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Antimicrobial, antibiofilm and antiplasmid activity of fruit peel extracts on bacterial dental caries

Raghda S. Sultan[1,*] Muayad S. Shawkat[1] and Sabah M. Hadi[2]

[1] *Department of Biotechnology, College of Science, Baghdad University, Baghdad, Iraq.*
[2] *Central Environmental Laboratory, College of Sciences, Baghdad University, Baghdad, Iraq.*

* Corresponding author: Raghda S. Sultan; e-mail: strongabed@gmail.com

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ABSTRACT

Fruit peels are generally considered as waste and are normally thrown away by us. But different studies conducted on peels revealed the presence of important substance, which can be used for medicament purpose. The results of the chemical analysis of methanol and hot aqueous extracts of *C. lanatus* and *M. paradisiaca* peels showed the presence of (phenols, alkaloids, terpenoids, tannins, saponins, glycosides and flavonoids). Experiments were carried out to evaluate the antimicrobial activity of crude extracts against *Streptococcus mutans* and *Escherichia coli* isolated from mouth of patients with dental caries, well agar diffusion method was used to detect the inhibitory zone and inhibition of biofilm formation. The results of the inhibitory effect showed that the methanol extract of *C. lanatus* and *M. paradisiaca* peels recorded the highest inhibition zone at concentration 100% against *S. mutans* and *E. coli* followed by hot aqueous extract in the inhibition capacity. On the other hand the hot aqueous extract also had the strongest effect in preventing the formation of the biofilm than methanol of the peels which appeared clearly through a decrease in the absorption value as the anti-biofilm effect increase. The study also exhibited the effect of the hot aqueous extracts of the studied plants on curing resistant plasmid, the effect of the *C. lanatus* peels was limited to concentration 3% and 6% against *S. mutans*, while against *E. coli* at concentration 6%, *M. paradisiaca* peels was negative in all concentrations. The result of this study shows that the methanol and hot aqueous extracts of peels were more potent against both the Gram-positive and gram -negative bacteria used for the present study.

Keywords: herbal extracts; dental caries; plasmid curing; MDR bacteria; *C. lanatus*; *M. paradisiaca*.

1. INTRODUCTION

Fruit are a good source for men health, they are known to reduce risk of several chronic diseases and are generally used for their nutritive value and bioactive compounds [1]. Fruit peels are formed in massive amounts through industrial processing, therefore causes harmful impact on environment and they need to be managed or they can be utilized [2]. The natural bioactive compounds in fruits are originally found in the peels with higher concentration towards the flesh [3]. Jeong *et al.* (2004) reported that the peel usually contains higher bioactive compounds in order to protect the inner materials from deterioration by insects and microorganism [4]. The importance of natural bioactive compounds has led to the development of a large and potential market for natural sources in pharmaceutics and food products [5].

M. paradisiaca is a tropical fruit belonging to *Musaceae* family, and its peel is a waste product, all parts of this plant such as flower, pulp, stem, and leaves have a medicinal application [6,7]. Bioactive compound such as flavonoids, tannins, alkaloids, glycosides, and terpenoids are present in peels, This bioactive compound is reported to exert pharmacological and pharmaceutical effect, especially as an antioxidant, antidiabetic, anti-inflammatory, and antibiotic [7,8].

Citrullus lanatus is an annual herbaceous plant belong to cucurbitaceae family, it is widely distributed in the

tropics that serves as a thirst-quencher owing to its high (92%) water content [9,10]. *C. lanatus* contains active compounds like alkaloids, flavonoids, tannins and amino acids, rind are also waste of watermelon and to prevent solid waste related hazards to the environment, effort should be made to increase the utilization of food wastes which requires studies on their properties and pharmacologic activities [11]. Studies were reported on watermelon and banana fruits, but mainly on the juice/pulp [12,13] and a little on the peel/rind [14,15].

