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Identification of glucosyl transferase inhibitors from *Psidium guajava* against *Streptococcus mutans* in dental caries

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ABSTRACT

Dental caries is a multi factorial disease that starts with microbiological shifts affected by salivary flow, composition, exposure to fluoride, consumption of dietary sugars, and preventive behaviours. The *Streptococcus mutans* (*S. mutans*) is an initiator of caries because there is a variety of a virulence factor unique to the bacterium that has been isolated and plays an important role in caries formation. The aim of the present study is to identify the beneficial effect of bioactive compounds in *Psidium guajava* (*P. guajava*) and its inhibitory role against *S. mutans* in dental caries. The methanolic extract was used for analysis of GC-MS for the identification of bioactive compounds. The results confirm the existence of 7 different compounds. The identified bioactive compounds were corynan-17-ol, 18,19-didehydro-10-methyoxy-acetate, Copaene, 3Bicyclo(5.2.0)nonane, 2-methylene-4,8,8-trimethyl-4-vinyl,Azulene,1,2,3a,4,5,6,7-octahydro-1,4-dimethyl-7-methylethenyl) [1R- (1a,3aa′,4a′,7a′)], α-Caryophyllene, Alloaromadendrene oxide-(1) and Androstan-17-one, 3-ethyl-3-hydroxy-, (5a). The saliva of dental caries during and after treatment of aqueous leaf extract was used for the analysis of bacterial load and determining the activity of Glucosyl transferase (GTF). The result obtained at different time intervals, showed significant decrease ($P < 0.01$) in the bacterial load of saliva on *P. guajava* treatment. The molecular docking studies identified the interaction between GTF and the bioactive compounds of *P. guajava*. The anticariogenic active compounds interacted through active sites of sucrose and inhibit the formation of glucan. The study suggested that it could be maximized the anticariogenic effect of the selected medicinal plant, and further focus is needed to identify the combined plant extract to explore the additional protection against dental caries.

1. Introduction

Dental caries is an infectious disease caused by the interaction of specific bacteria with the constituents of the diet in a host system. The formation of dental biofilm facilitates the attachment of microbial communities on the surface of the tooth. The disease becomes chronic as a result of the breakdown of biofilm homeostasis, and is related to frequent sugar exposure and a low biofilm pH[1]. The factors include salivary, dietary, and genetic, cause complex interactions among the microbial species attaching to the tooth surface of dental caries. The interactions of metabolic microbes that take place in the dental biofilm result in acid production and extracellular glucan formation which promotes microbial attachment to the teeth.[2] The major causative microorganism in the oral microbiota includes the bacterial species *Streptococcus mutans* (*S. mutans*) which became the main focus of caries research, assumed to the specific cariogen.[3]

Streptococcus mutans are gram positive bacteria that are the major cariogenic organisms with their ability to produce large quantities of glucans as well as acid, altering the salivary buffering capacities, so that the bacteria have the advantage to outcompete noncariogenic commensal species at low pH environments.[4–6] The bacteria can survive in an acid environment and its metabolic pathway in the oral cavity is a major step in the pathogenesis of *S. mutans*. Further, *S. mutans* accumulates with other microbial species, leading to the multiplication and spreading into other area of oral mucosa and altered by the action of specific genes and signalling molecules. Glucans are polymers of glucose units

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synthesised from sucrose by the enzymatic action of glucosyl transferase (GTF). They serve as a matrix for the biofilm by promoting bacterial adherence, bacterial accumulation, provide structural carcass to the biofilm matrix.[7–10] In the later stage, the microbial biofilm enters a steady state which changes the equilibrium balance of the oral ecology resulting, the bacteria to access into the deeper tissues, ultimately causing disturbance of hydroxyapatite crystals in enamel and dentin which result in cavitations within the tooth.[2] Prevention and control of this cavitations results in ecological changes, where microorganisms protect themselves by biofilm, enabling caries to progress gradually.[1]

Phytochemicals have been focused for their ability to chemically control dental biofilm, to explore the antibacterial effects, including tea, red grape, green grape, and cocoa extracts, on *S. mutans*. Recently most studies of bioactive compounds against oral pathogens attempt to verify their antibacterial properties and mechanisms of action *in vitro*.[11] Guava (Scientific name: *Psidium guajava;* Common name: Apple guava) have good potential effects against various health problems such as cough, fever, diarrhoea, constipation, bad breath, gum problems and numerous other problems. Guava leaf extracts are used for the treatment of the disorders related with liver, digestive system, heart, kidney and intestine. In further guava is used to treat chronic diseases such as diabetes and pulmonary disease.[12] Guava is considered as a good source of dietary fibre, protein, calcium, phosphorus, potassium, copper, iron, vitamin A, vitamin B_1, vitamin C, vitamin B_2, vitamin B_3 and folic acid.[13] It is rich in antioxidant and protects cell damage. Guava plant leaves are considered as a natural pain reliever. The studies have proved the benefits of guava leaves in controlling blood pressure, lowering cholesterol, battling diabetes, combating cancer and protecting prostrate.[11] Leaf and bark extracts associated with flavonoids such as morin glycosides, quercetin and quercetin glycosides act against wide range of gram + ve and gram − ve human pathogens including *Escherichia coli, Vibrio cholera, Giardia lamblia* and *Shigella* species as well as *Staphylococcus aureus* and *Pseudomonas aeruginosa*.[12] Previous studies identified that Guava leaf extracts possess potential effect against the growth of *Staphylococcus mutans*.[14,15] So far, no studies have been undertaken to explore the anticariogenic activity of phytochemicals in the medicinal plant *P. guajava,* and it has become imperative to investigate and examine the potentials of the plant against cariogenic *S. mutans*.

2. Materials and methods

2.1. Collection of plant material

The fresh leaves of *P. guajava* were randomly collected from different mature plants growing in and around Mohamed Sathak College, Chennai, Tamil nadu, India. The collected leaves were cleaned with running water and cut into small pieces, shade dried at room temperature and pulverized. The coarse powder was stored in air tight plastic container.

2.2. Preparation of extract

The pulverized powder was soaked in the solvents of water, ethanol, methanol, diethyl ether and acetone (10 g of dried powder in 100 mL); (SRL chemicals-AR grade) and extracted for 24 h at room temperature kept in orbital shaker at 150 rpm. The extracts were separated from the residues by filtering through Whatmann No:1 filter paper. The residues were extracted twice with the same solvent. The combined extracts were decanted into pre weighed glass vials. Filtrates of the extracts were concentrated and freed of solvent under reduced pressure at 40 °C. The crude extract thus obtained was stored in an air-tight container and used for further analysis. The extracts were subjected to the qualitative phytochemical analysis, GC-MS analysis, and antibacterial activity.

2.3. Qualitative analysis on phytochemical constituents

The presence of bioactive compound was tested by the qualitative analysis using different solvent extracts. The extracts were screened for alkaloids, carotenoids, flavanoids, saponins, phenolic compounds, tannins, steroids and glycosides by the standard methods.[16,17]

2.4. GC-MS analysis of Methanolic extract of P. guajava

GC-MS analysis was carried out on a GC clarus 500 Perkin Elmer system (USA) comprising a AOC-20i auto sampler and gas chromatograph interfaced to a mass spectrometer instrument employing the following conditions: column Elite-1 fused silica capillary column (30 × 0.25 mm ID × 1μ Mdf, composed of 100% Dimethyl polysiloxane), operating in electron impact mode at 70 eV; Helium gas (99.999%) was used as carrier gas at a constant flow of 1 mL/min and an injection volume of 0.5 μl was employed (split ratio of 10:1) injector temperature 250 °C, ion-source temperature 280 °C. The oven temperature was programmed from 110 °C (isothermal for 2 min), with an increase of 10 °C/min, to 200 °C, then 5 °C/min to 280 °C, ending with a 9 min isothermal at 280 °C. Mass spectra were taken at 70 eV; a scan interval of 0.5 s and fragments from 40 to 450 Da. Total GC running time is 36 min. The relative percentage amount of each component was calculated by comparing its average peak area to the total areas. TurboMass Ver 5.2.0 software was employed to handle mass spectra and chromatograms.[18]

2.5. Collection of saliva sample

Human saliva sample was collected from normal and dental caries subjects (N = 10) who were within the age group of 30–50 years including both sexes and free from other diseases and drug usage. Non-stimulated whole saliva was collected in the morning between 8 and 9a.m. No oral hygiene was allowed before collection. Approximately 3 mL of saliva were collected from each participant in sterile plastic tubes. All volunteers were fully informed on the study purposes and signed an informed consent form.

1 g of fresh *P. guajava* leaves was allowed to be chewed by the persons who were affected with dental caries daily for a period of 30 days. The saliva was collected at the interval of 10 days, (0th, 10th, 20th, 30th days) and further used for the identification of the bacterial load.

2.6. In vitro antibacterial activity of P. guajava

2.6.1. Culturing of S. mutans

(i) Preparation of Brain Heart Infusion (BHI) Broth: 4.5 g of BHI broth powder (SRL chemicals-AR grade) was dissolved in 125 mL of distilled water and heated for 5 min, distributed as 5 mL in the test tube, sterilized under standard pressure, temperature and cooled gently. The pointed top of the bacterial culture ampoule was carefully removed and added about

0.3–0.4 mL of BHI liquid medium to make suspension of the culture. Avoid frothing and aerosols. 0.5 mL of suspension was added to 5 mL of BHI broth taken in a test tube and incubated at 37 °C for 24 h.[19]

(ii) Preparation of BHI Agar: About 4.5 g of BHI agar was dissolved in 100 mL of distilled water and boiled for 5 min. 24 h grown culture from the broth was inoculated in the plate by streak method. The inoculated plate was incubated at 37 °C for 24 h. The grown culture was sub cultured as slants and maintained carefully for further purposes.[20]

2.6.2. Measurement of bacterial count

0.5 mL of saliva was collected from normal persons and dental caries persons during different time interval, was used in liquid broth (5 mL) and agar plates for culturing the bacteria *S. mutan* by incubation at 37 °C for 24 h. The bacterial growth was measured by the following methods. All the analysis was carried out in triplicates.

