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Effect of Selected Plant Extracts on Plaque Associated Bacterial Isolates and LC-MS/MS based Characterization of their Bioactive Compounds

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Oral isolates, antibacterial activity, column chromatography, LCMS/MS, Naringin.

ABSTRACT: Oral health is an integral part of well-being and plants have a huge contribution in the maintenance of oral health and hygiene. We report in this work for the first time, the potent antibacterial activity of methanolic leaf extract of *Citrus maxima* against the plaque associated bacterial isolates and have identified the bioactive compound responsible for its activity. In this study, four plants *Saraca asoca (Sc), Cassia tora (Ct), Ricinus communis (Rc)* and *Citrus maxima (Cm)* claiming their traditional use in maintaining oral hygiene were subjected to Soxhlet extraction using ethyl acetate and methanol solvents. Four bacterial strains namely, *Bacillus paramycoides, Pseudomonas aeruginosa, Staphylococcus epidermidis* and *Enterobacter hormaechei* were isolated and identified from the patients with visible plaque. Isolates were tested for their antibiotic sensitivity using Hexa discs and all were found to be sensitive to Gentamicin at 10mcg, showing an average inhibition of 30mm inhibition zone. The antibacterial activity of the plant extracts was screened against the four oral isolates by agar well diffusion method at 100 mg/ml and 25mg/ml concentration where *Cm* was found to be effective against all the four oral isolates with an average zone of inhibition of 10mm at 25mg/ml concentration. In order to identify the bioactive compound responsible for its activity, *Cm* leaf extract was subjected to TLC, Column chromatography and the eluted fractions were screened for their antibacterial potential against the isolates at 10mg/ml concentration wherein the second fraction was found to be effective and hence was subjected to LC-MS/MS analysis to identify the bioactive compound. The bioactive compound was identified as Naringin, belonging to the class of flavonoids.

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INTRODUCTION

Oral health is the reflection of our total body health. The human microbiome is a very complex yet a stable environment, harboring a diverse range of microorganisms from viruses to protozoan [1]. In spite of the presence of antibacterial agents like lysozyme and lactoperoxidase in our saliva, the oral microorganisms are able to colonize and turn pathogenic under favorable conditions [2].

Dental caries and periodontal infections are the most common dental diseases. Fermentation of the dietary carbohydrates by the oral microorganisms results in acidic end products, which cause the dissolution of the tooth enamel surface, resulting in caries commonly caused by *Streptococcus mutans* and

Lactobacilli sp. It is the plaque that is the primary etiological agent of caries. Plaque is a biofilm formed by the various inter and intra species interactions within the host, which allows the microbes to adhere and colonize on the tooth surface firmly. Most often, this biofilm is harmless, but when bacterial and environmental conditions prevent equilibrium, caries and periodontal disease followed by endodontic infection may occur [3]. Periodontal disease is characterized by the degeneration and inflammation of gums and includes gingivitis and periodontitis. The most common pathogens involved here are *Porphyromonas gingivalis, Prevotella*

intermedia, Fusiobacterium nucleatum, Tannerella forsynthus, Treponema denticola, etc. [4]. The term endodontic infection refers to the progressive untreated dental carious lesions through the dentin and into the pulp, which becomes infected and eventually dies, this involves anaerobic proteolytic bacteria [5].

Although the greatest achievements of the 20th century are credited to the development of antibiotics, its misuse has led to the dissemination of high-level tolerance to all the existing broad-spectrum antibiotics among the microbes and also the increasing use of chemicals in cosmetics, food, medicines, personal hygiene and other products have led to an irreversible damage to mankind and the environment [6]. Toxic chemicals like sodium lauryl sulfate, titanium dioxide, Triclosan, polyethylene glycol, artificial sweeteners, etc. used in our mouthwash and toothpaste can cause various adverse effects to our body from headache, allergies, kidney failure to even cancer and damage brain cells [7]. In realization with the above and with the pros of plant-based products, it has become a trend to add the prefix 'Herbal' to every product launched to the market and changing the lifestyle towards organic and herbal based products.