Dental caries, is one of the most common chronic, multifactor disease in worldwide, that start with microbiological shift within the complex biofilm (dental plaque). Plaque is an oral biofilm that stick to the teeth and consists of many species bacteria such as (*Streptococcus mutans*) and *E. coli*. The accumulation of microorganisms subjects the teeth and gingival tissues to high concentrations of bacterial metabolites which results in dental disease [16,17].

Gene responsible for resistance to antibiotic are located in extra chromosomal DNA (plasmid) and often transferable to the other bacteria. This resistance is one of the most upcoming problem in the treatment of infection diseases [18]. One of the way to minimize plasmid transmission of the antibiotic resistance is to eliminate it by process called (plasmid curing), the use of antiplasmid chemical agents are unsuitable due to their toxic and mutagenic nature [19]. Therefore, alternative agents based on herbal extracts are being the particular interest [20], and these natural substances are used for control of caries disease in terms of antimicrobial response and lower associated risks [21]. Various herbal extracts from plant containing secondary metabolites could be used for plasmid curing [22].

The aim of this study to evaluations the antimicrobial, anti-biofilm and antiplasmid activity of fruit peels extracts on bacterial dental caries also support the recycling of fruit waste.

2. MATERIALS AND METHODS

2.1 Collecting of plant material

Peels of *Musa paradisiaca* and *Citrullus lanatus* were collected from local market in Baghdad and identified kindly by the herbarium in Department of Biology, College of Science, University of Baghdad. Plant parts were washed with tap water and dried at room temperature, then the samples were grounded into powder by electrical grinder. The powdered parts were kept in plastic bags at 4C° until use [23].

2.2 Preparation of hot aqueous extracts of *M. paradisiacal* and *C. lanatus* peels

Peels were dried by oven at 60°C. extracts were prepared by weighting (100 gm) of dried grinded peels, then mixed with 500 ml of boiled D.W., the mixture was homogenized for 2 hrs. then were filtered and centrifuged at 8000 rpm for 10 min. extracts were dried using the oven at 45°C then kept at 4°C until

2.3 preparation of methanol extracts

A quantity of 100 gm of peels of *M. paradisiaca* and *C. lanatus* powder were soaked in 500 ml of methanol, left for 48 hrs then filtered and concentrated at room temperature then few drops of DMSO was added to the dried extracts and stored in 4°C until use [23].

2.4 Preparation of test microorganism

The microorganism used in this study include gram positive bacteria *Streptococcus mutans* and gram negative bacteria *Escherichia coli*. The microorganisms were clinical isolated from Department of microbiology, collage of Dentistry, University of Baghdad.

2.5 Phytochemical analysis of peels extracts

The extract was subjected for phytochemical investigations by qualitative chemical test. Standard phytochemical methods were used to test for the presence of active compounds.

2.6 The standard antibiotic used for antimicrobial assay

The following antibiotic discs were used as positive control in this study by using the Kirby-Bauer standardized single disc method [24] presented in table (1).

2.7 Well diffusion method

Antibacterial activity of plant extracts were evaluated using well diffusion method on Mueller-Hinton agar (MHA). The inhibition zones were reported in millimeter (mm) using *E. coli* and *S. mutans* bacteria as references. MHA agar plates were inoculated with bacterial strain under aseptic conditions and wells (diameter=6mm) were filled with 50 µl of the test samples and incubated at 37°C for 24 hours. After the incubation period, the diameter of the growth inhibition zones was measured [25,26].

