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Research Article

PHYTOCHEMICAL SCREENING BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC) AND ANTIMICROBIAL ACTIVITY OF DIFFERENT SOLVENT FRACTIONS OF ARECA NUTS AGAINST *BACILLUS SUBTILIS* BIOFILM

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ABSTRACT

Areca nut commonly known as Supari is in use since 15th century. Plaque formation is one of the main reasons for deterioration of teeth. In this research work, the effect of areca nut on plaque formation was checked. For this, plaque forming microorganisms were isolated from healthy human teeth. A total of 100 samples were collected from individuals possessing healthy teeth. From these samples, five microorganisms were isolated which included *Staphylcoccus* sp., *Streptococcus* sp., *Bacillus* sp., *Pseudomonas* sp. and *Klebsiella* sp. Only *Bacillus* sp. was found common in teeth of all subjects. It was able to grow biofilm in the Luria Bertani (LB) broth after 24 hours of incubation at 37 °C. *Bacillus* sp. was revealed to be *Bacillus subtilis* by 16s rRNA sequencing (Macrogen®). The extracts of areca nuts were prepared in ethanol, methanol, chloroform and water. Both disc and well diffusion methods were employed to check the antimicrobial activity of these extracts. Areca nuts exhibited antimicrobial activity in all extracts except chloroform. High Performance Liquid Chromatography (HPLC) confirmed the presence on antimicrobial compounds i.e. quercetin and cinnamic acid. The biofilm growing ability of *B. subtilis* was interrupted up to 40 % by using 100 % ethanol and water extracts. For demolishing the established biofilm, 50 % of all extracts were found to be effective. It can be concluded that areca nut was effective for combating the biofilm formation by *B. subtilis*. It can be used in mouth wash formulations to get rid of plaque formation on teeth.

Keywords: *Bacillus subtilis*, areca nuts, antimicrobial activity, biofilm formation, HPLC, plaque

INTRODUCTION

Areca nut known as supari in Pakistan is an important component of paan which was part of Mughal civilization back in 15th century. Areca nut is the seed of Areca catechu, an oriental palm of the Palmaceae family that grows in South and Southeast Asia, East Africa and parts of Tropical Pacific. The nut is not a fruit but a drupe and can be consumed in raw, processed, roasted, sun dried forms. Different forms of areca nut contribute to variance in taste and appearance[1]. It is considered to be the fourth most commonly used addictive and psychoactive substance preceded by tobacco, alcohol, and caffeine respectively. Approximately 10 % of the world's population are involved in the consumption of areca nut. Its use is habitual in countries like India, Sri Lanka, Pakistan, Maldives, Bangladesh, Myanmar, Taiwan, Thailand, Indonesia, Cambodia, Vietnam, Philipines, Laos, and China[2]. Additionally, the practice of chewing pan masala with or without the use of tobacco is common in the subcontinent from thousands of years and currently an estimated 600 million people are inflicted in the consumption of betel quid with areca nut globally[3]. Normal oral microflora consists of many organisms reside in the mouth. Overproduction of microbes on the surfaces of teeth and gum are called plaques and it can lead to gingivitis, caries, septicemia, and endocarditis[4]. Various studies have demonstrated that the alkaloids extracted from areca nut contribute to the cytotoxic and genotoxic property and carcinogenicity to humans. Several reports have shown the association of chewing areca nuts with the development of various disorders, such as cardiovascular disease, metabolic syndrome, and hypertension[5]. Areca nut has been categorised as Group 1 human carcinogens by the World Health Organisation[6]. Areca nut has a wide range of adverse health effects reported in recent literature and various studies are further cementing this previously thought to be false notion. It is reported to be carcinogenic and to cancers of the pharynx, oesophagus, liver, oral cavity, and the uterus[7]. Pan Masala, also called betel quid, is a mixture of *Acacia catechu* with slaked lime, areca nut and other flavoring agents. It is widely available and used famously by inhabitants of the subcontinent. Betel chewing produces an increase in the blood pressure, a spike in the heart rate and body temperature and sweating. It also leads to an increase in the plasma concentrations of norepinephrine and epinephrine, which suggests that betel chewing affects the central and autonomic nervous systems[8]. It is reported to be hepatotoxic that results in an increase level of enzymes and disturbed lipid and carbohydrate metabolism. Additionally, it is harmful to kidneys with reported cases of increased creatinine[7].

This study was conducted to study the antimicrobial activity of areca nut on the formation of biofilms by oral flora and phytoconstituents of areca nuts by high performance liquid chromatography (HPLC).

