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Manufacturing of Medicate Chewing Gum against *Streptococcus Mutans* by the Extract of Grape Juice Wastes (*Vitis vinifera L.*)

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Abstract: The grape juice wastes (GJW) including seeds, peels and flesh of the fruit .The results determination of total phenols in GJW extract 140.13mg/g also flavonoids 25.75mg/g. The zone of inhibition of S. mutans showed the range 16-20mm for different concentration of GJW extract, while GJW extract had MIC values less than 12.5 mg/ml and MBC values 50 mg/ml. The calculated value of median lethal dose (LD50) of GJW extract was assigned to the test mice, which was 6442 mg/kg body weight. Medical gum was manufactured from the following ingredients gum base, glycerine, xylitol, sun flower oil, flavor and color with the addition of the extract tested with different concentration (3.0 and 6.0 mg/g), the release of phenols compounds of chewing gum samples were estimated after each period of crushing the sample of gum in phosphate buffer. It was found that the ratio of the release of tis group of active compounds (phenols) increased with the increase of the duration of crushing gum samples until reaching 100% for all samples after 20 min. The results showed sensory evaluation that samples of gum added to the extract was acceptable and no significant differences was found in comparison to the control treatment.

Key points: S. mutans, Grape juice wastes, Phenols, Flavonoids, Antibacterial, Chewing gum, phenol release.

Introduction

Oral disease such as dental caries, oral mucosal lesions and periodontitis are prime cause public health problems world-wide and their effects on general health and quality of life are substantial (1). *Streptococcus mutans* is one of primary dental plaque colonizers which provide the aggregation of other microorganisms (2). These bacteria synthesize polysaccharides that are present in dental plaque and causes dental caries (3). Dental caries prevention is preferable to treatment. Convention preventive methods such as the use of alcohol or antibiotics, like chlorhexidine, erythromycin, ampicillin and penicillin, have proven effective in preventing dental caries (4). However, excessive used of these chemicals has been reported to change the oral and intestinal flora, and can cause other problems such as vomiting, tooth staining or oral cancer, importantly antibacterial agents can also promote the development of resistant bacteria strains (5,6). For these reasons alternative methods such as the use of medicinal plants are of increasing interest. In recent years, research is developing new methods of dental disease prevention, releasing natural bioactive compounds included in traditional or innovative medical devices in the mouth to improve tooth protection.

Chewing gums is particularly effective means for delivering and maintaining bioactive compounds, included in the gum formulation able to have an anti-cariogenic effect (7). Gum advantages over the other delivery methods include no needing to water or liquids to eat, increasing the systemic effects, low dose administration, faster onset of action and good stability relieving of dry mouth strengthening the mastication muscles and prevention of dental caries (8).

Grape is one of the older fruit crops domesticated by humans (9). It is a phenol rich plant and these phenolics are mainly distributed in the seed, skin, leaf and stem of grape (10,11). Grape seeds are considered rich sources of poly phenolic compounds mainly monomeric gallic acid and polymeric, catechin and epicatechin and oligomeric procyanidins (12). Grape pulp is contain only 4% of grape polyphenols, while in grape skin there is another type of polyphenol called anthocyanins (13). Moreover, Grape seeds is a rich source of diverse bioflavonoids, collectively known as grape seed proanthocyanidins extract (14). Polyphenols are well documented to have microbicidal activities against huge number of pathogenic bacteria (15,16).

Therefore this study aimed to manufacturing of Medicate Chewing Gum Against Oral Pathogens by the Extract of Grape Juice Wastes as source of low-cost

Material and Method

Plants collection

The grape juice wastes of *Vitis vinifera L.* (GJW) was obtained from local grape juice shop in Baghdad, The obtained waste was washed using tap water and dried in shade for 3-4 days.

Figure (1): Grape juice wastes of *Vitis vinifera L.*

Extraction

Applied method according to method described by (17) to 10 g of GJW added 300 ml of distilled water at boiling point and the mixture was left for 30 min on magnetic stirrer. After that, the mixture was filtered through filter paper (What man No.1), then concentrated by rotary evaporator under reduced pressure at 50˚C, the concentrated extracts was dried in oven at 40˚C, scraped and stored powder in labeled sterile screw capped bottles at 5˚C in the refrigerator, until when required for use.