Table 1: Antibiotic disc used in this study

Antibiotics	Disc content (µg)	Company and origin
Amoxicillin	25 µg	Sigma, USA
Ampecillin	10 µg	
Azothromycin	15 µg	
Ciprofloxacin	10 µg	
Tetracycline	30 µg	

2.8 Biofilm formation inhibition

Biofilm formation assays were performed using 96-well microtiter plate, based on the protocol by Goh *et al*., (2013), with minor modifications. Briefly *E. coli* and *S. mutans* were cultured in TS Broth overnight and the resulting culture was adjusted to 0.5 McFarland. Each well of microtiter plate was loaded with 100 ml of medium and 100 µl of plant extracts except a control well without plant extracts solutions, each concentration was tested triplicate then the plate was incubated at 37°C for 24 h. The plank tonic bacteria were removed by shaking the dish over a waste tray filled with sterile distilled water, subsequently 0.1% w/v crystal violet solution was added to each well and the plate was left to stain for 10 min at room temperature. Next the crystal violet solution was removed by submerging the plate in a water tray, then inverted and topped on paper towels to remove excess liquid and left to air dry. The stained wells were treated with 95% v/v ethanol for 10 min at room temperature to solubilize the dye and optical density (OD) was measured in a micro plate reader at 630 nm [27].

2.9 DNA extraction

The DNA was extracted by using alkaline lysis methods [28], 10 ml of fresh culture from the selected isolate was grown in brain heart infusion broth at 37°C for 24 hrs then transferred to 1.5 ml tubes, the cells were harvested by centrifugation at 14000 rpm for 1-3 min at room temperature. 150 µl of resuspend solution was added and the bacterial pellet was resuspend by vortex, 200 µl of lyses solution was added to bacterial suspension (freshly made) and mix by inverting the tube 4-6 times then 300 µl of neutralization was added and mix the contents thoroughly by inverting the tube 4-6 times. The supernatant was Centrifuged at 14000 rpm for 5 min and it was transferred to a new 1.5 ml tube, 300 µl of isopropanol was added to the supernatant and mix it by inverting the tube couple of times, then incubate in ice for 30 min. Centrifugation at 14000 rpm for 5 min, the supernatant was removed and 600 µL of EtOH 70% was added, then centrifugation at 14000 rpm for 5 min.The supernatant was removed, the pellet was dried for 10-30 min, and then dissolves the DNA in (50-100) µl TE buffer.

2.10 Agarose Gel Preparation and Electrophoresis

Agarose gel was prepared by dissolving 1 g of agarose powder in 100 ml of 1 x TBE buffer and melted then cooled to 50- 60°C. 5 µL of ethidium bromide dye was added to the gel with mixing, the solution was poured into a tray, several wells were carefully made with a comb at one side of the gel. After solidification, the comb was carefully removed and jar was put in the electrophoresis tank [29]. Each well was loaded with 3 µl of loading dye mixed with 5µl of total DNA, the electrical power was turned on after closing the tank lid then adjusted at 70 volt for 1 hr, the migration of DNA was from cathode (-) to anode (+) poles. Agarose gel tray was placed in gel documentation system for visualization of DNA under ultraviolet light (350 nm) and photographed [30].

2.11 Curing of plasmid mediated antibiotic resistance

This method was used to describe the ability of plant extracts in elimination of plasmid mediated antibiotic resistance. Bacterial cells were incubated with different concentration of these extract in shaker incubator for 24hr at 37°C. After 24hrs culture were placed on Muller Hinton agar plates to obtain isolated colonies, then Isolated colonies were then replica plate to Muller Hinton agar plates with antibiotic. The colonies grew in Muller Hinton plates but failed to grow in the presence of antibiotics were considered as cure derivatives [31].

3. RESULTS AND DISCUSSION

3.1 Phytochemical screening

The hot aqueous and methanol extracts were subjected to preliminary photochemical investigation for the detection of secondary metabolites [32]. Result obtained by chemical detection of hot aqueous extract for *M. paradisiaca* indicated the presence of tannins, phenols, flavonoids and terpenoids, while the results were negative for alkaloids, saponins and glycosides as shown in table (2).

