial infections also useful for identification of bacterial pathogens in patients prepared with antibiotics

Antibiotic sensitivity screening for isolates: The antibiotic sensitivity test was performed for 80 isolates of *Streptococcus mutans* demonstrated in Table (3) which illustrates that the resistant isolates of *S. mutans* were different and shown wide range of resistant to antibiotics which used in this study, the resistance started from 15% as low percentage of all isolates against each of MY, VA, N and NA antibiotics while, the high percentage of resistance was 95% toward Methicillin (ME) and Doxycycline(DO) antibiotic for all isolates (75%) isolates were resistant to SXT and(E) (70%) isolates were resistant to (TE) and (FOX) table 4. The antibiotics MY, VA and N are more effective against cariogenic *S. mutans* among another drug tested.

Antibacterial activity of *J. regia* against *S. mutans:* Minimum Inhibitory Concentration (MIC) of alcoholic and aqueous extracts of *J. regia* leaves were measured through the optical density which reads at 600 nm by UV/Light Spectrophotometer as clarified in table (5), which shown that the MIC of aqueous, ethyl alcoholic and methyl alcoholic extracts of *J. regia* barks are 1800 µg/ml, 1400 µg/ml and 800 µg/ml, the spectrophotometric reading were 0.092, 0.044 and 0.002 respectively, while the MIC of aqueous, ethyl alcoholic and methyl alcoholic extracts of *J. regia* leaves were 1600 µg/ml, 800 µg/ml and 600 µg/ml, the spectrophotometric reading were 0.121, 0.192 and 0.175 respectively. The results obtained demonstrate that the ethyl and methyl alcoholic extracts of both parts most effective against *S. mutans* than aqueous extract.

Table 1. Antibiotics name, abbreviations, standard diameter of inhibition zone[15,16]

No.	Antibiotics name	abbreviations	Final concentration (µg/ml)	Zone diameter		
				R	I	S
1	Trimethoprim - Sulfamethoxazole	SXT	1.25+23.75	≤ 15	16-18	≥ 19
2	Oxacillin	OX	1	≤ 19	20	≥ 21
3	Methicillin	ME	5	≤ 9	10-12	≥ 13
4	Lincomycin	MY	10	≤ 12	13-16	≥ 17
5	Ofloxacin	OF	5	≤ 12	13-15	≥ 16
6	Tetracycline	TE	30	≤ 18	19-22	≥ 23
7	Ampicillin	AMP	10	≤ 13	14-16	≥ 17
8	Clindamycin	DA	2	≤ 14	15-17	≥ 18
9	Vancomycin	VA	30	≤ 14	15-16	≥ 17
10	Gentamicin	G	10	≤ 13	14-15	≥ 16
11	Erythromycin	E	15	≤ 13	14-22	≥ 23
12	Tobramycin	TO	10	≤ 12	13-14	≥ 15
13	Doxycycline	DO	15	≤ 13	14-22	≥ 23
14	Neomycin	N	10	≤ 13	14-15	≥ 16
15	Rifampicin	RA	5	≤ 16	17-18	≥ 19
16	Cefoxitin	FOX	30	≤ 14	15-17	≥ 18
17	Carbenicillin	CAR	100	≤ 13	14-16	≥ 17
18	Cephalothin	KF	30	≤ 14	15-18	≥ 18
19	Nalidixic acid	NA	30	≤ 13	14-18	≥ 19
20	Amikacin	AK	30	≤ 14	15-16	≥ 17

Table 2: Biochemical Characteristics of *S. mutans*

Tests	Results
Blood Hemolysis	α – Hemolysis
Urea Hydrolysis	–
Voges Proskauer Test	+
Growth on 6.5% NaCl	–
Catalase	–

-: Negative result, +: Positive result, α: Partially hemolysis of blood.

Fig.1: Agarose gel electrophoresis is graphic of PCR amplification for 16S rRNA gene in *S. mutans*. Lane M: DNA ladder 100 bps, lane NC: negative control, lane (1-24)) were positive for 16S rRNA gene at 1500 bps band.

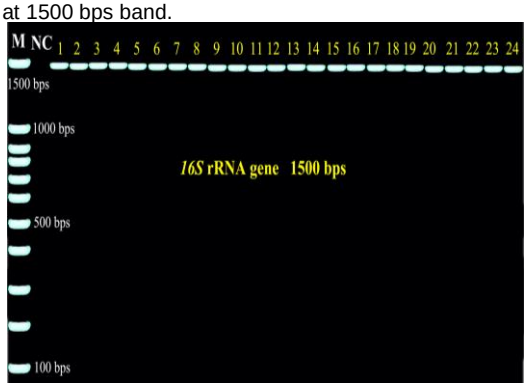

Fig. 2: Electrophoresis results of *S. mutants* Sm479 gene (433 bp) on agarose gel size markers. Lanes: M, 100 bp marker ladder; NC, negative control (distilled water); 1-24, positive results for Sm479 gene with amplicon size 433bps.