MATERIALS AND METHODS

Chemicals

All chemicals used in this study were of analytical grades. The solvents for fractionation were purchased from Merck, Germany. Microbiology media used were of Oxide, Thermofisher UK.

Isolation of Plaque Forming Microorganisms

To isolate plaque forming microorganisms, dental swabs were taken from subjects possessing healthy teeth. The subjects were requested to rub the sterilize swab on plaque forming area of tooth, after waking up in the morning before brushing their teeth. Total 100 subjects were selected for this study (volunteer university students, age group 20-30 years, males only, no medical history of any disease, not in habit of any addiction like smoking, paan chewing etc). The swabs were immediately brought to Microbiology Laboratory for isolation of microbial flora. The swabs were processed on Luria Bertani (LB) agar plates till pure colonies were obtained. The Gram staining and biochemical characterization were performed[9,10]. The bacterial isolate found common in all samples was characterized on molecular level by 16s rRNA sequencing (Macrogen®, South Korea).

Extraction and Fractionation of Plant Material

The method of Saleem *et al*.,[11] was followed with slight modifications. Areca nuts were purchased from local market and converted into powdered form. Powder was kept into clean dried container for further procedure. Four different types of solvents i.e. ethanol, methanol, chloroform and water were used for preparation of areca nuts extract separately. The dry powder (30 grams) of areca nuts was mixed in 100 ml of respective solvent and mixture was then kept in blue capped bottle at room temperature for 4 days with occasional shaking. The filtrate was filtered with Whatman no. 1 filter paper. To obtain the dried and concentrated form, filtrate was processed at rotary evaporator (Heidolph, Germany) at 55 °C and vaccum pump (V700, H-9230, BUCHI, Switzerland) connected with refrigerated circulator (DLSB 5/20, ZGSI, China). Finally the extract in all different solvents was placed in refrigerator at 4 °C for further experiments.

Antimicrobial Activity of Extracts

Antimicrobial activity of extracts was checked by both well and disc diffusion methods. It was performed by dissolving the dried fraction areca nuts in different ratios in respective solvents (ethanol, methanol, chloroform and water). Four different concentrations of each solvent were prepared 100 mg/ml, 50 mg/ml, 25 mg/ml and 10 mg/ml respectively. Ciprofloxacin was used as positive control, whereas 10 % respective solvent was used as negative control.

Well Diffusion Method: Luria Bertani (LB) medium plates were prepared. Pure colony of *B. subtilis* was swabbed on LB plates. Wells were formed by pressing the backside of Pasteur pipette into the agar medium. About 20 µl of each concentration of each extract was added in wells. The plates were incubated at 37 °C for 24 hours. Zone of inhibition (mm) was measured after 24 hours. The experiment was repeated with methanol, chloroform and water extracts.

Disc Diffusion Method: For this, Luria Bertani (LB) medium plates were prepared. *B. subtilis* pure colony was streaked on the plate in the form of a mat. Filter paper was pinched to form discs which were dipped in the extract, dried to remove extra extract and placed on the plate. It was incubated for 24 hours at 37 ºC. After 24 hours, the zone of inhibition (mm) was recorded[12].

Biofilm Experiments

Biofilm Production by *B. subtilis*: *B. subtilis* (18 hours old) culture (1 %) was inoculated in the wells of 96 well plate containing 100 µl nutrient broth (Oxoid) followed by incubation at 37 °C for 24 hours. The biofilm of bacterial growth was observed and OD was taken at 600 nm. Biofilm production was also confirmed in flask by giving the 1 % inoculum of *B. subtilis* in 100 ml nutrient broth (Oxoid) followed by incubation at 37 °C for 24 hours. After 24 hours, biofilm was observed, it was mixed with the broth medium and OD was taken at 600 nm[12].

Interruption of Biofilm Formation: The biofilm of *B. subtilis* was grown in microtitre plate as mentioned above. After 24 hours, 100 µl of ethanol, methanol and water extracts was added in wells and then placed in incubator for 24 hours. After one day, the adhered bacterial growth was determined by following method: 100 µl 1 % crystal violet stain was added in each well. In the next step, each well was emptied then washed three times with distilled water. In the next step, 150 µl of methanol was added for 15 minutes. When methanol was completely dried, 160 µl of glacial acetic acid was added to it. The biomass was then made ready for microtiter reader. Readings were taken at 630 nm. The percentage of inhibition was calculated[13].