Table 2: Active compounds detected in *M. paradisiaca* peels

Active compounds	Methanol extract	Hot aqueous extract
alkaloids	+	-
Phenols	+	+
Terpenoids	+	+
Tannins	+	+
Saponins	+	-
Flavonoids	+	+
glycosides	+	-

C. lanatus chemical analysis indicated the presence of tannins, flavonoids, terpenoids saponins, alkaloids and phenols but it is negative for glycosides as shown in table (3). The methanol extracts of *M. paradisiaca* and *C. lanatus* contain all active compounds and this is due to the high polarity of methanol. The variation in the presence of these compounds may be due to the genetic nature of the plants beside the time of plant collection and the conditions of extraction.

Table 3: Active compounds detected in *C. lanatus* peels

Active compounds	Methanol extract	Hot aqueous extract
alkaloids	+	+
Phenols	+	+
Terpenoids	+	+
Tannins	+	+
Saponins	++	+++
Flavonoids	+	+
glycosides	+	-

3.2 Susceptibility to antibiotic test

The effect of plant extracts on the bacteria is close or more than the effect of disc of antibiotic (positive control), except ciprofloxacin, which the bacteria showed high sensitivity towards it as shown in table (4).

Table 4: Susceptibility of test bacteria to antibiotics

Antibiotic name (positive control)	Zone of inhibition/mm *S. mutans*	Zone of inhibition/mm *E. coli*
Amoxicillin	6	5
Ampecillin	10	8
Tetracycline	15	19
Azothromycin	14	13
Ciprofloxacin	30	35

3.3 Antibacterial activity of extracts

Results showed that the higher diameter of inhibition was obtained by the methanol extract of *M. paradisiaca* at (100%) concentration against *E. coli* (16 mm) and (18 mm) against *S. mutans*. While the diameter of inhibition at (70, 50 and 25)% was (17, 17 and 10) mm and (15, 15 and 10) mm for *S. mutans* and *E. coli* respectively. The inhibition diameter for hot aqueous extract of *M. paradisiaca* at concentration (100,75 and 50)% against *S. mutans* (10, 9 and 7) mm but *M. paradisiaca* did not show antimicrobial activity against *S. mutans* at 25% and *against E. coli* at concentration (50 and 25)% as shown in table (5).

Table 5: Inhibition zone of *M. paradisiaca* peels extracts on different concentration.

Bacterial species	Hot aqueous extract Conc. %				Methanol extract Conc.%			
	100	75	50	25	100	75	50	25
S. mutans	10	9	7	-	18	17	17	10
E. coli	9	8	-	-	16	15	15	10

Note: diameter of well (6mm), (-) no inhibition zone, water (negative control), DMSO (negative control), Zone of inhibition (mm)

Methanol extract of *C. lanatus* were active against *S. mutans* and *E. coli*, the diameter of inhibition obtained by *C. lanatus* at (100, 75, 50 and 25)% against *E. coli* was (18, 17, 15 and10) mm and against *S. mutans* was(18,17,15 and10) mm. The inhibition diameter of aqueous extract of *C. lanatus* at concentration (100, 75, 50 and 25)% against *S. mutans* (10, 10, 9 and 8) mm and (9, 9, 8 and 5) against *E. coli* at concentration (100, 75, 50 and 25)% as shown in table (6).

Table 6: Inhibition zone of *C. lanatus* peel extracts on different concentration.

Bacterial species	Hot aqueous extract Conc. %				Methanol extract Conc. %			
	100	75	50	25	100	75	50	25
S. mutans	10	10	9	8	20	19	17	11
E. coli	9	9	8	5	18	17	15	10

Note: diameter of well (6mm), (-) no inhibition zone, water (negative control), DMSO (negative control), Zone of inhibition (mm).

3.4 Biofilm formation inhibition

The effect on biofilm formation showed that hot aqueous extract of peels of both plants *C. lanatus* and *M. paradisiaca* had the highest inhibition effect on *S. mutans* and *E. coli* followed by methanol extract as shown in table (7).