Determination of total phenolic compounds

Folin-ciocalteu's reagent colorimetric method was used as described by (18) add 0.5 ml of the extract (1mg/ml) to 2.5 ml of Folin-ciocalteu's reagent, and 2ml of sodium carbonate 7.5% then left the mixture for 30 min at room temperature, the absorbance was recorded at 760 nm, the total phenolic compounds were determined according to gallic acid standard curve fig (2).

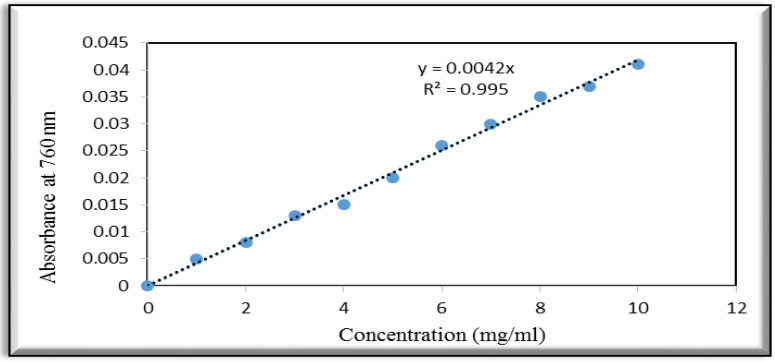


Figure (2): Standard curve of gallic acid

Determination of total flavonoids compounds

The total flavonoids in aqueous extract was determined according to (19) mixing 1ml of the extracts (1mg/ml) in 10ml volumetric flask with 5ml of distilled water and 0.3ml of NaNo2 5%. After 5 min added 0.6 ml of AlCl3 10% . After another 5 min added 2ml of 1M NaOH and the volume was made up to 10ml with distilled water. The mixture was mixed thoroughly and the absorbance was measured at 510 nm. The total flavonoids compounds were determined according to catechin standard curve fig (3).

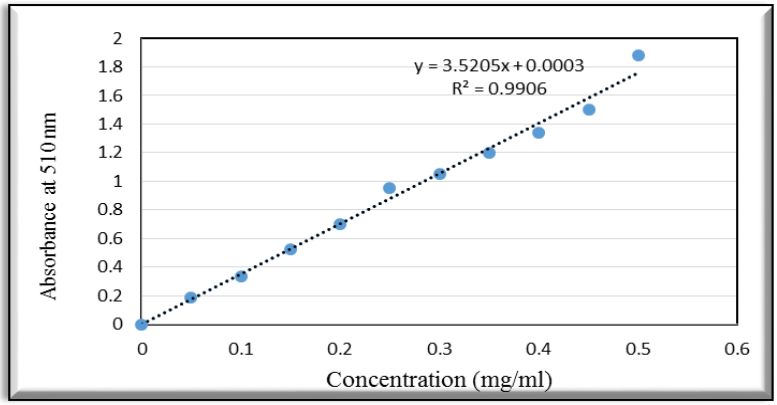


Figure (3): Standard curve of catechin

Antibacterial activity

Agar well diffusion assay

Antimicrobial activity of GJW extract was screened against *Streptococcus mutans*,. Use the method of well diffusion according to (20). For the bacteria an overnight culture (1×10^6 cfu / ml), one ml of inoculum was aseptically spread on the surface of Mueller Hinton agar plates using spreader. A well of about 6 mm was aseptically punctured using sterile cock borer, 100 µl of extract of different dilution was poured into wells. Each concentration (50, 100, 150, 200 and 250) mg/ml of the extracts was tested, and was sterilized by Millipore filter 0.45µm . Sterilized distilled water was used as control , The plates were kept for 30 min in laminar flow for pre-diffusion to occur and then incubated at 37C˚ for 24 hr anaerobic. The zone of inhibition was measured.

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) determination

The minimum inhibitory concentration was determined by using broth dilution method in 96-well plates. The extract powder were dissolved in 20% dimethylsulfoxide (DMSO) at different concentrations were assayed (0.05, 0.1, 0.5, 1.0, 2.5, 5.0, 7.5, 10.0, 12.5, 25, 50, 150 and 250, mg/ml). The bacterial suspension was regulated to (1×10^6 cfu /ml) were then added to each concentration of extract. Microplate reader was estimated optical density (O.D) at 600 nm before incubation (T_0), then 96-well plats were incubated with constant shaking at 37˚C for 24 hr. After 24 hr. the plates were again estimated (T_{24}), the growth inhibition was determined using the formula:

$$\%\ \text{inhibition} = \frac{1 - (\text{O. D test well at T24} - \text{O. D test well at T0})}{(\text{O. D control well at T24} - \text{O. D control well at T0})} \times 100$$

The MIC showed 100% growth inhibition. Aliquots from each concentration were incubated in BHA by subcultured at 37˚C for 24hr., the highest concentration that showed on bacterial colonies was taken as MBC (21).