2.6.2.1. Turbidity measurement. The growth of bacteria from saliva sample was measured by their turbidity at 540 nm optical density in colorimeter.

2.6.2.2. Counting in Neubauer chamber. A drop of liquid culture was stained with methylene blue, diluted with diluting fluid using WBC pipette, and counted in Neubauer chamber under light microscope, the total number of cells was calculated applying the following formula.

$$\text{Number of Bacteria} = \frac{\text{Bacteria Counted} \times \text{Dilution factor} \times \text{Chamber Depth}}{\text{Area at Chamber counted}}$$

$$\text{Number of Bacteria} = \frac{\text{Bacteria Counted} \times 20 \times 10}{4}$$

2.6.2.3. Counting in agar plate. Bacterial growth in agar plates was counted by dividing the plates as four divisions and multiplied with four and the overall count is calculated.

2.7. Assay the activity of glucosyl transferase

About 3 mL of sodium acetate buffer (100 mM; pH 6.2), from 0.5 mL saliva, 5% sucrose was added with 1.5 mL of crude bacterial enzyme, again 0.5 mL of potassium phosphate buffer was reacted with the above mixture. The crude mixture was incubated at 37 °C for 2 h. The water insoluble glucan was separated by centrifugation at 10,000*g* for 5 min and it was quantitated by phenol sulphuric acid method.[21] The activity of GTF was calculated by the formula

$$\text{GTF Activity} = \frac{\mu\text{g of Glucon liberated/ml}}{\text{Molecular Weight of Glucon}} \times \frac{1000}{\text{Incubation time}}$$

The activity of GTF was expressed in terms of IU/L. The analysis of GTF activity was carried out in triplicates.

2.8. Molecular docking studies

2.8.1. Preparation of protein

The three dimensional structure of GTF was retrieved from RCSB protein data bank. Its PDB code is 3AIB. The possible binding sites for 3AIB were searched using 3D LIGANDSITE an online tool. The binding sites which are more flexible were selected for this study.[22]

2.8.2. Preparation of ligand

The seven different compounds obtained in GC-MS analysis and the primary substrate of GTF, sucrose was used as ligands. The ligands were saved in mol 2 format. The OPEN BABEL software (www.vcclab.org/lab/babel/start.htmL) was used to convert mol format to pdb format. Rapid virtual screenings of these compounds were performed in the docking tool iGEMDOCK v2.0. A population size of 200 is set with 70 generation and one solution for quick docking.[22]

2.8.3. Protein-ligand docking

iGEMDOCK is used for the integrated virtual screening through post-screening analysis with pharmacological interactions. First, iGEMDOCK provides interactive interfaces to prepare both the binding site of the target protein and the screening compound library. Each compound in the library is then docked into the binding site by using the in-house docking tool GEMDOCK.[23]

2.8.4. Post docking analysis

iGEMDOCK generates protein-compound interaction profiles of electrostatic, hydrogen-bonding and Vander Waals interactions. Based on these profiles and compound structures, iGEMDOCK infers the pharmacological interactions and clusters the screening compounds for the post-screening analysis. Finally, iGEMDOCK ranks and visualizes the screening compounds by combining the pharmacological interactions and energy-based scoring function of GEMDOCK. After the completion of the docking the post docking analysis was performed to find the docking pose and its energy values.

2.9. Statistical analysis

All data were analyzed with SPSS 12 student software. Hypothesis testing methods included two tailed student's *t*-test. The values were expressed as mean with standard deviation (SD). P-values of less than 0.05 were considered to be statistically significant.

Table 1
Phytochemical analysis of *P. guajava* leaves.

S. No.	Phytochemicals	Water	Acetone	Ethanol	Ether	Methanol
1.	Carbohydrates	+	−	+	−	+
2.	Fatty Acids	−	−	−	−	−
3.	Proteins	−	+	−	+	−
4.	Amino Acids	−	+	−	+	−
5.	Saponins	−	−	−	−	+
6.	Tannins	+	−	+	−	+
7.	Carotenoids	+	−	+	−	−
8.	Flavonoids	+	+	+	−	+
9.	Alkaloids	−	+	+	−	+
10.	Glycosides	−	−	−	+	+
11.	Polyphenols	+	+	+	−	+
12.	Sterols	+	−	+	−	+

+ Present, − Absent.

3. Results

3.1. Phytochemical analysis of P. guajava

The qualitative analysis of phytochemicals has been listed in Table 1. The results indicate the presence of carbohydrates, proteins and amino acids. Additionally carotenoids, flavonoids, alkaloids, polyphenols, saponins, tannins, glycosides and sterols were also present.

3.2. GC-MS analysis of P. guajava

The phytochemical analysis of the aqueous and organic extracts indicates that the availability of various bioactive compounds can be effectively extracted by the solvents used. On comparing solvents, organic solvents are found to be the better candidate for the extraction of phytochemicals. Among the organic solvents methanol shows effective extraction of various phytochemicals such as polyphenols, flavanoids, alkaloids and glycosides. Further the methanol extract was analyzed under GC-MS and the chromatogram showed that the available bioactive phytochemicals can be separated within 40 min (Fig. 1).

The results of GC-MS chromatogram indicate the presence of seven different compounds. The detectable compounds were identified with the retention time 6.17 (peak 1), 18.8 (peak 2), 19.38 (peak 3), 19.85 (peak 4), 21.22 (peak 5), 23.87 (peak 6) and 25.60 (peak 7). Of all the peaks, peak 2 was considered as the major peak due to its higher concentration and its relative percentage.

The seven different major and minor peaks identified from the methanolic extract were further subjected to GC-MS analysis for their structural characterization and analysis. The GC-MS peaks of methanolic extract of *P. guajava* are indicated in Fig. 2A–G.

The identified peak in GC-MS analysis confirms the presence of the compounds as corynan-17-ol, 18,19-didehydro-10-methyoxy-acetate (ester) (Compound 1–8.95%), Copaene (Compound 2–40.30%), Bicyclo(5.2.0)nonane, 2-methylene-4,8,8-trimethyl-4-vinyl (Compound 3–11.94%), Azulene,1,2,3a,4,5,6,7-octahydro-1,4-dimethyl-7-(methylethenyl)-,[1R- (1a,3aa′,4a′,7a′)] (Compound 4–10.45%), α-Caryophyllene (Compound 5–7.46%), Alloaromadendrene oxide-(1) (Compound 6–13.46%) and Androstan-17-one, 3-ethyl-3-hydroxy-, (5a) (Compound 7–7.46%).

The methanolic extract of *P. guajava* is rich phytochemical constituents which in turn resulted in identification of 7 different compounds by GC-MS analysis. The components retention time (RT), molecular formula, molecular weight (MW) concentration (%) and structure are presented in the following Table 2.

3.3. In vitro antibacterial activity of P. guajava

To identify the growth inhibitory effects of the leaf *P. guajava*, the bacterial count was carried out using the saliva sample collected at different time interval, during and after treatment. The bacterial count were measured by its turbidity in colorimeter and given in Table 3. The results clearly shows that the turbidity of the bacterial growth was significantly decreased in the saliva collected at the end of 30th day by the continuous treatment with the guava leaves, indicating the bacterial growth inhibitory action of *P. guajava* compared with 0 day. The broth culture was further diluted and counted under light microscope using Neubauer chamber and listed in Table 4. As the earlier result showed decreased turbidity on treatment with *P. guajava*, the bacterial counting of the present findings was also correlated with that. The numbers of cells were statistically reduced on the treatment of *P. guajava* within 20 days of treatment, which indicates the bacterial multiplication was effectively arrested by some of the bioactive compounds existing in the selected medicinal plant *P. guajava.* Further to identify the growth inhibitory effects of *P. guajava,* the bacterial count was also carried out in the cultured agar plates