Table 7: Effect of plant extracts on biofilm formation

Plant extracts		O.D (630nm)	
		S. mutans	*E. coli*
		0.412 un treated	0.460 un treated
C. lanatus	Aqueous	0.180	0.212
	Methanol	0.287	0.367
M. paradisiaca	Aqueous	0.193	0.233
	Methanol	0.293	0.271

3.5 Antiplasmid activity

the high concentrations of *C. lanatus* extract (3 and 6) % in lanes (9 and 10) inhibited the plasmid repeating by showed no bands of plasmid DNA, also the aqueous extract of *M. paradisiaca* peels showed no curing activity against the *S. mutans* bacteria by observed all the bands of plasmid DNA in lanes (2, 3, 4 and 5) which refers to the extract concentrations at (0.5, 1. 3 and 6) % as show in the figure (1). The hot aqueous extract of *M. paradisiaca* at the concentrations (0.5, 1, 3 and 6) % didn't have any plasmid curing activity against the *E. coli* bacteria through the existence of all the bands of plasmid DNA in lanes (2, 3, 4 and 5) as shown in figure (2), while the band of plasmid DNA in lane (10) observed that the high concentration (6) % from the aqueous extract of *C. lanatus* showed plasmid curing activity against the *E. coli* bacteria only.

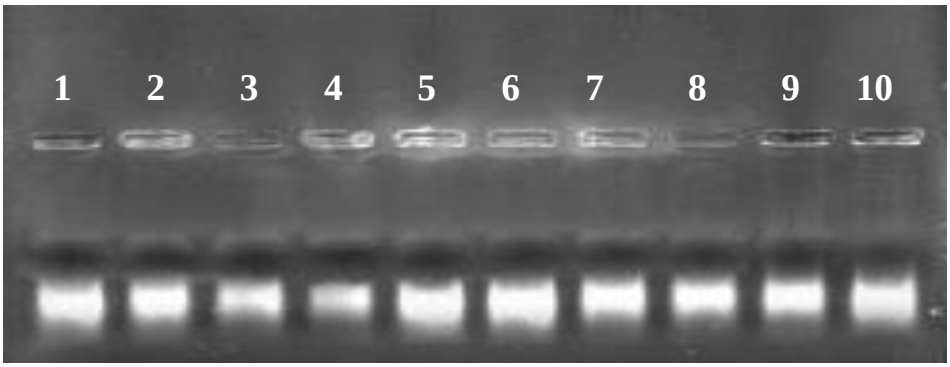

Figure 1: Gel electrophoresis of *E. coli* plasmid DNA treated with different concentrations of *M. paradisiaca* and *C. lanatus* aqueous extract: lane (1) control, lane (2) plasmid DNA treated with 0.5 % of *M. paradisiaca*, lane (3) plasmid DNA treated with 1% of *M. paradisiaca*, lane (4) plasmid DNA treated with 3% of *M. paradisiaca*, lane (5) plasmid DNA treated with 6% of *M. paradisiaca*, lane (6) control, lane (7) plasmid DNA treated with 0.5 % of *C. lanatus*, lane (8) plasmid DNA treated with 1% of *C. lanatus*, lane (9) plasmid DNA treated with 3% of *C. lanatus*, lane (10) plasmid DNA treated with 6% of *C. lanatus*