Determination of Median Lethal Dose (LD_{50})

1. Preparation of the stock solution of extracts

Extract of GJW is available in powder form. All doses were prepared by dissolving powder of extract in distilled water (1000, 3000, 5000, 7000 and 10000 mg/kg) at the time of administration for the determination of LD_{50} .

2. The experiment of animal house

The experiment has been carried on the laboratory mice at Biotechnology Research center – University of Al-Nahrain.

3. The laboratory mice

Male mice have been supplied by the animal house- Biotechnology Research center – University of Al-Nahrain. The mice weighing about (25-27 g) and all are allowed to acclimatize for one week in animal house conditions (22 ± 3 ˚C, relative humidity 50-55%, and 12 hr light/dark cycle) prior to the experiment. A standard nutritionally balanced diet (manufactured by Grain and Flour Mills Organization, India) was supplied by Biotechnology Research center and according to (22).

4. Median lethal dose (LD_{50}) experiment

The study was conducted in total of 40 mice were used, they were divided in to 5 groups (8 mice). The range of doses of each type were detected by doing a pilot study, LD_{50} depend on percentage of dead animals during 48 hr., then converted to probit number. A plot of logarithm dose was constructed against obtained probit number (23).

Chewing gum preparation

Chewing gum was formulated of the gum base, oil, glycerin, xylitol , color and flavor. The combination of the base were softened in a water bath at 60°C. Oil, glycerin and xylitol, finally color and flavor and GJW extract (3.0 and 6.0 mg/g) were added at 40 °C table (1). Homogeneous mixture was extended on a glass plate. Then it was cooled and cut in small pieces and kept for 48 hours at room temperature.

No.	Ingredient	g
1	Gum base	75
2	Glycerine	15
3	Xylitol	1.5
4	Sun flower oil	2.5
5	Flavor and color	0.7

Table (1): Ingredient of medicated chewing gum

***In vitro*: phenol release test**

One gram of the formulation was taken in mortar, added to it 50 ml of pH 6.8, 0.2M phosphate buffer and for 20 min crushed. Temperature was maintained at 37˚C by water bath. 0.5 ml aliquots of mixture were removed at the times of 0, 5, 10, 15 and 20 min since the start of crushed. The aliquots were replaced by 0.5 ml fresh phosphate buffer subsequently. After that, added the solutions of total phenols determination (2.5 ml folin-cicalteu's reagent and 2ml sodium carbonate 7.5%) and absorbance was recorded at 760nm by using spectrophotometer.

Evaluating the organoleptic characteristics

To evaluate organoleptic features of this product, 10 healthy volunteers were asked to chew the gum and give comments on the hardness/softness, adhesion to teeth, the volume of the gum mass and taste according to the Likert scale on the evaluation forms (24) Table (2).

Table (2): Sensory evaluation form

No	Organoleptic properties	Samples						
		1	2	3	4	5	6	7
1	Chewing gum volume							
2	Softness							
3	No adherence							
4	Taste							
5	Persistence of taste							

1. The bulk volume of gum was evaluated as Huge=5, much=4, right=3, little=2, very little=1.
2. The softness/Hardness was evaluated as very hard=5, hard=4, suitable=3, soft=2, very soft=1.
3. The adherence to the teeth was evaluated as never adheres=5, rarely adheres=4, sometimes adheres=3, often adheres=2, always sticks=1.
4. The taste was evaluated as excellent=5, good=4, fair=3, poor=2, very poor=1.
5. The persistence of the taste was evaluated as strong persistence=5, good persistence=4, intermediate persistence=3, weak persistence=2, very weak persistence=1

Statistical analysis

The Statistical Analysis System- SAS (25) program was used to show the effect of difference of treatments in study parameters . Least significant difference –LSD ($p > 0.05$) was used to significant compare between means in this study.

Result and Discussion

The GJW extract had total phenols and flavonoids content 140.13, 25.75 mg/g respectively. These methods are of great utility for screening of plant materials for phenols as a way to measure gross phenolic content (26). The results agree with that obtained by (27) the total phenols content in grape pomace extract for different varieties of grapes like cabernet Sauvighon and Syrah 132.229 and 102.592 mg/g respectively.