The knowledge of plants to maintain a healthy life and to treat an array of infections is not new to mankind [8]. Nature is our constant source of hope and plants offer various bioactive compounds in the form of secondary metabolites, which include flavonoids, phenolics, quinones, terpenoids and alkaloids, which are a rich source of therapeutic agents [9].
There are several studies on the antibacterial activity of various plant extracts however, sufficient evidence to support their active compounds and their effect on oral pathogens is limited. Hence in the current study an attempt has been made to identify the potent bioactive compound exhibiting antibacterial activity against the plaque associated bacteria. For this piece of work, we have chosen the plants that are claimed to be traditionally used for maintaining oral health and hygiene but not commercialized till date.

MATERIALS AND METHODS

Collection, preparation and extraction of the plant material

The study was initiated with the collection of fresh flowers of *Saraca asoca* (Sa) *(*Ashoka flower), leaves of *Cassia tora* (Ct) known as Sogata or sickle pod, *Ricinus communis (Rc)* the Castor oil plant and *Citrus maxima* (Cm) called as Chakkotha or Pomelo from GKVK, Bangalore, India. The plant materials were washed under clean running tap water and were then shade dried at room temperature for 4- 5 days. The dried material was later powdered using a mixer grinder and stored in air tight bottles.

Soxhlet method was employed for extraction [10, 11] using ethyl acetate and methanol solvents.

Isolation of the oral isolates

Oral samples were collected from Bowring and Lady's Curzon Hospital, Bangalore. Five patients with visible Plaque and dental caries with no exposure to antibiotics for atleast three months and with a minimum, six hours of the gap from the last brushing were selected for the study. A sterile tooth pick was used for disturbing the plaque area, The disturbed surface was then swabbed with a sterile cotton bud which was inoculated into a 10ml of sterile Trypticase Soya broth, taken in a sterile 15 ml centrifuge tubes as described by Borty *et al, 2015* with a slight modification. Soon after transfer to the laboratory, the tubes were incubated at 37º C overnight under shaker conditions. 100 µl of the overnight culture was spread plated on solidified Trypticase soya agar plate with 1% sucrose. An uninoculated control plate was also maintained. Following the incubation, for 24 h at 37º C, the plates were observed for visible colonies.

Identification of the oral isolates

The oral isolates were characterized based on cultural characteristics, staining, biochemical properties and molecular method. Gram's staining was employed for initial identification followed by the biochemical tests consisting of IMViC, catalase, oxidase, starch hydrolysis and carbohydrate fermentation [30]. We used 16S rRNA gene sequencing for molecular characterization of the oral isolates. The genomic DNA of oral isolates was extracted and the 16S rRNA was subjected for amplification in Polymerase chain reaction (PCR) using genomic DNA as a template and bacterial universal primers and sequenced.
16S Forward Primer: 5'- CDGGHCTANCAVATGCWAGTS -3'
16S Reverse Primer: 5'- GMCGGRTGKGTACHAGGY -3'
The sequences were then subjected to a homology search using BLAST against NCBI 16S ribosomal DNA sequence database. The sequences were deposited in NCBI Gen Bank for an accession number [13].

Antibiotic susceptibility test

The oral isolates were screened for their antibiotic sensitivity using Hexa discs (Himedia) impregnated with Bacitracin **(B)**, Chloramphenicol **(C)**, Penicillin G (**P**), Polymyxin B **(Pb),** Gentamycin (**G**) and Neomycin **(N)**. Kirby Bauer disc diffusion method was followed as described by Bauer *et al.,* 1966. The tests were performed in triplicates.

Screening for antibacterial activity

The ethyl acetate and methanol extracts of the collected plants were screened for their antibacterial activity using the agar well diffusion method. The wells in each plate were loaded with 20 µl of extract (100mg/ml prepared in DMSO), Gentamycin (10ug/ml), and DMSO as positive and negative controls respectively. The plates were incubated at 37° C for 24 h. All the tests were performed in triplicates. The extracts which showed activity with more than 8 mm were further screened for their activity at 25mg/ml concentration as described above [15].

Characterization of Bioactive compounds

- Thin Layer Chromatography

The phytoconstituents of the potent plant extract were separated by TLC using commercially available TLC plate [Merck] Silica gel 60F254, 7×6 cm [Merck]. The separated bands were identified under the short and long wavelength of UV light. The R_f value of each band was determined [16].