Disturbing the Established Biofilm: After incubation of the plate the non-adherent cells were removed by washing it three times with distilled water. Each concentration of extracts (100 µl) was added to each well respectively, and then incubated for 24 hours at 37 °C. The disruption of biofilm was determined by using crystal violet. Then the percentage of reduction in biofilm structures was calculated[12].

Quantification of Phytocomponents by HPLC

The phytocomponents of areca nuts were determined by HPLC.

Sample Preparation: Phenolic compounds were identified and quantified from ethanol, methanol and water extracts of areca nuts by reverse phase HPLC (Aligent HPLC system). Briefly, 25 milligrams of each extract was dissolved in 5 mL of 6 M HCl, 12 mL methanol with 8 mL of distilled water. The resultant solution was incubated at 90 °C for 2 hours and filtered with 0.2 mm millipore membrane filter before injection into HPLC column.

High Performance Liquid Chromatography: The HPLC separation was performed using HPLC system with column 20 RBAX ECLIPSE, XDB-C18, (4.6 × 150 mm; 5µm, Agilent USA), UV–VIS Spectra-Focus detector and injector-auto sampler. The isocratic mobile phase, consisting of A (water: AA-94:6, pH=2.27), B (ACN, 100%)**,** The flow of mobile phase was adjusted as 0-15 min. (15 % B), 15-30 min. (45 % B), 30-45 min. (100 % B) at a flow-rate of 1 mL/ min. Prior to use, the mobile phase was filtered through 0.2 mm millipore membrane filters and degassed by sonication in an ultrasonic bath. Detection wavelength was set at 280 nm and the column temperature was maintained at room temperature (37 °C) with injection volume of 10 µL.

RESULTS

Isolation and Identification of Plaque Forming Microorganisms

On the basis of Gram staining and biochemical characterization, five microorganisms including *Staphylococcus* sp., *Streptococcus* sp., *Bacillus* sp., *Pseudomonas* sp. and *Klebsiella* sp. were isolated from 100 subjects. Only *Bacillus* sp. was found common in teeth of all subjects. The 16s rRNA sequencing revealed it *Bacillus subtilis*. It was selected for further experimental work.

Antimicrobial Activity of Areca Nuts

Disc diffusion method showed more prominent zones as compared to well diffusion method (Table 1, Figure 2).

Chloroform did not show any zone of inhibition (results not shown here).

HPLC

HPLC chromatogram of ethanol, methanol and aqueous extracts are given in Figure 3. The phenolic acids which are represented by each peak, their retention time, area in mV.s and % are given (Table 2, Figure 3). Among the various components, quercetin and cinnamic acid were found to possessed antimicrobial properties.

Biofilm Experiments

Growth of Biofilm in Flask: *B. subtilis* was found to form a layer in form of film at the surface and edges of LB broth in flask after 24 hours (Figure 1).

Growth of Biofilm in Microtitre Plate: The growth of biofilm in microtitre plate after 24 hours incubation at 37 °C was observed.

Growth of Biofilm in Presence of Extracts: The growth of biofilm in the presence of ethanol, methanol and aqueous extracts at concentrations 100 %, 75 %, and 50 % each respectively. The % inhibition of biofilm by each extract is shown in Figure 4.

Demolishing the Biofilm: The graphical representation of % inhibition of biofilm by each extract is given in Figure 5.

Table 1: Zones of inhibition by ethanol, methanol and aqueous extracts of nuts

Extracts	Conditions	Zones of inhibition (mm)				Control 1	Control 2
		100 mg/ml	50 mg/ml	25 mg/ml	10 mg/ml		
N+E	**Wells**	7.5±0.1	7±0.1	1±0.1	0.5±0.1	10	-
	Disc	9±0.05	8±0.05	3.5±0.1	3±0.05	10	-
N+M	**Wells**	8±0.05	6±0.1	2.3±0.05	2±0.1	10	-
	Disc	7.5±0.1	7±0.05	3±0.1	2±0.05	10	-
N+W	**Wells**	7.5±0.05	6±0.1	4±0.05	1±0.05	10	-
	Disc	9.5±0.1	8±0.05	6±0.1	2±0.1	10	-

N+E = Nuts in ethanol, N+M = Nuts in methanol, N+W = Nuts in water, Control 1 = ciprofloxacin, control 2 = solvent (ethanol, methanol or water depending upon the experiment)

Table 2: Major phenolic acids found in ethanol extract of nuts by HPLC chromatogram