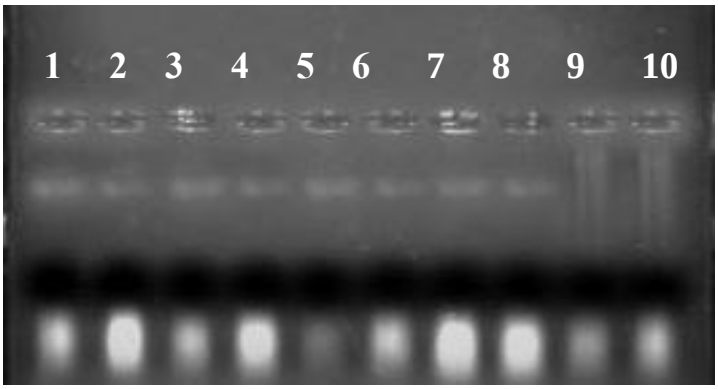

Figure 2: Gel electrophoresis of plasmid DNA for *S. mutans* bacteria with different concentrations of *M. paradisiaca* and *C. lanatus* aqueous extracts: lane (1) control, lane (2) plasmid DNA treated with 0.5 % of *M. paradisiaca*, lane (3) plasmid DNA treated with 1% of *M. paradisiaca*, lane (4) plasmid DNA treated with 3% of *M. paradisiaca*, lane (5) plasmid DNA treated with 6% of *M. paradisiaca*, lane (6) control, lane (7) plasmid DNA treated with 0.5 % of *C. lanatus*, lane (8) plasmid DNA treated with 1% *C. lanatus*, lane (9) plasmid DNA treated with 3% of *C. lanatus*, (10) plasmid DNA treated with 6% of *C. lanatus*.

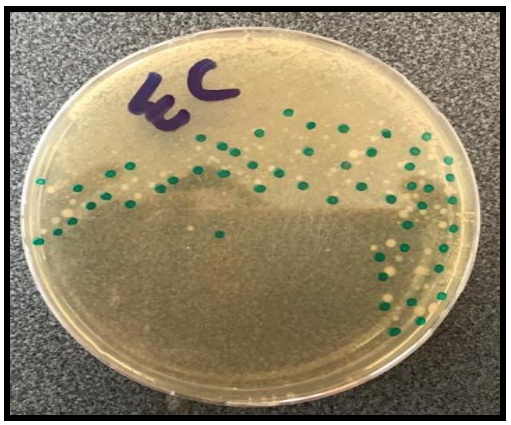

Figure 3: Growth of *E. coli* bacteria on the medium contained the antibiotic, after treated with 6% of *C. lanatus* hot aqueous extract.

Results showed that no growth of bacteria observed on the medium contained the antibiotic, after treated with plant extracts, except *E. coli* few number of colonies grew in this medium as shown in figure (3).

Phytochemical screening of *M. paradisiaca* and *C. lanatus* peels extracts revealed the presence of active compounds, these compounds are known to be biologically active. The high percent of saponins and flavonoids in hot aqueous and methanol extracts of *C. lanatus* dried peel may be responsible for antimicrobial activity, saponins have specific ability to form pore in membranes has contributed to their common use in physiological research, while The inhibitory activity of terpenoids and flavonoids is due to formation of complex with extracellular and soluble protein and bacterial cell well disruption [33,34]. Tannins have been found to form irreversible complex with prolin-rich proteins resulting in the inhibition of cell protein synthesis, inactivate microbial enzyme and complex with polysaccharide [35]. Plant extracts have greatly affected biofilm formation by damaging microbial membrane structure [36], inhibiting peptidoglycan synthesis, and/or modulating quorum sensing [37]. Flavonoids are among the compounds that exert anti-biofilm effects by quorum sensing inhibition [38].

The hot aqueous extract of peels for both plants showed contains various active compounds may affect the enzymatic system of bacteria specially those required for plasmid replication or may affect cell membrane and specially on mesosome which consider as attachment point for plasmid. The mechanism of antimicrobial action of alkaloids is attributed to their ability to intercalate with DNA, inhibition of enzyme (esterase, DNA-RNA polymerase), inhibition of cell respiration [39].

4. CONCLUSION

The results of the present study showed that the hot aqueous and methanol extracts of peels of *C. lanatus* and *M. paradisiaca* have antibacterial activity and biofilm inhibition against *S. mutanus* and *E. coli* as well as anti-plasmid activity. The study also reveals the fact that the use of this natural drug in the optimum dosage can replace the use of antibiotics.

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