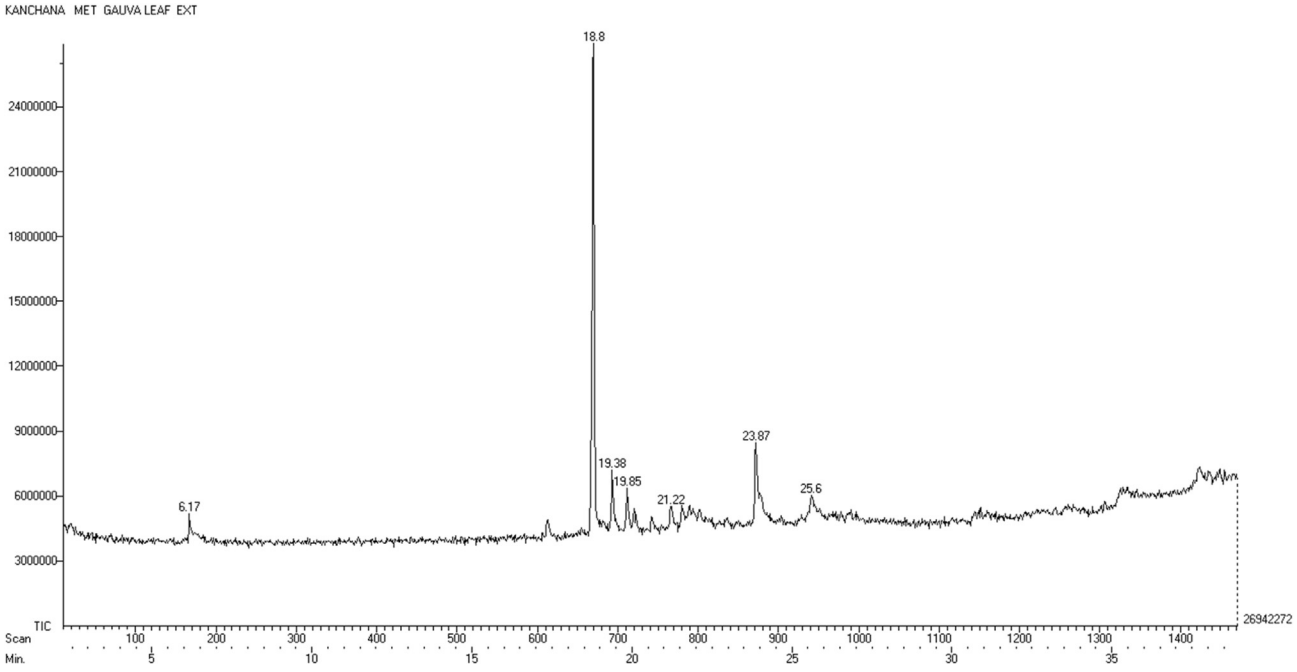
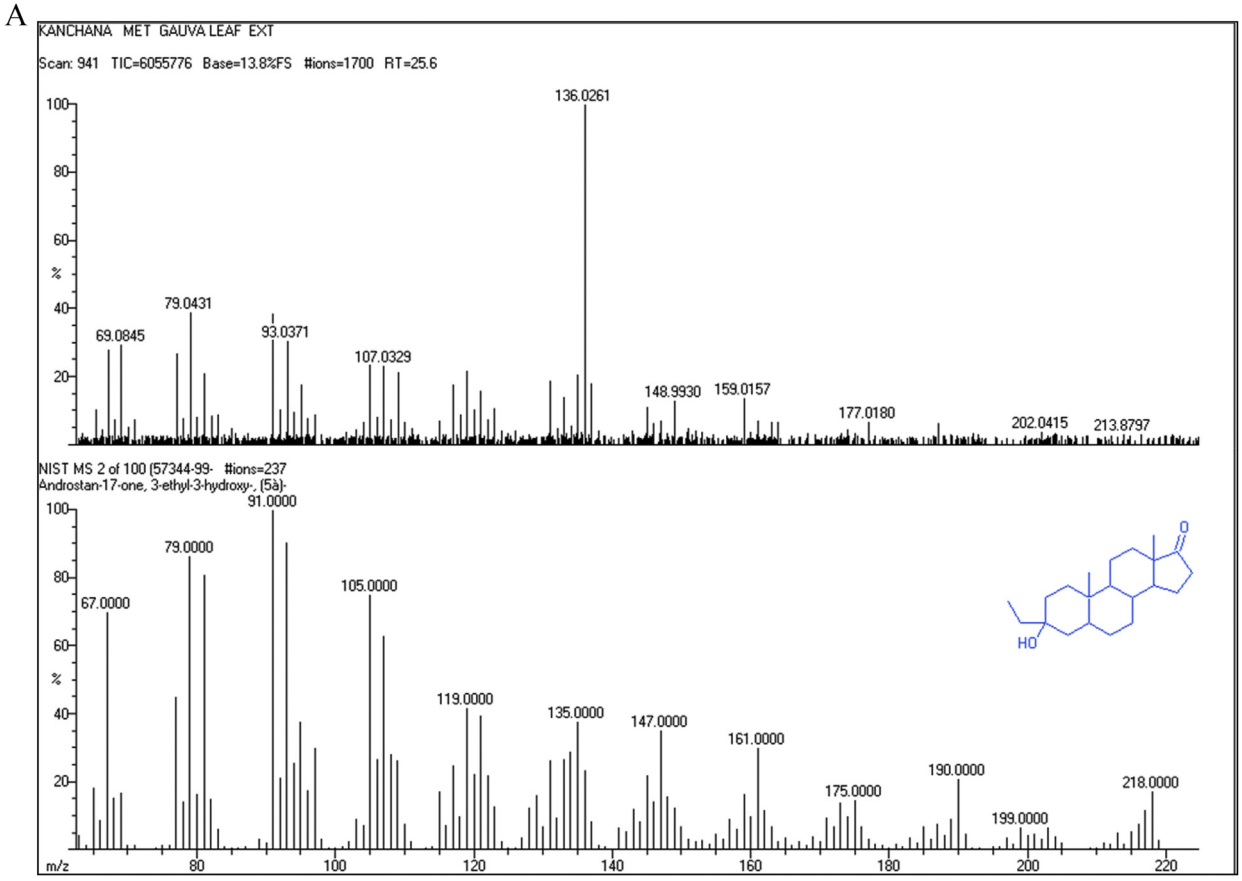


Fig. 1. GC-MS Analysis of methanolic extract of *Psidium guajava.*



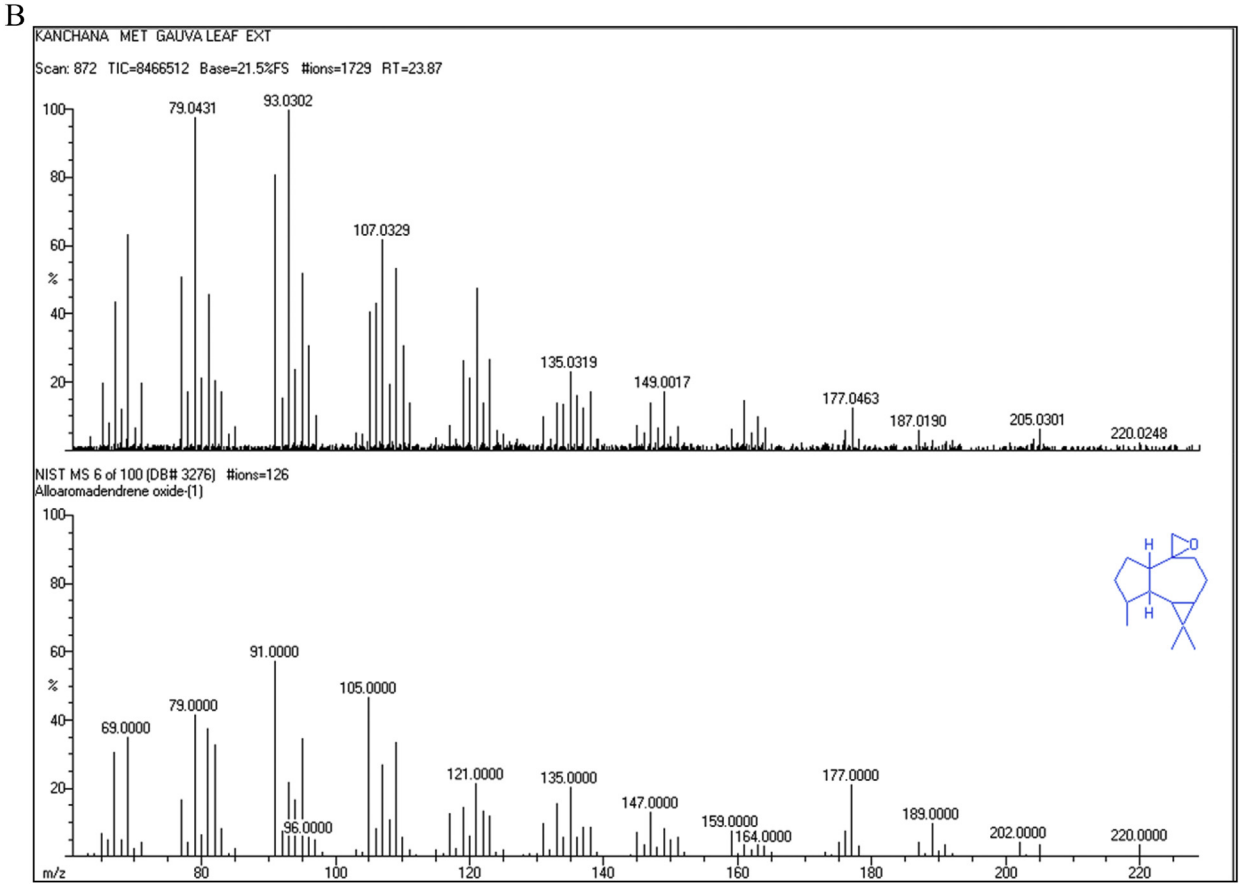
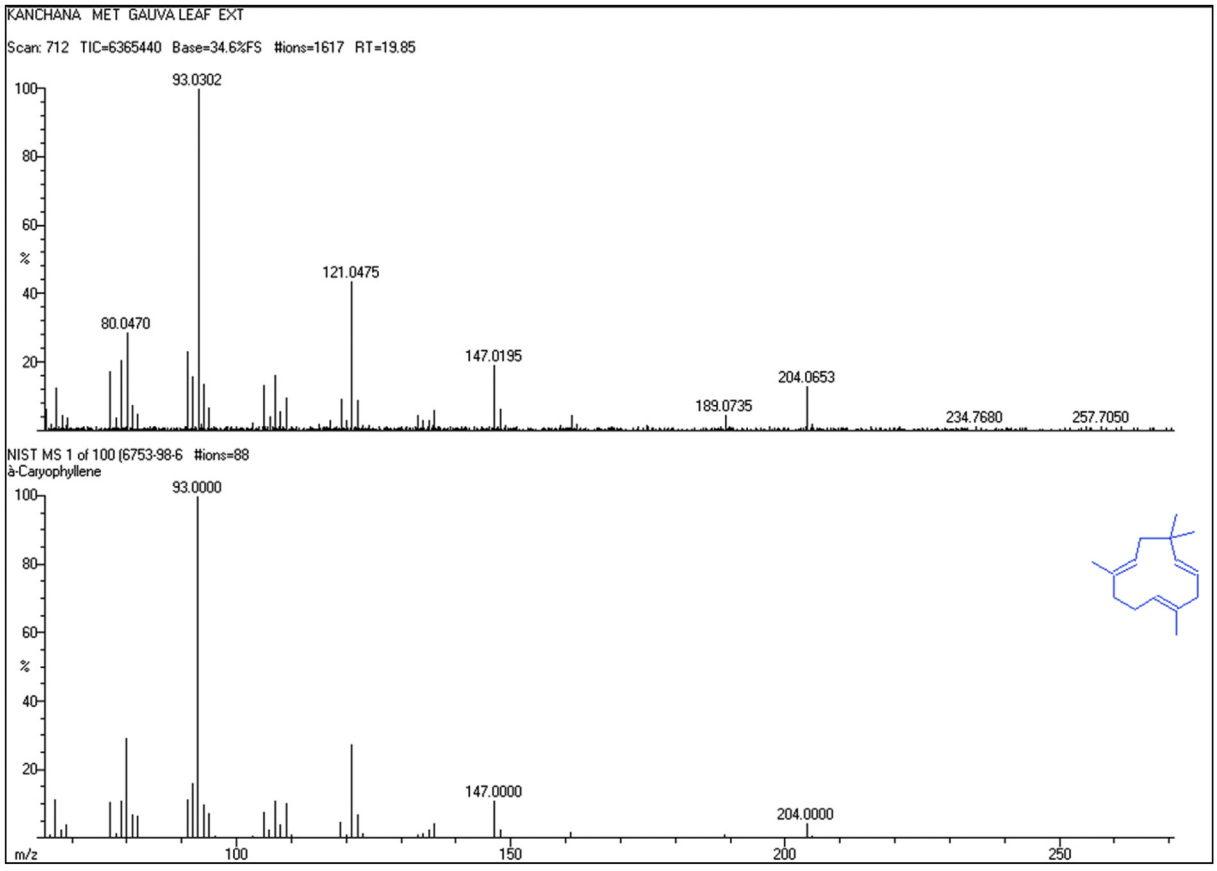