ETHANOL EXTRACT				
Phenolic acids	**Retention time**	**Area (mV.s)**	**Area (%)**	**Bioactivity**
Quercetin	2.820	2.166	0.2	Yes
Gallic acid	4.580	103.125	9.0	-
Caffeic acid	12.913	79.060	6.9	-
Syringic acid	16.600	12.654	1.1	-
Cinamic acid	24.627	181.265	15.9	Yes
Sinapic acid	26.867	181.265	15.9	-
METHANOL EXTRACT				
Phenolic acids	**Retention time**	**Area (mV.s)**	**Area (%)**	**Bioactivity**
Vanillic acid	12.927	43.256	2.3	-
m-Coumaric acid	19.600	186.864	10.1	-
Cinamic acid	24.813	190.412	10.3	Yes
WATER EXTRACT				
Phenolic acids	**Retention time**	**Area (mV.s)**	**Area (%)**	**Bioactivity**
Quercetin	3.040	4.516	0.1	Yes
Gallic acid	4.347	642.122	20.5	-
Caffeic acid	12.127	100.404	3.2	-
Vanillic acid	13.467	247.206	7.9	-
m-Coumaric acid	19.960	153.488	4.9	-
Ferulic acid	22.413	117.971	3.8	-
Cinamic acid	25.080	175.714	5.6	Yes