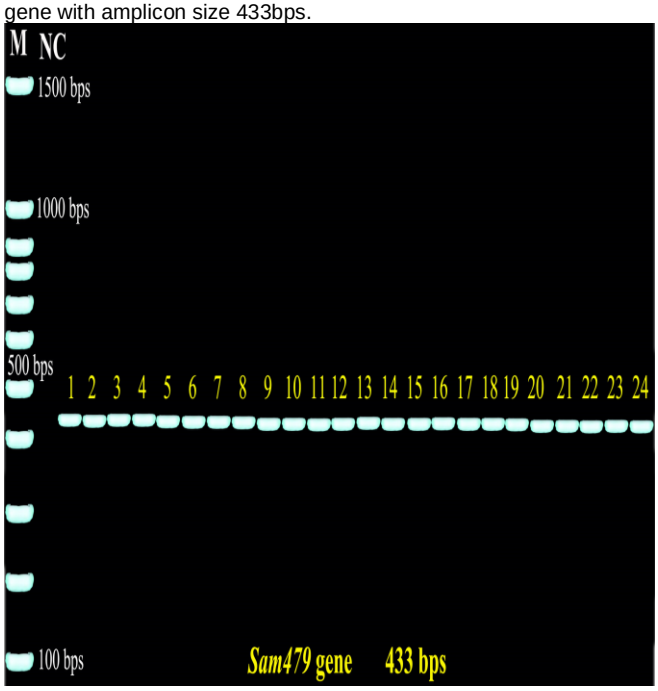

Table 3: Sensitivity percentage of *S. mutans* isolates to different types of antibiotics

Antibiotics	Resistant%	Intermediate%	Sensitive%
AK	34	8	38
AMP	24	12	44
CAR	24	12	44
DA	16	0	64
DO	76	0	4
E	60	12	8
FOX	56	8	16
G	34	8	38
KF	16	0	64
ME	76	0	4
MY	12	0	68
N	12	0	68
NA	12	16	52
OF	21	7	52
OX	28	0	52
RA	21	8	52
SXT	60	12	8
TE	56	8	16
TO	28	0	52
VA	12	16	52

Table 4: Antibiotic Susceptibility Results of *S. mutans*.

Antibiotics*	No. of resistant isolates	% of resistant
SXT	60	75
OX	28	35
ME	**76**	**95**
MY	12	15
OF	20	25
TE	56	70
AMP	24	30
DA	16	20
VA	12	15
G	48	60
E	60	75
TO	28	35
DO	**76**	**95**
N	12	15
RA	20	25
FOX	56	70
CAR	24	30
KF	16	20
NA	12	15
AK	34	60

* Abbreviations are given in table (1).

Table 5: The MIC of *J. regia* parts extracts on *S. mutans* ISO 37

MIC methods		Concentration (µg/ml)												
Plant parts	**Types of Extracts**	100	200	400	**600**	**800**	1000	1200	**1400**	**1600**	**1800**	2000	2200	2400
Barks	**Aqueous**	0.621	0.605	0.596	0.582	0.526	0.555	0.488	0.422	0.157	**0.092**	0.032	0.000	0.000
	Ethyl Alcoholic	0.583	0.471	0.414	0.365	0.335	0.326	0.302	**0.044**	0.044	0.02	0.00	0.00	0.00
	Methyl Alcoholic	0.48	0.39	0.263	0.183	**0.002**	0.112	0.082	0.062	0.062	0.038	0.007	0.00	0.00
Leaves	**Aqueous**	0.686	0.601	0.586	0.536	0.524	0.445	0.337	0.304	**0.121**	0.019	0.00	0.00	0.00
	Ethyl Alcoholic	0.434	0.419	0.399	0.385	**0.192**	0.158	0.144	0.066	0.047	0.012	0.00	0.00	0.00
	Methyl Alcoholic	0.503	0.421	0.385	**0.175**	0.117	0.075	0.02	0.00	0.00	0.00	0.00	0.00	0.00

DISCUSION

The findings of this study provide information about the impact of 20 different antibiotics on *S. mutans*. It is evident that antibiotic resistance was varied from strain to strain of S mutans. The current study is in agreement with other study, as[1] observed that all isolates of *S. mutans* sensitive 100% to AUG, AMX, CXC, G and COT, while 85.1% of isolates was sensitive to each of ER, TET and C. Likewise,

In a study[1] of found that 72% of cariogenic *S. mutans* isolates resist to AMX, 17%, AMC, 65%, C, 40%, DO, 46%, E, 32%, of and 61% to TE respectively. It may be suggested that due to the lack of appropriate knowledge of prescribing antibiotics for the treatment of dental caries. The microbial flora responsible for the development of dental caries has developed antimicrobial resistance. The strong biofilm formation capability and antibiotic resistant isolates indicate the powerful contribution in the

pathogenesis of dental caries[2]. Most of the Resistance microorganisms to antibiotics of gram-positive organisms is through efflux and methylase enzyme production, then modifies the ribosomal binding site for the antibiotics[3].

Another aspect of our results is the antibacterial assay of *J. regia* L. extracts found that the (MIC) for alcoholic and aqueous extracts of *Juglans regia* was 1400, and 1800 µg/ml respectively. the ethyl and methyl alcoholic extracts of both parts most effective against *S. mutans* than aqueous extract. These results are correspondent to those founded by[6,21] the research included six walnut antimicrobials (*J. regia* L.) develop capacity against gram positive (*B. cereus*, *B. subtilis*, *S. aureus*) and gram-negative bacteria (*P. aeruginosa*, *E. coli*, *K. pneumoniae*). The study revealed that the aqueous extract inhibited gram-positive bacteria at very low concentrations with MICs of 1 mg/mL 0.1 mg/mL. While Gram negative bacteria were also sensible for some walnut extracts. Medicinal plants produce slow recovery; the therapeutic use of medicinal plant is becoming popular because of their lesser side effects and low resistance in microorganisms[1].

It can be concluded that the regular and unscientific method of intake of antibiotics can make the dental caries pathogen, *Streptococcus mutans* more antibiotic resistant. And spread to other bacteria. Antibiotic alternatives such as Juglans regia are most likely preferred to avoid future bacterial resistance to the antibiotic.

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