Fig. 2. A: Corynan-17-ol, 18,19-didehydro-10-methyoxy-acetate (ester), **B**: Copaene, **C**: Bicyclo(5.2.0)nonane, 2-mthylene-4,8,8-trimethyl-4-vinyl, **D**: Azulene,1,2,3a,4,5,6,7-octahydro-1,4-dimethyl-7-(methylethenyl)-,[1R- (1a,3aa′,4a′,7a′)], **E**: α-Caryophyllene, F: Alloaromadendrene oxide-(1), **G**: Androstan-17-one, 3-ethyl-3-hydroxy-, (5a).

C



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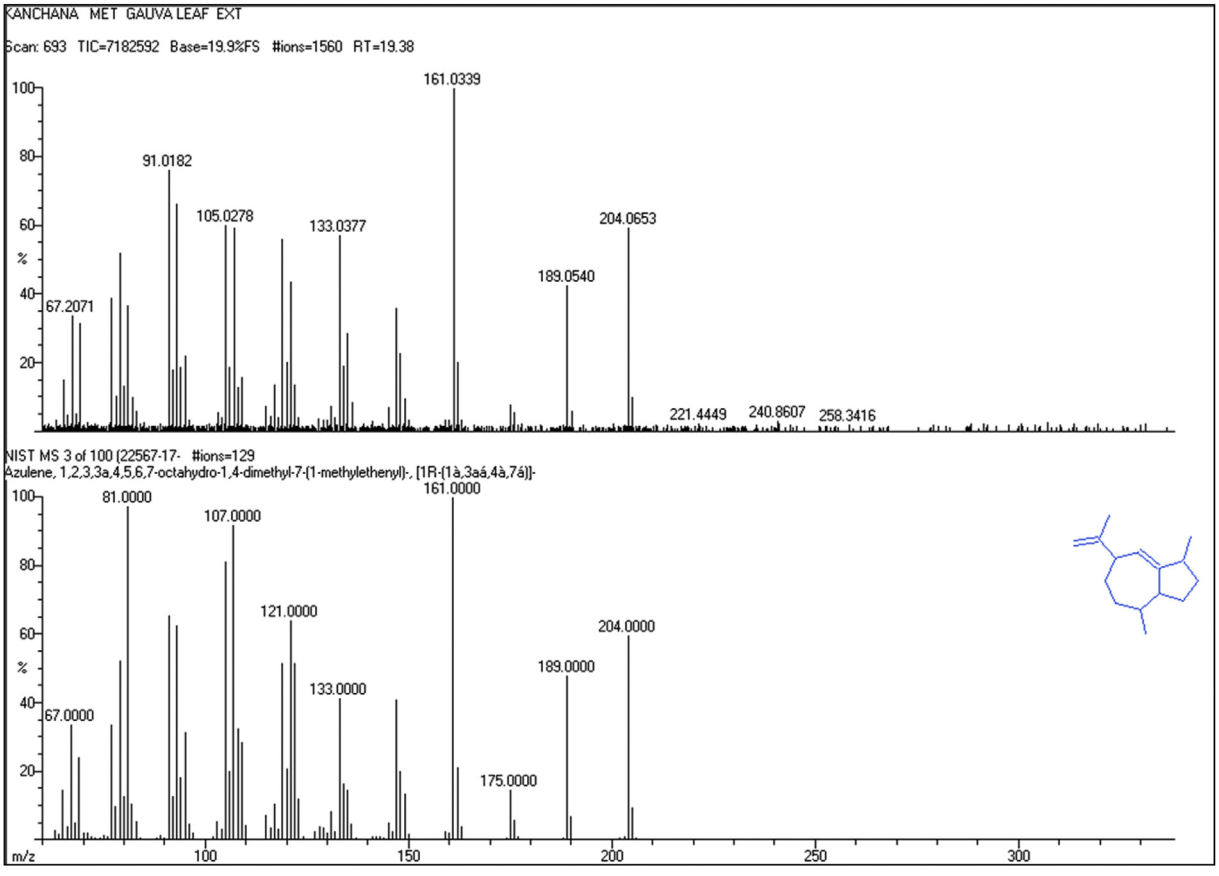
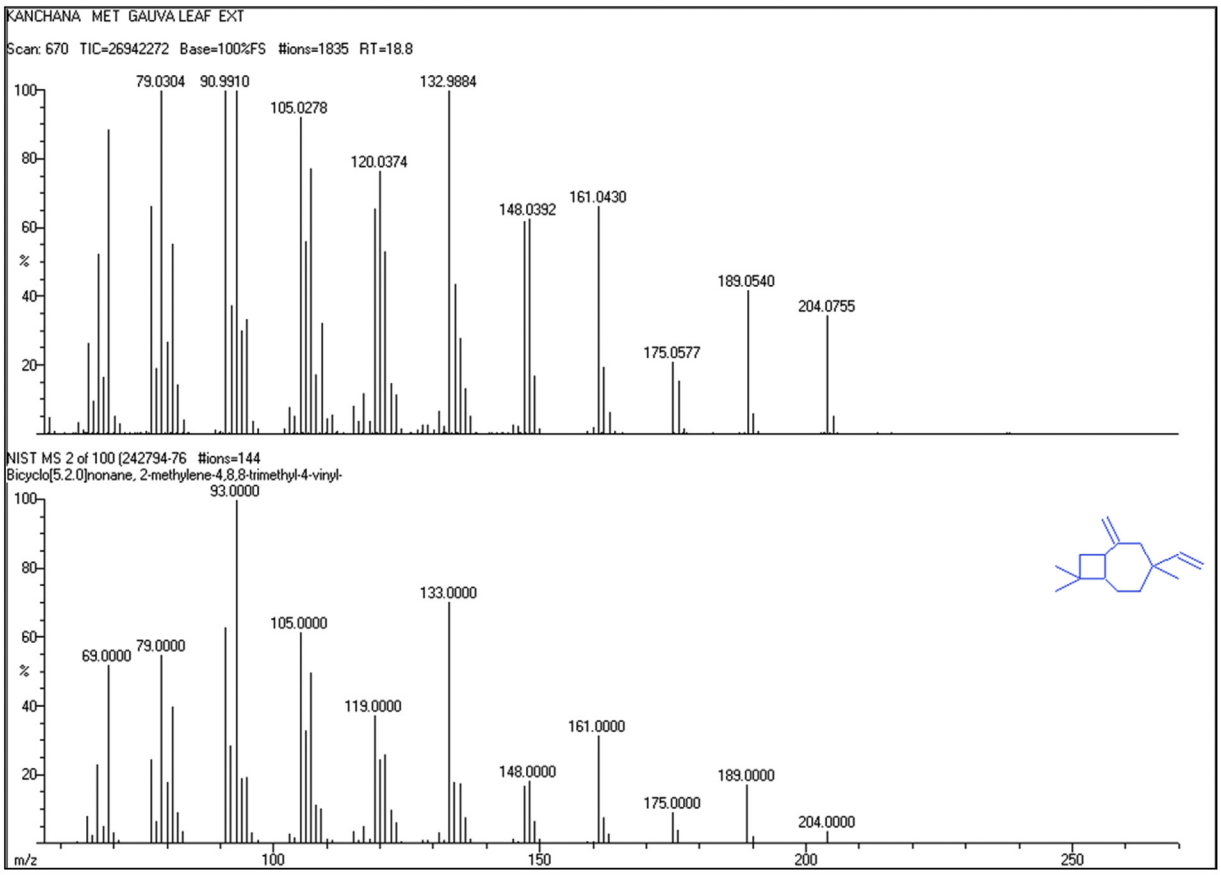


Fig. 2. (*continued*).