Figure 1: Formation of biofilm by *B. subtilis* on broth medium

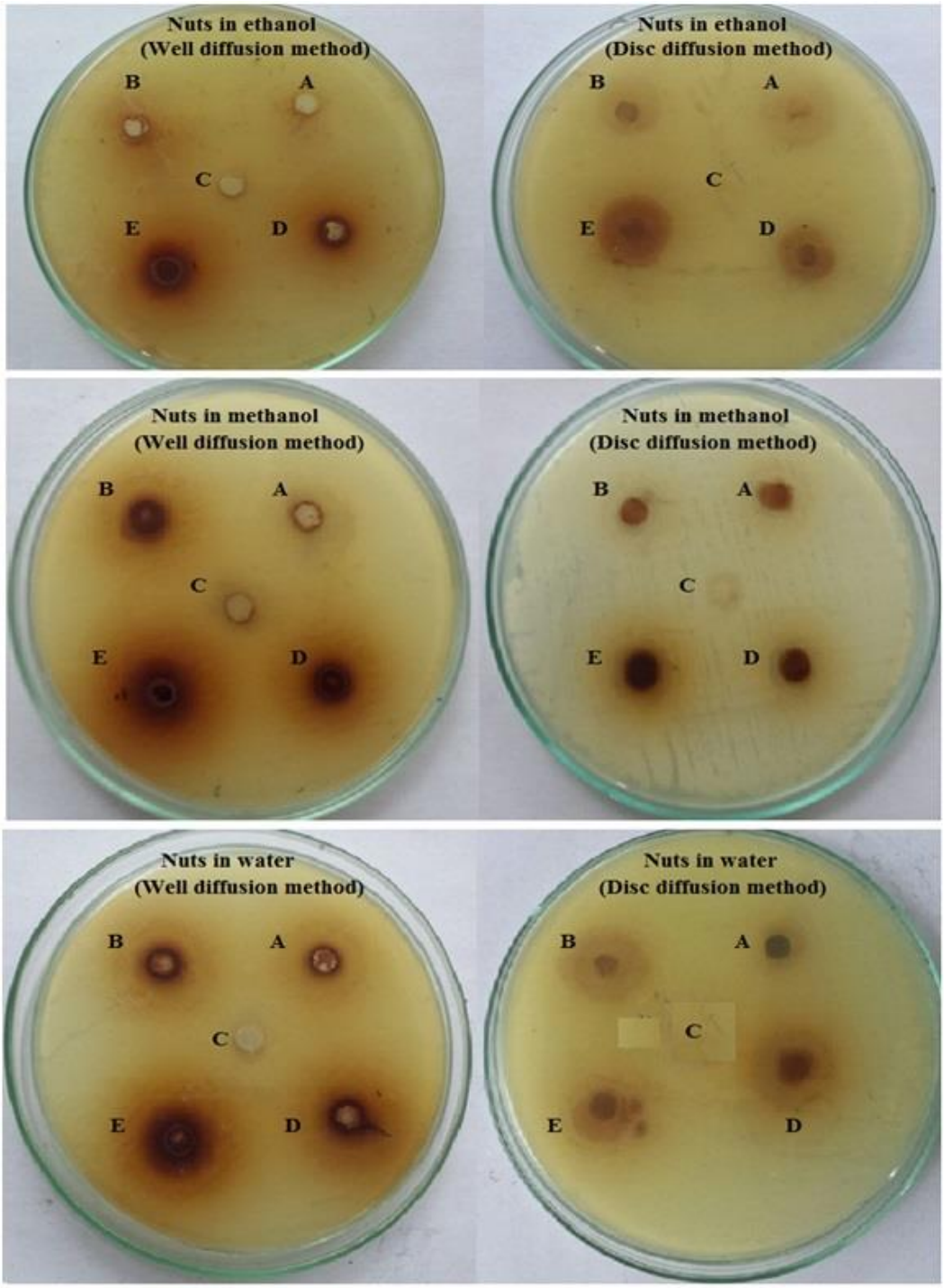


Figure 2: Well and disc diffusion assays to check the zones of inhibition by nuts in ethanol, methanol and water