Fig (4) show antimicrobial activity of an extract is measured in the terms of zone of inhibition exhibited by an extract against *S. mutans* 16, 16, 18, 20 and 20 mm at concentrations 50, 100, 150, 200 and 250 mg/ml respectively. Organic extract from seeds of grape showed bacteriostatic activity against anaerobic bacteria responsible for periodontal diseases (28) and they were also able to inhibit of glucosyltransfereases B and C in *S. mutans* (29).

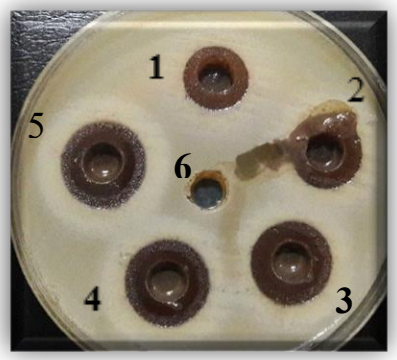


Figure (4) : The effects of aqueous extract of GJW against *S.mutans.* 1= 50mg/ml, 2=100mg/ml, 3=150mg/ml, 4=200mg/ml, 5= 250mg/ml 6= Control (sterilized distilled water).

This study shows the relation between the total content of phenolic compounds and antibacterial activity, which is explained by the highest range of inhibition diameter of GJW extract, may return to highest content of phenols and flavonoids. The mechanism of poly phenols toxicity against

microbes may be related to inhibition of hydrolytic enzymes (proteases and carbohydrolases) or other interactions to inactive microbial adhesions, transport proteins of cell envelope and nonspecific interactions with carbohydrates (15). It means that antibacterial activity of phenols is due to enzyme inhibition by the oxidized compounds possibly through more nonspecific interactions with the proteins (15,30). Furthermore, flavonoids and tannins can bind or form precipitates with various proteins (31).

The MIC and MBC values, aqueous extract of GJW had MIC values less than 12.5 mg/ml. While these extract demonstrated MBC values 50 mg/ml, it was supported by the study of (32), which focused that grape seed extract with lower concentration has not showed any bactericidal or bacteriostatic effect against *S. mutans*. *S. mutans* must tolerate rapidly harsh environmental fluctuations and exposure to various antimicrobial agents in order to survive (33). Furthermore, negative results have not indicated that the bioactive constituents are absent, active compounds may be present in insufficient quantities in the crude extracts, therefore, the dose levels used would not be sufficient enough to exhibit the inhibitory activity, alternatively even if the active principle is present in high enough quantities, there antagonistic effects on the positive effects of the bioactive agents, thus zeroing the antibacterial activity (34). It is also possible that the extracts may be active against other bacterial species that were not tested (35).

The calculated values of LD_{50} of the extract GJW was estimated by probit method results which were obtained from plotting the curve of logarithm doses of extract with the probit number of death according to (23), and was 6442 mg/kg table (3) and fig. (5). The results showed a positive relationship between the concentration of the extract and the mortality rate of the mice. The negative effects of phenolic compounds were related to the synergistic of some molecules, and the concentration was not always a crucial factor, therefore the dose and composition of plant extract should be investigated further for secure healthy application of plant products (36).

Table (3): The number of dead mice and their ratios in the medium lethal dose test for GJW extract.

Sub-group	Dose (mg/kg)	Log of dose	Total mice	Dead mice	Survived mice	Mortality %	*Corrected %	Probit No.
1	1000	3.0	8	0	8	0	3.0	3.12
2	3000	3.47	8	1	7	12.5	12.5	3.84
3	5000	3.69	8	2	5	25	25.0	4.33
4	7000	3.84	8	5	3	26.5	26.5	5.32
5	10000	4.0	8	6	2	75	75.0	5.67