E



F

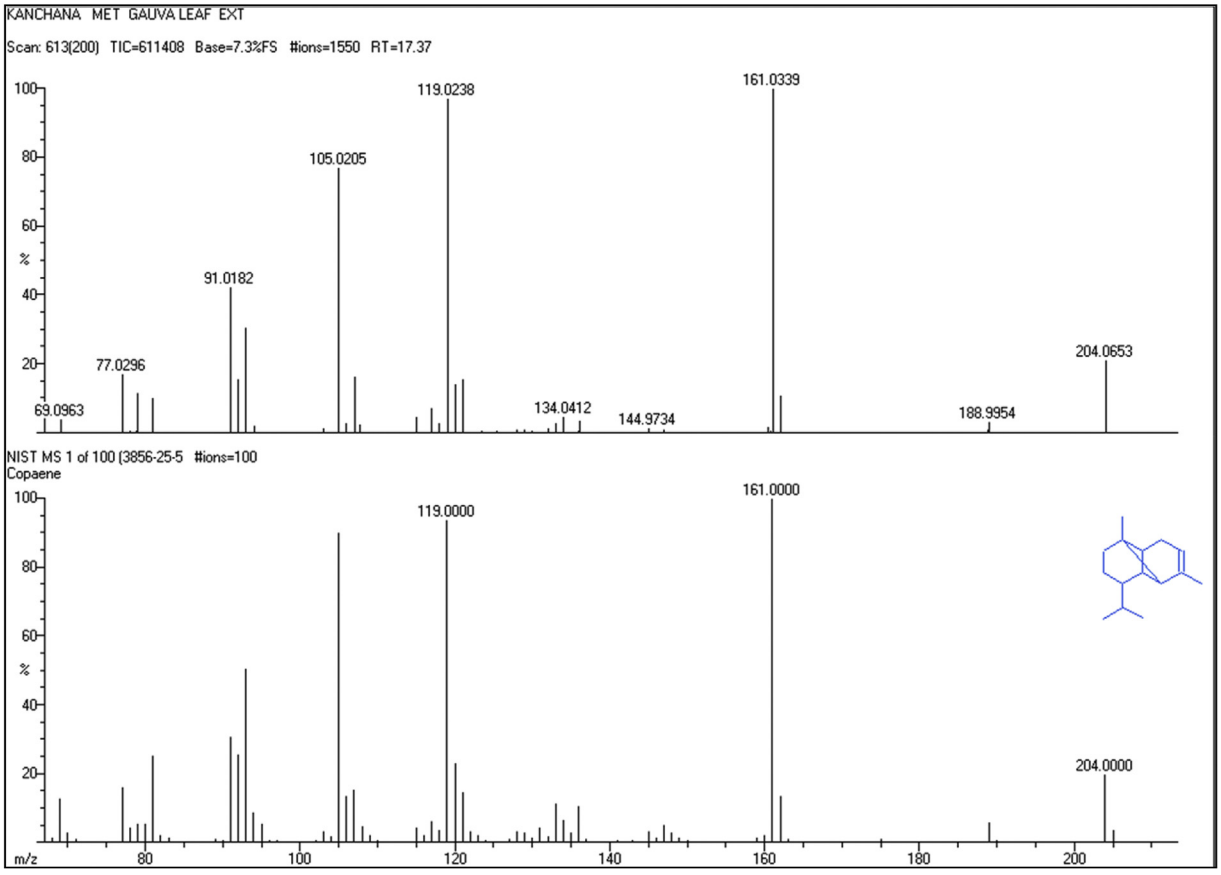


Fig. 2. (*continued*).

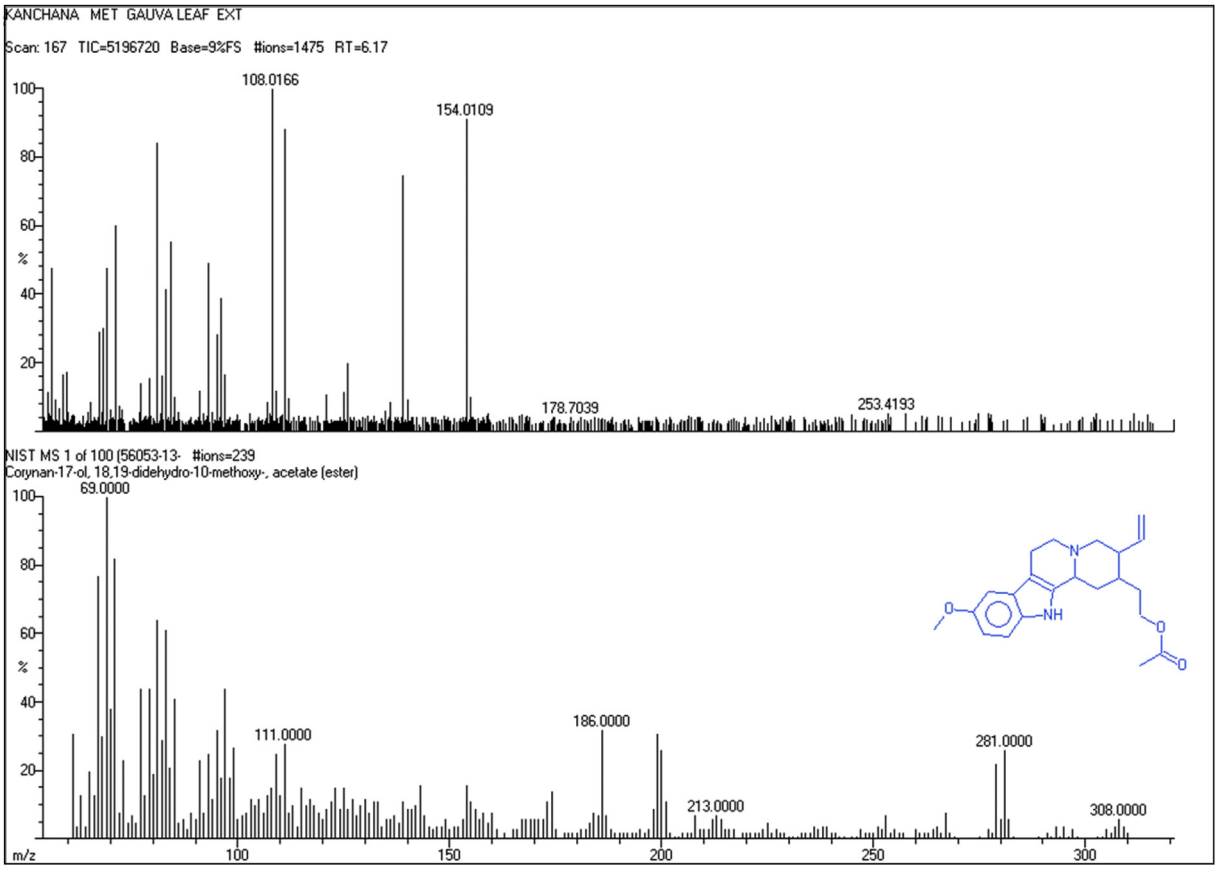


Fig. 2. (*continued*).

(Table 5; Plate 1). The bacterial counting results clearly indicate the significant reduction of the number of bacteria by the action of bioactive compounds present in the leaves *P. guajava.* This reduction was showed by the total number of bacterial cells counted in agar plates in saliva samples collected at different time interval on continuous treatment of *P. guajava* leaves.

3.4. Activity of glucosyl transferase

The present study was further focused to know about the antibacterial activity of *P. guajava,* on preventing the GTF activity and inhibition of glucan biofilm formation. So the GTF activity was also determined in the collected saliva sample at various time points during and after treatment of *P. guajava* (Fig. 3).

3.5. Molecular docking analysis

In the present study, the phytochemical existence in methanol extracts of *P. guajava* indicates that methanol is considered as a best solvent to extract bioactive compounds. The GC-MS analysis of methanolic extract shows the presence of 7 different compounds. These 7 compounds were used in molecular docking studies, to know the binding interaction with enzyme GTF. Fig. 4A and B indicates the primary structure, and the tertiary structure of GTF.

The primary structure of glucosyl transferase was identified from protein data bank (PDB ID: 3AIB). The enzyme constitutes about 844 amino acid residues. In docking analysis, the primary substrate sucrose, effectively binds to the active site of GTF by interacting through the amino acids including Thr, Ile, Gly, Gly, Thr, Gln and Tyr placed in the position 426, 427, 428, 429, 430, 553 and 978 respectively. The energy requirement was calculated which shows, overall binding needs −89.71 KCal/mol which was shared by Vander Waals forces and hydrogen interaction (−63.35 KCal/mol and −26.35 KCal/mol). The docking studies were further preceded with the seven identified ligands from the methanolic extract of *P. guajava*. The energy score estimated for each ligand has been indicated in Fig. 5, Table 6.

Docking simulation of compound 1 showed −69.76 KCal/mole binding energy. The compound 1 show Vander Waals forces and hydrogen interaction which involves the amino acids Leu, Asn, Gly and Tyr. Docking simulation of compound 2 shows −48.46 KCal/mole binding energy, includes Vander Waals force and hydrogen interaction with the involvement of amino acids Ile, Thr and Gln at the position 427, 426 and 553. Docking simulation of compound 3 showed −51.67 KCal/mole binding energy. The compound 3 showed VanderWaals forces and hydrogen interaction through the amino acids Thr and Ile. Docking simulation of compound 4 showed −52.03 KCal/mole binding energy. The compound 4 showed Vander Waals forces and hydrogen interaction. The interacted amino acids are from the position 426, 427 and 553 Tyr, Ile and Gln respectively.

Docking simulation of compound 5 showed −51.21 KCal/mole binding energy. The compound 5 showed Vander Waals forces and hydrogen interaction with the involvement of amino acids Ile and

Table 2
GC-MS Analysis of Methanolic extract of *P. guajava* leaves.