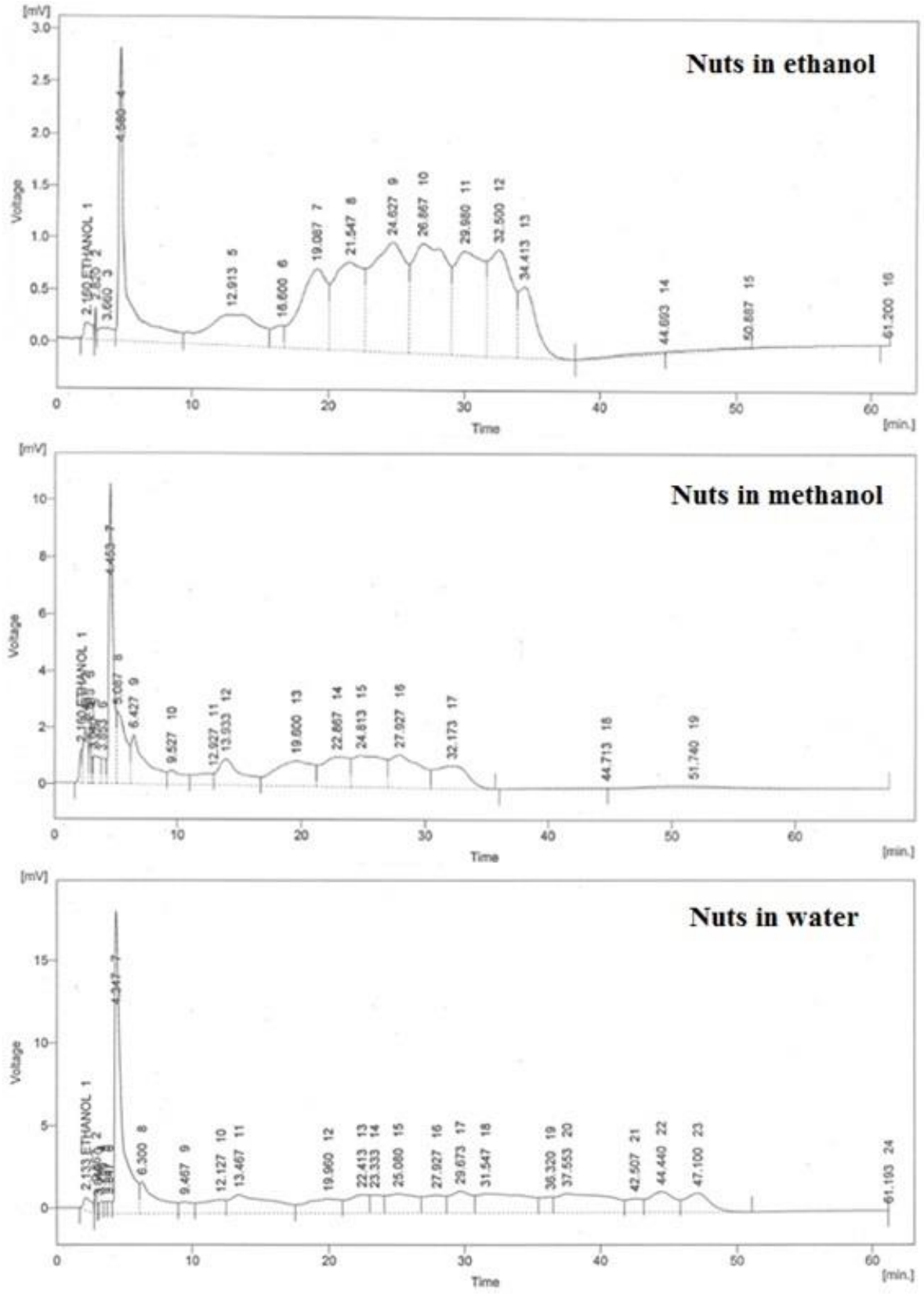


Figure 3: HPL C chromatogram of nuts in ethanol, methanol and water

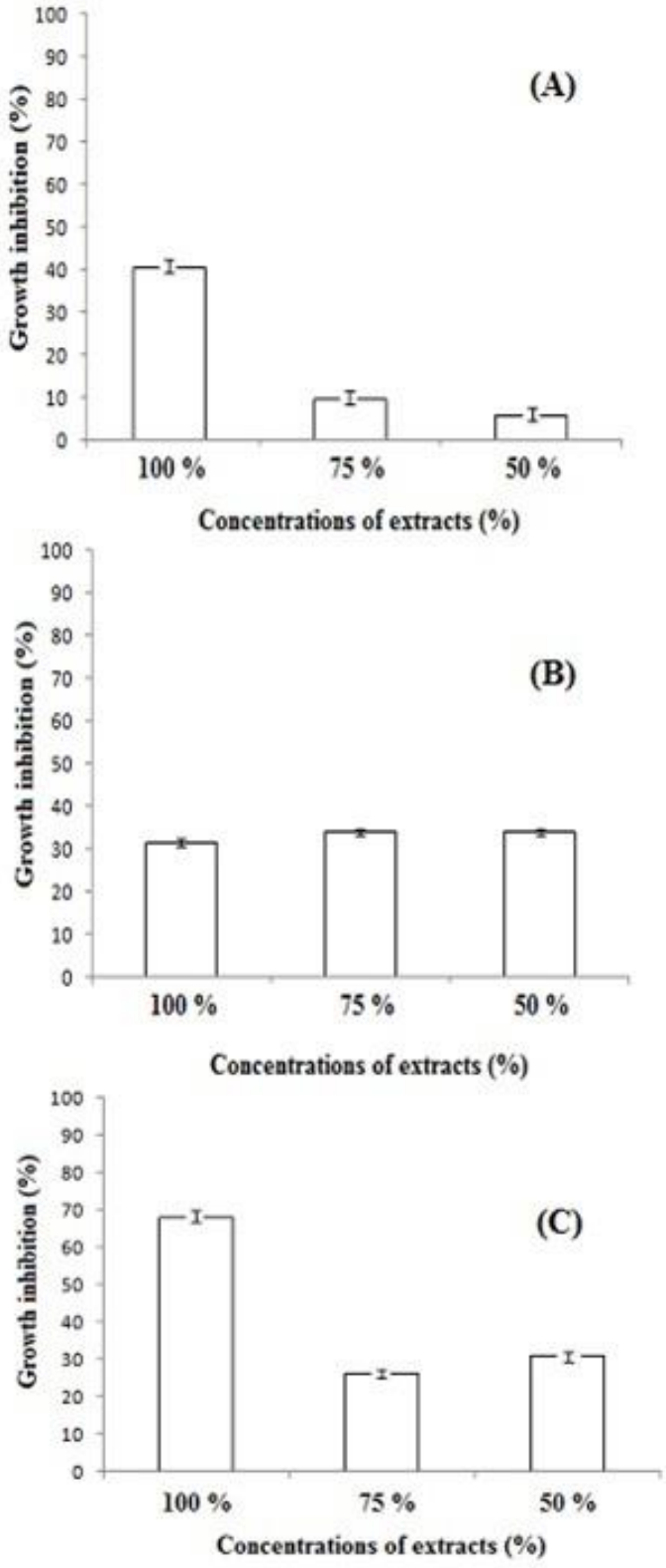


Figure 4: Percentage of growth inhibition by using different concentrations of ethanol, methanol and water extracts