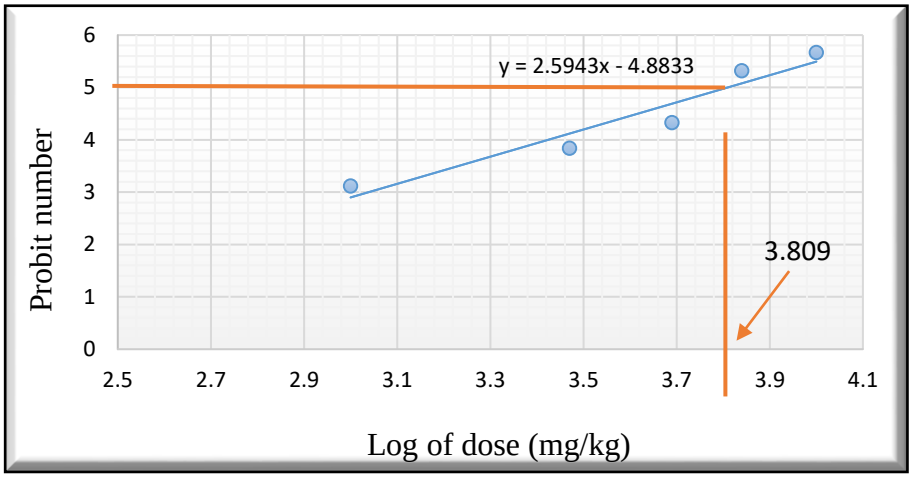
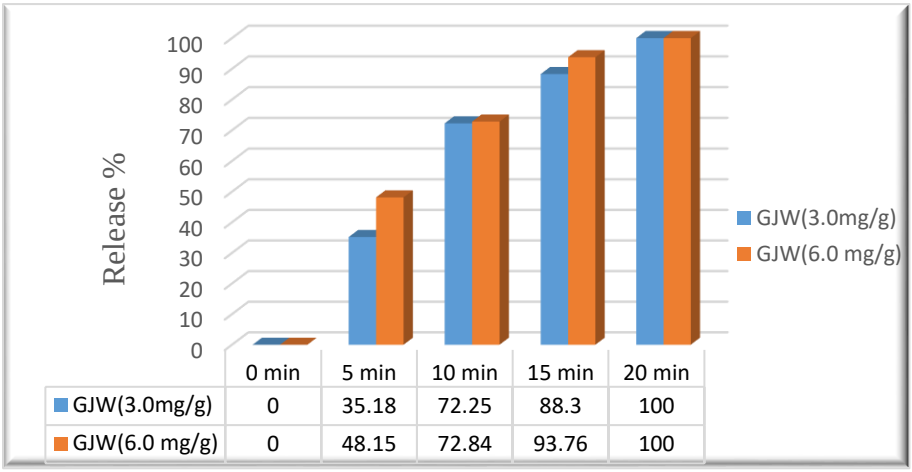


Figure (5): Curve of median lethal dose (LD50) by GJW extract (LD50 =6442 mg/kg of body weight).

Extract was applied in the industry of chewing gum, and the following testes were performed on the resulting gum. Fig(6) show the rate of release of phenols compounds found in the chewing gum to the phosphate buffer solution, which represents saliva in the manner of estimating phenols compounds. The results of the current study showed that the phenols release to the solution increased by crushing time of the chewing gum samples. The phenols release rate were 35.18 and 48.15% after 5 min of use of GJW extract at 3.0 and 6.0 mg/g respectively. The tested formulations released approximately 100% of their active agents after 20 min.



	0 min	5 min	10 min	15 min	20 min
GJW(3.0mg/g)	0	35.18	72.25	88.3	100
GJW(6.0 mg/g)	0	48.15	72.84	93.76	100

Figure (6): Invitro release of phenols from chewing gum formulations in pH 6.8 phosphate buffer at 37˚C with various concentration of GJW extract.

In general, the results showed that the sample of chewing gum with higher concentration of extract had a higher phenols release rate than the sample with a lower concentration. This may be due to increased concentration of phenols polymerized in the high concentration of the extract. Phenols are either small molecules such as phenolic acids or high polymerization compounds such as tannins (37). The drug-polymer ratio was found to affect the drug release characteristics of the prepared chewing gums, at higher drug-polymer ratio, the drug release from the chewing gum was faster as compared to lower drugpolymer ratio, this was because the high drug-polymer ratio promotes the increase saliva uptake and lead to the greater solubilization of the drug present in the polymer matrix causing faster diffusion of drug through the gum base (38).

Shows the results of the statistical analysis of the organoleptic properties of the chewing gum, which are treated with different concentrations of the GJW extract compared with the control (without adding the extract). The results showed no significant differences ($p< 0.05$) for the chewing gum samples added to the extracts of GJW at concentrations (6.0 and 3.0 mg/g) compared to the treatment of the control in the qualities of chewing gum volume, softness, hardness, no adherence and persistence of taste. Fig (7) shows the resulting chewing gum forms for GJW extract treatment with control.

Figure (7): Experimental chewing gums produced.

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