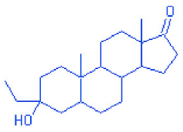
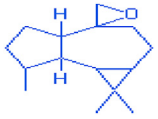
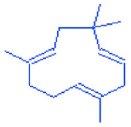
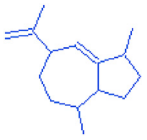
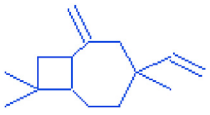
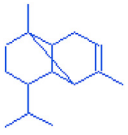
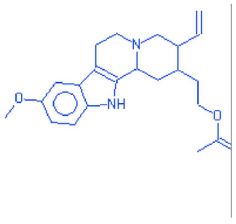
Compound	Retention Time	Peak Area (%)	Name of the Compound	Molecular Formula	Molecular Weight	Structure
1.	6.17	8.95	Corynan-17-ol, 18,19-didehydro-10-methyoxy-acetate (ester)	$C_{22}H_{28}N_2O_3$	368.46932	
2.	17.37	40.30	Copaene	$C_{15}H_{24}$	220.35046	
3.	18.8	11.94	Bicyclo(5.2.0)nonane, 2-mthylene-4,8,8-trimethyl-4-vinyl	$C_{15}H_{24}$	204.35106	
4.	19.38	10.45	Azulene,1,2,3a,4,5,6,7-octahydro-1,4-dimethyl-7-(methylethenyl)-,[1R- (1a,3aa′,4a′,7a′)]	$C_{15}H_{24}$	204.27732	
5.	19.85	7.46	a-Caryophyllene	$C_{15}H_{24}$	204.27391	
6.	23.87	13.43	Alloaromadendrene oxide-(1)	$C_{15}H_{24}O$	204.91892	
7.	25.6	7.46	Androstan-17-one, 3-ethyl-3-hydroxy-, (5a)	$C_{21}H_{34}O_2$	308.29838	

Table 3
Antibacterial activity of *P. guajava* in saliva samples (Turbidity measurement).

Saliva sample	Cell Turbidity (OD_{540}nm)			
	0 days	10 days	20 days	30 days
1.	1.21 ± 0.02	0.62 ± 0.03***	0.54 ± 0.03***	0.42 ± 0.01*
2.	1.40 ± 0.02	0.56 ± 0.01*	0.41 ± 0.02*	0.40 ± 0.02***
3.	1.07 ± 0.02	0.74 ± 0.03***	0.45 ± 0.01***	0.37 ± 0.02***
4.	1.64 ± 0.02	0.72 ± 0.02*	0.54 ± 0.02**	0.41 ± 0.02*
5.	1.05 ± 0.01	0.85 ± 0.02***	0.67 ± 0.01***	0.52 ± 0.03*
6.	0.85 ± 0.03	0.73 ± 0.03*	0.44 ± 0.02***	0.30 ± 0.02***
7.	0.87 ± 0.02	0.75 ± 0.02*	0.42 ± 0.03***	0.38 ± 0.01***
8.	0.99 ± 0.02	0.72 ± 0.02***	0.53 ± 0.03***	0.45 ± 0.01***
9.	1.43 ± 0.02	0.96 ± 0.02*	0.74 ± 0.03***	0.60 ± 0.02*
10.	1.43 ± 0.03	1.03 ± 0.03***	0.84 ± 0.02***	0.66 ± 0.02*

The values are mean ± SD (n = 3).
***P < 0.001, **P < 0.01, *P < 0.05 – Statistically Significant compared with 0 day treatment.

Table 4
Antibacterial activity of *P. guajava* in saliva samples (Bacterial count – Liquid Broth).

Saliva sample	Number of Cells × 10^3/mL			
	0 days	10 days	20 days	30 days
1.	9.4 ± 0.2	6.4 ± 0.1***	1.6 ± 0.1**	1.5 ± 0.1***
2.	6.3 ± 0.1	3.1 ± 0.1*	1.6 ± 0.2*	1.5 ± 0.2***
3.	9.6 ± 0.1	6.3 ± 0.1***	3.6 ± 0.1***	2.5 ± 0.3***
4.	12.0 ± 2.0	9.4 ± 0.2***	4.5 ± 0.3**	3.2 ± 0.2*
5.	6.3 ± 0.1	3.2 ± 0.2***	2.3 ± 0.2**	1.7 ± 0.2*
6.	9.5 ± 0.1	6.5 ± 0.1*	2.4 ± 0.2*	1.7 ± 0.2**
7.	9.4 ± 0.2	6.3 ± 0.1*	2.3 ± 0.2***	1.6 ± 0.2**
8.	6.5 ± 0.1	3.5 ± 0.3***	2.4 ± 0.3**	1.2 ± 0.2*
9.	6.2 ± 0.2	3.2 ± 0.3***	1.3 ± 0.3**	1.2 ± 0.2***
10.	9.5 ± 0.2	6.4 ± 0.1*	1.5 ± 0.1***	1.5 ± 0.3**

The values are mean ± SD (n = 3).
***P < 0.001, **P < 0.01, *P < 0.05 – Statistically Significant compared with 0 day treatment.

Table 5
Antibacterial activity of *P. guajava* in saliva samples. (Bacterial count − Agar plates).

Saliva samples	Number of Bacteria/500 μl			
	0 days	10 days	20 days	30 days
1.	283 ± 18	192 ± 9**	145 ± 4**	86 ± 3**
2.	295 ± 19	196 ± 10**	153 ± 2**	82 ± 4*
3.	275 ± 18	185 ± 7**	145 ± 3**	76 ± 3**
4.	285 ± 18	197 ± 12**	157 ± 4*	93 ± 3**
5.	265 ± 10	181 ± 11**	131 ± 2**	66 ± 4**
6.	255 ± 18	185 ± 10*	137 ± 3*	75 ± 3**
7.	280 ± 14	197 ± 12***	142 ± 4***	86 ± 4***
8.	270 ± 15	190 ± 9***	137 ± 3**	90 ± 3**
9.	260 ± 18	176 ± 11**	123 ± 3*	86 ± 3**
10.	255 ± 17	190 ± 11*	106 ± 3**	94 ± 3*

The values are mean ± SD (n = 3).
***P < 0.001, **P < 0.01, *P < 0.05 − Statistically Significant compared with 0 day treatment.

Gln. Docking simulation of compound 6 showed −53.16 KCal/mole binding energy. The compound 6 showed Vander waals forces and hydrogen interaction. In the docking studies no electrostatic interactions were exhibited by all the six compounds. Compound 7 did not show any binding interactions with GTF.

The residues Gly 415 and Tyr 978 of GTF models present in the hydrophilic domain (−OH and =O groups) was found to have molecular interactions with high binding affinities of −5.6 KCal/Mol and −14.7 KCal/Mol, by compound 1 than other compounds. The binding energy of docked ligands confirms with experimentally observed inhibitory activities, indicating binding energy calculated from docking studies are reliable.

From the docking studies compound 1 is selected as a best compound based on number of highest H-bonds and binding energy. Among the compounds analyzed, docking showed the highest binding energy amongst the various lead compounds binding energy. So finally the best lead compounds were chosen depending on their binding energy and number of H-bonds interaction with 3D structure of the GTF as shown in Fig. 6. The active site is made up of 10 residues Leu 406, Asn 412, Asn 407, Gly 415, Thr 426, Ile 427, Gly 428, Thr 545, Gln 553 and Tyr 978. Templates were judged as per their 0, mZ score and top hits were considered for further evaluation. According to that, the best binding with lowest binding energy of the compounds was ordered as 1,6,4,3,5 and 2.

In the molecular docking studies the overall binding energy of bioactive compounds is comparatively less than sucrose on GTF. To elaborate the amino acid involvement in the interaction the results indicate that there were a variety of amino acids Ile, Gly, Gln, Tyr directly involved in the bonding interaction of bioactive compounds. In the helix, the backbone of GTF Ile 427 established an inter H bond with the backbone carbonyl oxygen, keeping the helical secondary structure stable. The ligand binding packet in the present model is more compact, surrounded mainly by the other residues. The side chains were pointing towards binding packet,

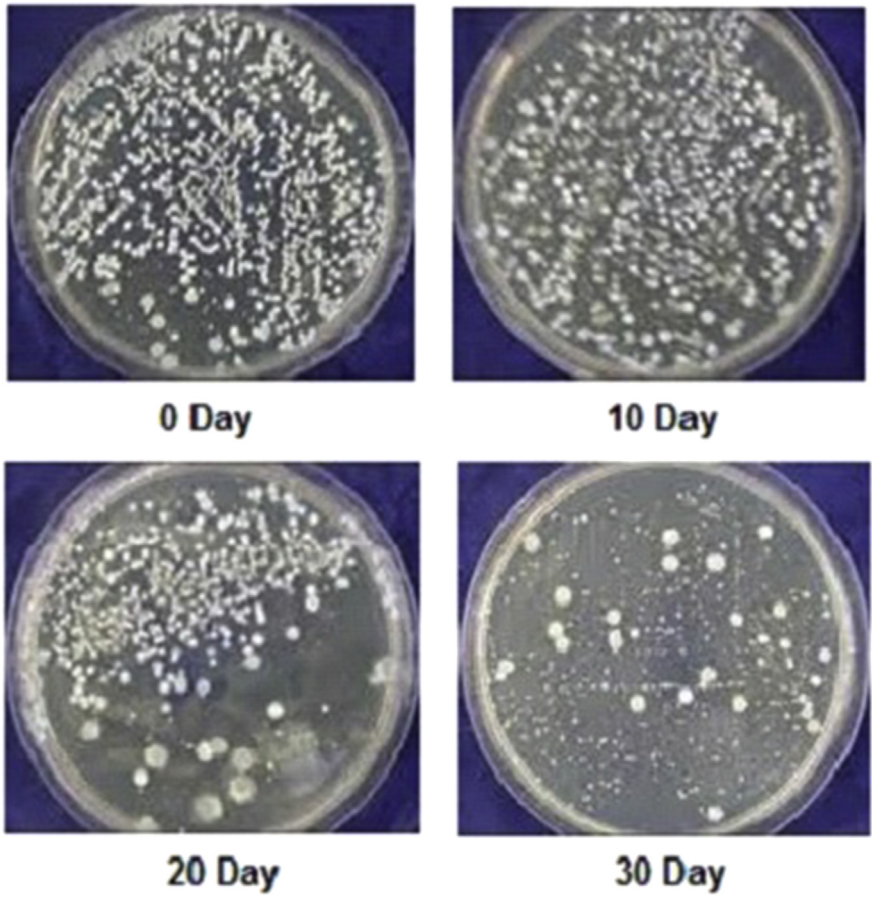


Plate 1. Antibacterial activity of *P. guajava* in saliva samples (Bacterial count−Agar plates).