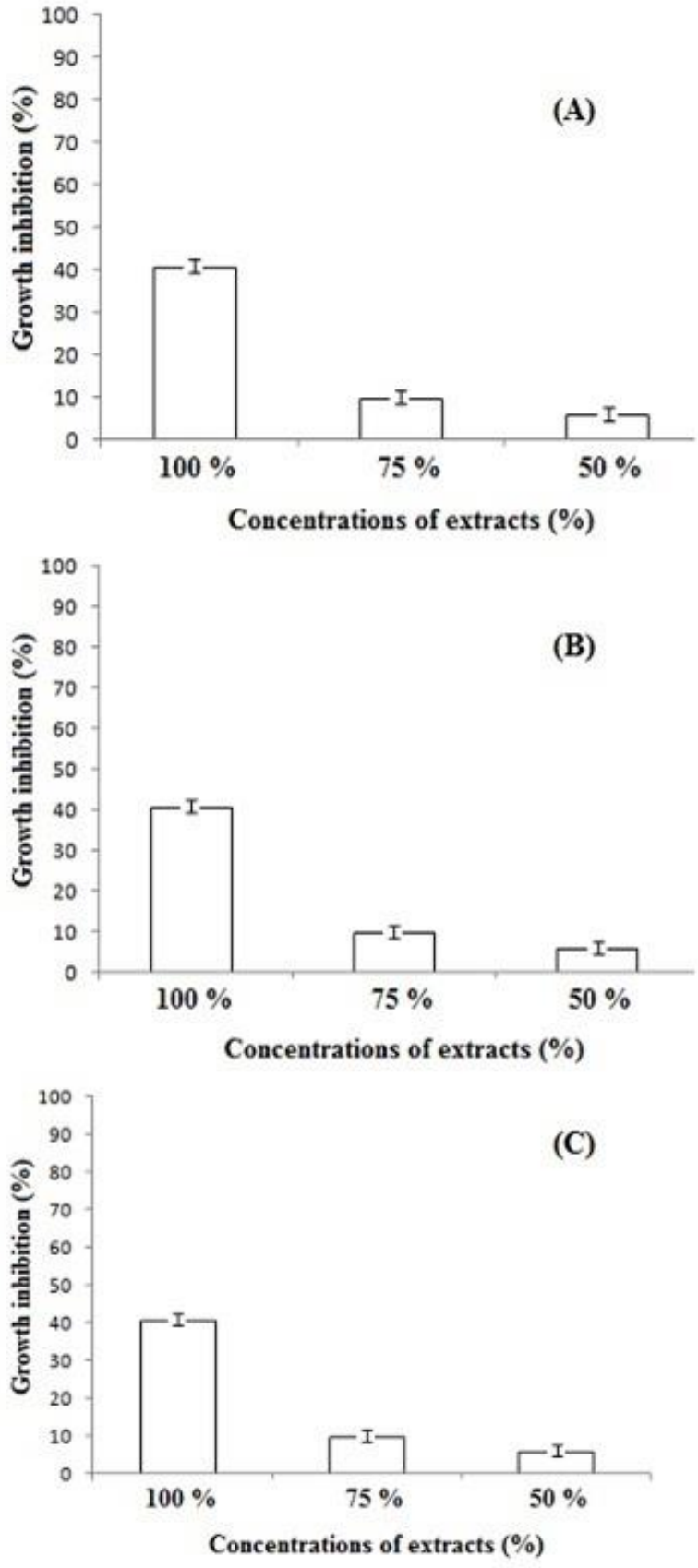


Figure 5: Percentage of growth inhibition by using different concentrations of ethanol, methanol and water extracts

DISCUSSION

Dental plaque is a community of microorganisms in form of biofilm. Tooth provides solid support which microorganisms use to bind with each other as well as with the tooth surface to form a film[14, 15]. If this biofilm is not removed on daily basis, it will lead to caries formation[16]. Various microorganisms are known to cause this biofilm formation e.g. *Streptococcus mutans*, *Lactobacillus casei*[17]. It is known that mouth inhabits 200-300 bacterial species[16] (Loesche, 1996). In this study, five microorganisms were isolated from healthy teeth of 100 individuals. They included *Staphylococcus* sp., *Streptococcus* sp., *Bacillus* sp., *Pseudomonas* sp. and *Klebsiella* sp. *Streptococcus* sp. were also reported by Li *et al*.,[17]. *B. subtilis* was found in all teeth of subjects. On this basis, it was selected for further experiments. *B. subtilis* is Gram positive, motile, rod shaped and spore forming bacterium[18]. Areca nut is considered an active and abundant source of bioactive compounds as compared to its other plant parts[19]. The antibacterial mode of action of areca nuts in betel quid was also explained by the fact that during betel quid chewing, polyphenols of areca nuts get oxidized which results in the formation of superoxide anion ($O2^{-}$). The presence of lime greatly increased the pH of the mouth during chewing process. This superoxide anion gets converted to H_2O_2 which further generates hydroxyl radicals. These radicals are toxic to microbial flora[20, 21]. Kenney *et al*., (1975) explained that alteration of pH and oxidation-reduction potential of oral region causes death of Gram positive oral flora due to which Gram negative bacteria caused dental caries thus, leads to deterioration of teeth in general[22].