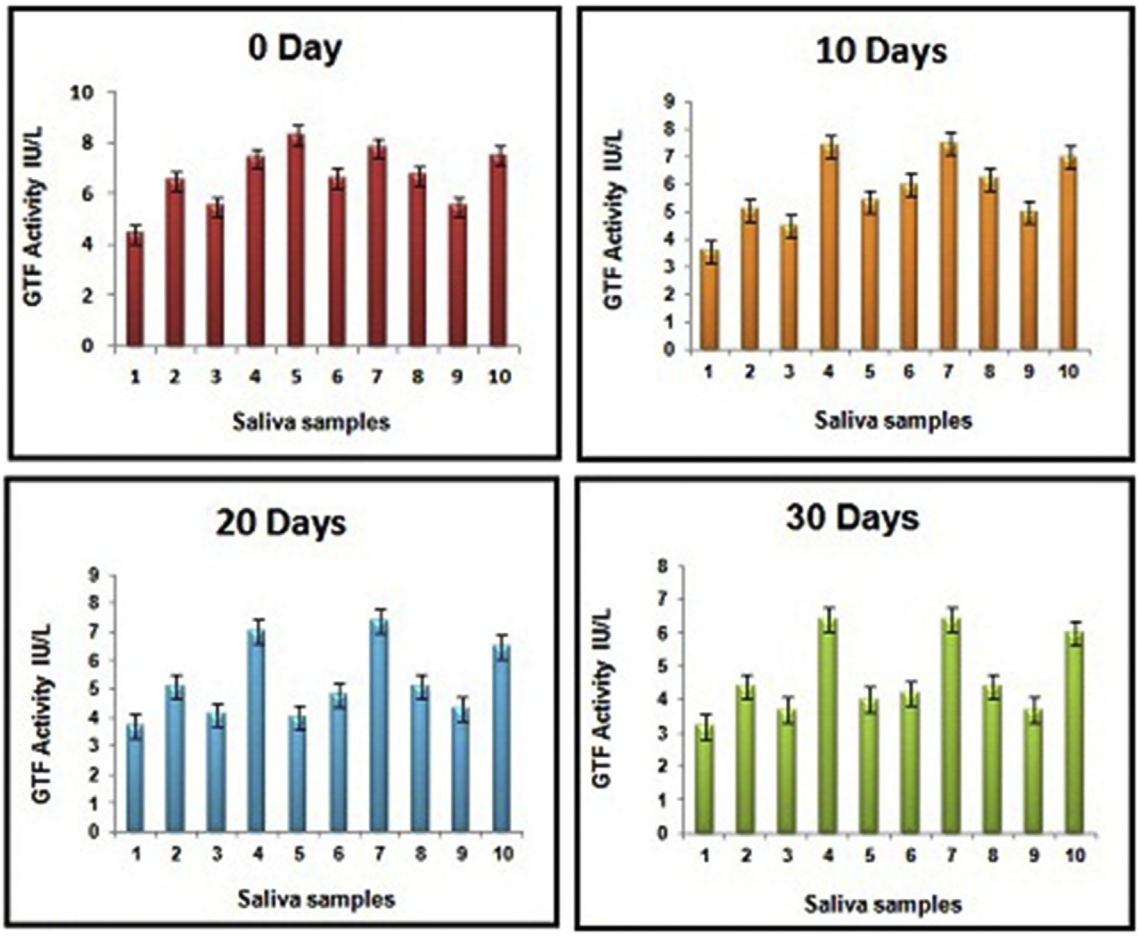


Fig. 3. Effect of *P. guajava* on GTF activity in saliva samples. The values are mean ± SD (n = 3).

indicating structurally their direct involvement. Additional hydrogen bonding interactions were noted between the carboxylate group of bioactive compounds and Gln 553 and Tyr 978 of GTF. In these interactions Thr, Ile, Gln and Tyr in the position 426, 427, 553 and 978 were directly involved in the bonding interaction of sucrose and the bioactive compounds with GTF. These findings clearly indicate that the phytochemicals isolated from *P. guajava* also interacts through the active centre of sucrose in GTF, and interferes with the binding of sucrose. Through that these compounds inhibits the formation of glucan which is the ultimate cariogen. Present investigation clearly shows that these six residues were in close proximity neither to the substrate nor to any other residue essential for the activity.

The overall result of present investigation clearly demonstrates medicinal potentiality of *P. guajava* leaves by its effective role against the cariogenic pathogen *S. mutans.*

4. Discussion

The present findings of phytochemicals in *P. guajava* confirms the existence of bioactive components especially tannins, alkaloids, polyphenols and flavanoids which are known to support the bioactivities of the selected medical plant and these are considered as very important elements responsible for the antibacterial activities of *P. guajava* leaf extract against pathogenic microbes. Tannins in general have good activity against bacterial growth, because these compounds contain titanic acid. These act against the bacteria through the ability of these compounds to dissolve the fatty layer of bacterial wall that causes leakage of cell fluid out of the cell and destroys it. Polyphenols show antimicrobial activity against human pathogens. Polyphenols act directly against microorganisms and by inhibiting virulence factors. Polyphenols act synergistically in combination with antibiotics against resistant pathogens.[24,25] Similar studies conducted by Buvaneswari and group,[26] proved that *P. guajava* leaves have long been recognized for their antibacterial activity, stating that the plant was rich in alkaloids, flavonoids, phenols and tannins exist as individual or in combination are responsible for their antibacterial activity.

The chemical compositions of *P. guajava* leaves were investigated using GC-MS analysis, while the mass spectra of the compounds found in the extract was matched by the National Institute of Standards and Technology (NIST) library. GC-MS analysis of ethanolic extract of *P. guajava* leaves revealed the existence of Alpha - bisabolol, 1, 2- Benzenedicarboxylic acid, buty, Hexadeca-2, 6, 10, 14-tetraen, Caryophyllene, Bis (2-ethylhexyl) phthalate, Nerolidol and Germacrene.[27] The GC-MS chromatogram performed by Okenwa and Abii[28] on isopropanol extract of the leaves of *P. guajava* showed fourteen peaks from the chromatogram of the extract.

A

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>3AIB:A|PDBID|CHAIN|SEQUENCE
SFAQYNQVYSTDAANFEHVDHYLTAESWYRPKYILKDGKTWTQSTEKDFR
PLLMTWWPDQETQRQYVNYMNAQLGIHQTYNTATSPLQLNLAAQTIQTKI
EEKITAEKNTNWLRQTISAFVKTQSAWNSDSEKPFDDHLQKGALLYSNNS
KLTSQANSNYRILNRTPTNQTGKKDPRYTADRTIGGYEFLLANDVDNSNP
VVQAEQLNWLHFLMNFGNIYANDPDANFDSIRVDAVDNVDADLLQIAGDY
LKAAKGIHKNDKAANDHLSILEAWSYNDTPYLHDDGDNMINMDNRLRLSL
LYSLAKPLNQRSGMNPLITNSLVNRTDDNAETAAVPSYSFIRAHDSEVQD
LIRDIIKAEINPNVVGYSFTMEEIKKAFEIYNKDLLATEKKYTHYNTALS
YALLLTNKSSVPRVYYGDMFTDDGQYMAHKTINYEAIETLLKARIKYVSG
GQAMRNQQVGNSEIITSVRYGKGALKATDTGDRITRTSGVVVIEGNNPSL
RLKASDRVVVNMGAAHKNQAYRPLLLTTDNGIKAYHSDQEAAGLVRYTND
RGELIFTAADIKGYANPQVSGYLGVWVPVGAAADQDVRVAASTAPSTDGK
SVHQNAALDSRVMFEGFSNFQAFATKKEEYTNVVIAKNVDKFAEWGVTDF
EMAPQYVSSTDGSFLDSVIQNGYAFTDRYDLGISKPNKYGTADDLVKAIK
ALHSKGIKVMADWVPDQMYALPEKEVVTATRVDKYGTPVAGSQIKNTLYV
VDGKSSGKDQQAKYGGAFLEELQAKYPELFARKQISTGVPMDPSVKIKQW
SAKYFNGTNILGRGAGYVLKDQATNTYFSLVSDNTFLPKSLVNP
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B

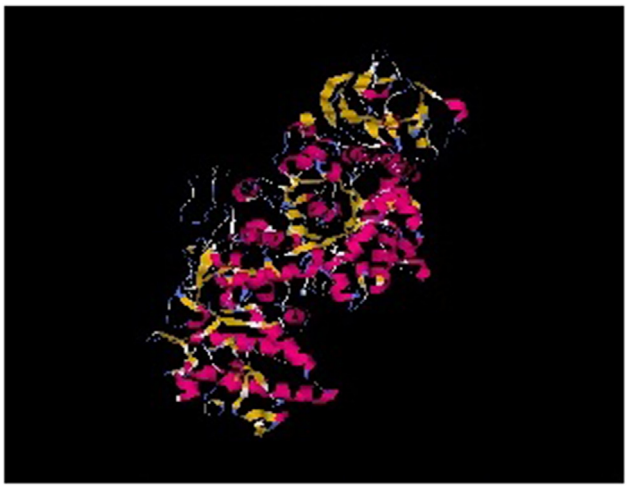

Fig. 4. A. Primary structure of GTF in FASTA format, **B**. The 3D structure of GTF viewed with Rasmol.

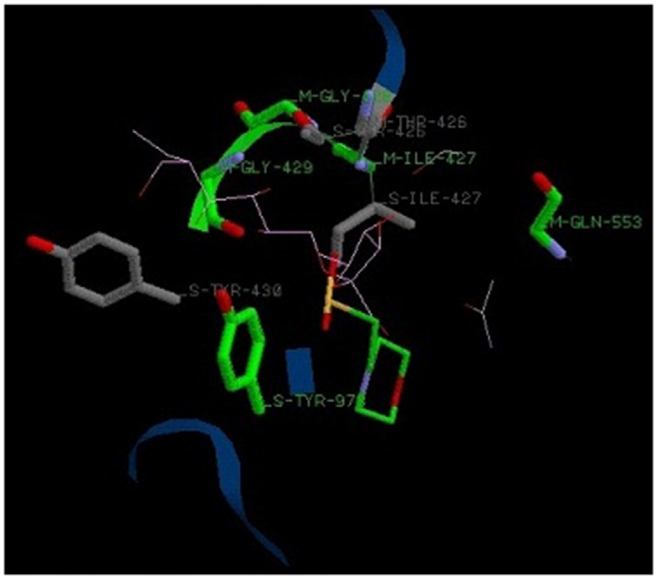

Fig. 5. Docking Poes of sucrose on GTF.

These peaks indicated the presence of fourteen compounds in the extract. This shows that in the present study the methanolic extract revealed different compounds rather than isopropanol extract. Methanol extract prevailed ester derivates and aromatic nitro compounds. The synergistic effect of these bioactive compounds in the leaves of *P. guajava* could be the reason behind its use for the treatment of dental caries.

Studies conducted by Tuney and groups[29] have proved that methanol extract have more antimicrobial activity than acetone and ethanol extracts. The reason for antibacterial activity of the methanolic extract may be due to the availability of antimicrobial compounds effectively extracted by the solvent which was analyzed by GC-MS analysis and in further mechanism of action is to be identified. The overall results of phytochemical existence and the antibacterial action of *P. guajava* clearly stated that there is some active mechanism behind, in the growth inhibition of bacteria by phytochemicals present in *P. guajava* against the formation of dental caries. The molecular mechanism should be further identified, through which the potential benefit of the medicinal plant could be achieved. The present findings were supported by previous antimicrobial study conducted by Shyla Jebashree and groups with different plant extracts includes *P. guajava* against *S. mutans*.[30]

The study shows the bacterial GTF activity was found to be decreased by the concurrent oral treatment of the leaf. While correlating with the bacterial load and the GTF activity, the overall results indicates both are dependent. This suggests that GTF activity is directly involved in the production of glucan based biofilm in the oral cavity that in turn facilitates to increase the microbial biomass. Similarly decreased GTF activity was also observed in the previous report studied using crude extracts of *P. guajava* and *Piper betle*.[31,32]

The present findings are in agreement with the previous work reported by Duarte and group.[33] However, it has been shown that the cell surface *S. mutans* is reduced in the presence of the plant extract. The previous studies, on *in vitro* evaluation of the anti-cariogenic potential against *S. mutans* proves that various phytochemical compounds are used to prevent the formation of dental bacterial plaque and are validated to be best ligand to inhibit the glucosyl transferase activity.[34]

In the docking simulation technique was used to preliminarily investigate the mode of interaction of these bioactive compounds with the active site of GTF. The findings clearly indicate that the phytochemicals isolated from *P. guajava* also interacts through the active centre of sucrose in GTF, and interferes with the binding of sucrose. Through that these compounds inhibits the formation of glucan which is the ultimate cariogen. Present investigation clearly shows that these six residues were in close proximity neither to the substrate nor to any other residue essential for the activity. The overall result of present investigation clearly demonstrates medicinal potentiality of *P. guajava* leaves by its effective action against the cariogenic pathogen *S. mutans.*

To conclude the results of the present study, there is a need to design a drug candidate against the protein target glucosyl transferase in order to inhibit the hazardous effect of *S. mutans* in host organism. The compounds isolated from *P. guajava* have the capability to interact and inhibit the activity of GTF which in turn reduces the oral infections. It could be a promising potential drug for oral infections using GTF site as a drug target. Therefore, inhibition of GTF activity is considered as an important mechanism in the drug discovery.

Table 6
Post Docking analysis of Glucosyl transferase inhibitors.

Substrate/ Compounds	IUPAC Name	Total Energy (Kcal/mol)	Vander Waals Interaction (Kcal/mol)	Hydrogen Bond (Kcal/mol)	Amino acid
Substrate	Sucrose	−89.71	−63.35	−26.35	Thr - 426, Ile - 427, Gly − 428, Gly − 429, Thr − 430, Gln − 553, Tyr - 978,
Compound 1	(Corynan-17-ol, 18,19-didehydro-10-methyoxy-acetate (ester))	−69.76	−63.76	−6.0	Leu - 406, Asn - 412, Asn - 407, Gly - 415, Ilu - 427, Tyr - 978, Gln - 553.
Compound 2	(Copaene)	−48.46	−45.97	−2.49	Thr - 426, Ilu - 427, Gln - 553.
Compound 3	3 (Bicyclo(5.2.0)nonane, 2-mthylene-4,8,8-trimethyl-4-vinyl)	−51.67	−51.67	0	Thr - 426, Ilu - 427, Tyr - 978, Tyr - 545, Gln - 553.
Compound 4	(Azulene,1,2,3a,4,5,6,7-octahydro-1,4-dimethyl-7-(methylethenyl)-,[1R- (1a,3aa′,4a′,7a′)])	−52.03	−52.03	0	Thr - 426, Ile - 427, Tyr - 978, Gln - 553.
Compound 5	(a-Caryophyllene)	−51.21	−51.21	0	Tyr - 426, Ile - 427, Tyr - 978, Gln − 553.
Compound 6	(Alloaromadendrene oxide-(1))	−53.16	−53.16	0	Thr - 426, Ile - 427, Tyr - 978, Tyr - 545, Gln - 553.

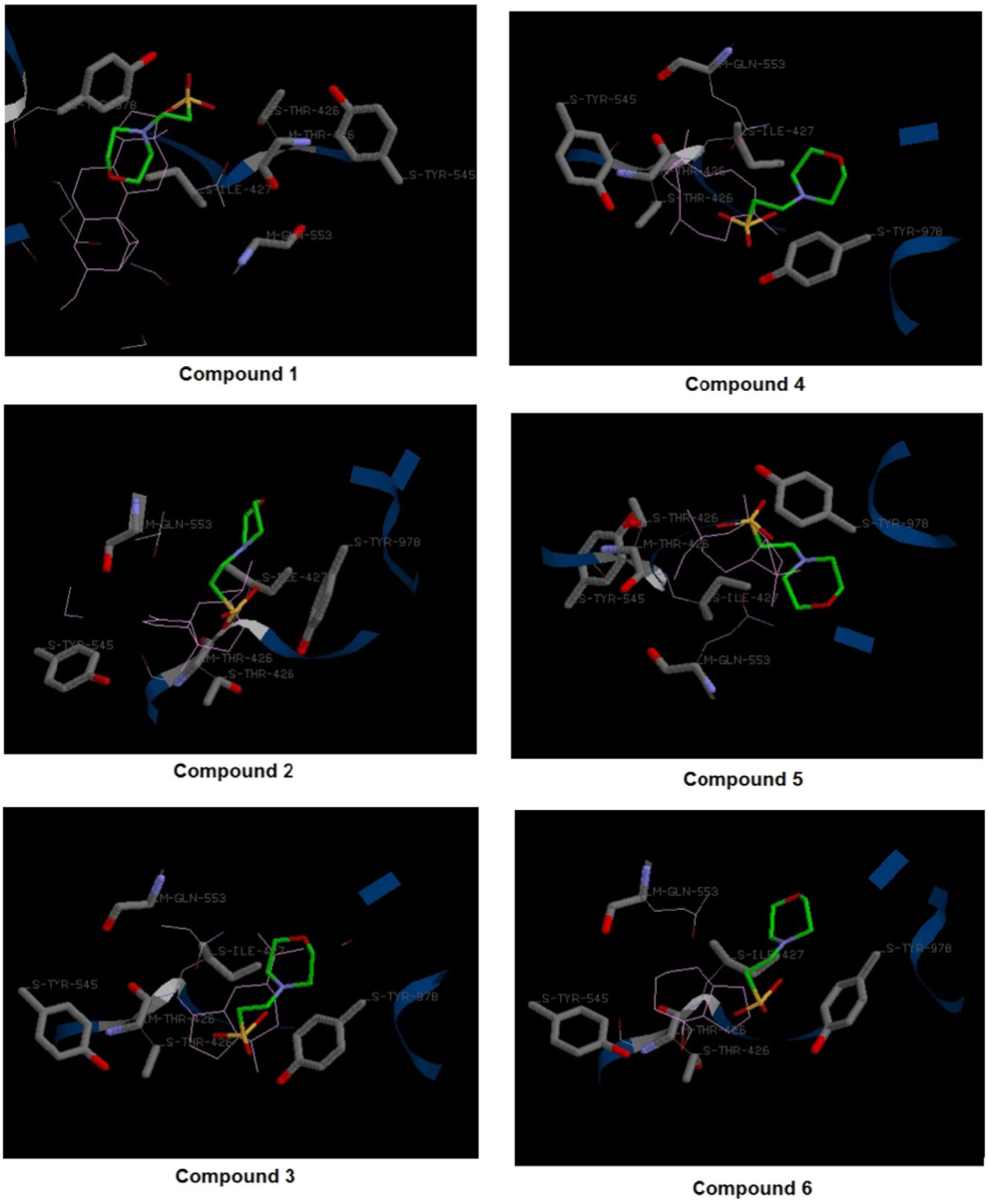

Fig. 6. Docking Poes of Glucosyl transferase inhibitors.

Conflicts of interest

The authors do not have any conflict of interest.

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