Previous workers used both disc and well diffusion methods methods to check the antimicrobial activity. Chin *et al*., [22] employed well diffusion method whereas Saleem *et al*.,[11] used disc diffusion method. In this study, both disc and well diffusion methods were used and compared. Overall, disc diffusion method showed more zones as compared to well diffusion method. In Figure 2, disc method showed more zones of inhibition as compared to well method. It was also observed that with increasing nuts concentration in ethanol, enhanced zone was observed. Similar was the case with methanol and water extracts. Overall, ethanol extract showed more zone followed by water then methanol. No zone was observed with chloroform (results not shown here). It showed that chloroform did not extract the bioactive compound from nuts. Anthikat and Michael,[23] observed 100 % growth inhibition of Gram positive bacteria at 16 µg/ ml areca nuts concentration. Rahman *et al*.,[24] also reported antimicrobial activity of areca nuts from ethanol and water extracts which was in accordance with our results. The mode of antibacterial action of nut extract is the immediate precipitation of bacterial proteins thus, inhibiting the signals molecules by denaturing the bacterial proteins[22]. When areca nuts are mixed with other ingredients to form betel quid, then decrease in microbial population was observed[25]. The antibacterial activity of areca nuts found in this study, confirmed the previous literature [22, 26].

Here HPLC chromatogram of areca nuts in ethanol, methanol and water extracts showed variety of phenolic acids namely quercetin, gallic acid, caffeic acid, syringic acid, cinnamic acid, sinapic acid, vanillic acid, coumaric acid and ferulic acid (Table 2, Figure 3). They were responsible for antibacterial, antihelminthic, antifungal, anti-inflammatory and antioxidative properties. Quercetin is a flavonoid. Flavonoids are natural bioactive compounds found in plants[27]. The bioactivity of quercetin is due to disruption of cell membrane and formation of complexes with soluble and extracellular proteins resulting in inactivation of proteins[28]. The antibacterial activity of quercetin against 11 microorganisms was reported earlier[29] which confirms our results. In this study, quercetin was found in ethanol and water extracts but not in methanol extract although it showed antimicrobial activity. Cinnamic acid was found in all three extracts. Cinnamic acid is already reported to possess antibacterial properties[30]. It can be concluded that the bioactivity of methanolic extract was due to cinnamic acid in our study (Table 2).

B. subtilis has ability to form floating biofilm or pellicle at liquid-air interface[18, 31] which is in agreement with this study (Figure 1). Bacterial biofilm formation is an important topic since last decade[32] as it provides shelter to bacteria residing inside and helps bacteria to become resistant to drugs and antibiotics. Biofilms dwelling bacteria are considered 1000 times more resistant to antibiotics than free floating cells[31]. Due to this reason, medical treatment often fails and disease condition persists. Many factors are responsible for biofilm resistance like expression of multidrug efflux pumps, type IV secretion systems, restricted diffusion of antibiotics in biofilm matrix, decreased permeability, and action of antibiotic modifying enzymes[33]. An antibiofilm agent of plant origin is a hot topic these days as they are considered safe and have no side effects[32]. In this study, all three extracts inhibited the biofilm formation of *B. subtilis* (Figure 4). The 100 % ethanol extract inhibited 40 % biofilm growth. The methanol extract inhibited 30 % whereas much inhibition was observed with water extract i.e. more than 60 % (Figure 4). Similarly, ethanol, methanol and water extracts demolished the established biofilm i.e. about 40 % growth inhibition of biofilm was observed with all extracts. The ability to demolish biofilm increased with increasing concentration of extract (Figure 5). Biofilm experiments showed that ethanol, methanol and water extracts of areca nuts possessed the antibacterial and antibiofilm ability. Further research can help in getting deep insight into the molecular mechanisms that are responsible for antibiofilm ability of areca nuts. Further research in this area can help in developing antibiofilm agent from areca nuts.

CONCLUSION

Areca nuts possess antimicrobial activity against *B. subtilis* biofilms. There is a need to check its antimicrobial activity against other microorganisms before it can be used in the formulation of mouth wash.

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