Column Chromatography

To isolate the bioactive compounds from the potent plant extract, they were fractionated using column chromatography with silica gel with a mesh size of 60- 120, as the stationary phase. The solvent mixture which showed maximum separation of phytoconstituents in TLC was used as a mobile phase following which the fractionated compounds were collected and quantified [17]. Each fraction was subjected to TLC followed by the screening of the antimicrobial potency of all the fractionated compounds [18, 19].

The bioactive compound exhibiting good activity was proceeded for further characterization.

UV-Vis Spectrophotometry

The potent fractionated compound exhibiting activity was subjected to UV-VIS spectrophotometric analysis (UV-1800 Shimadzu) [19, 20].

LC-MS/MS Analysis

The molecular mass of the compound was determined by Liquid Chromatograph Mass Spectrometer 8040 Make-Shimadzu using the instrument Agilent Series infinity rapid resolution LC system interfaced with electrospray ionization (ESI). The mass spectrometer performed different runs in positive and negative ionization modes over a mass range from 50 to 1000 M/Z at 4 GHz high-resolution mode with a scan duration of 2 spectra/s in centroid and profile mode. The analysis resulted in the determination of molecular weight of the compound extracted and purified [21].

RESULTS AND DISCUSSION

Soxhlet extraction of the plant material

The Soxhlet extraction was carried out for all the four plant materials for 6-10 cycles until the solvent in the beaker changed its colour. After evaporating the solvent, the percentage of the yield was calculated. *Ricinus communis* gave the highest yield of 43.12%, while *Cassia tora* gave the least yield of 24.64% for 50 g of the plant material.

Isolation of the oral isolates

Five oral samples incubated overnight in 10ml of sterile Trypticase Soya broth were swabbed on TSA plates and incubated at 37ºC for 24 h. 350 to 400 colonies were observed in each plate out of which four distinct colonies were identified and isolated into a pure culture. The colony characteristics of four oral isolates were recorded and were subjected to staining and biochemical characterization; the results of which are as tabulated in **Table 1**. The isolates were further identified as *Bacillus paramycoides, Pseudomonas aeruginosa, Staphylococcus epidermidis* and *Enterobacter hormaechei* by 16srRNA sequencing and by performing the BLAST similarity check. The sequences were deposited in NCBI Gen Bank to procure the accession number.

Table 1 Identification of the oral isolates by staining and biochemical characteristics

ORAL ISOLATE NO.	GRAMS STAINING	INDOLE TEST	METHYL - RED TEST	VP TEST	CITRATE TEST	OXIDASE TEST	CATALASE TEST	STARCH HYDROLYSIS
1	Gram positive bacilli	-	+	+	-	+	+	+
2	Gram negative bacilli	-	+	-	+	+	+	+
3	Gram positive cocci	-	+	-	-	-	+	-
4	Gram negative bacilli	-	+	+	-	-	+	-

Antibiotic susceptibility test

The oral isolates were screened for their sensitivity to the commonly prescribed antibiotics using the Hexa discs (Himedia). All the isolates were found to be sensitive to Gentamycin and Neomycin with the average zone of inhibition of 30mm and 25.86mm respectively, while all were found to be resistant to Penicillin G. Hence Gentamycin was used for further antibacterial activity studies as a positive control. The antibiotic sensitivity pattern against the four isolates is recorded in **Table 2**.

Screening for antibacterial activity

The flowers of *Saraca asoca*, leaves of *Cassia tora*, *Ricinus communis* and *Citrus maxima* were screened for their antibacterial activity using ethyl acetate and methanol solvents at 100mg/ml concentration. The results are as tabulated in **Table 3**; ethyl acetate flower extract of *Saraca asoca and* methanol leaf extract of *Citrus maxima* showed maximum inhibition against all the four isolates with an average zone of inhibition of 10.15mm and 13.76mm respectively. The two potent plant extracts were screened further for their antibacterial activity at 25mg/ml concentration, the results of which are tabulated in **Table 4**, in which the methanolic leaf extract of *Citrus maxima* was able to effectively inhibit the growth of all the isolates and hence was subjected to TLC and Column chromatographic techniques to separate and identify the bioactive compound responsible for its antibacterial activity.

Table 2. Antibiotic sensitivity pattern of the oral isolates against the six commercially available drugs by Kirby Bauer disc diffusion method.

Sl.No	Antibacterial drug	Concentration (µg)	Mean diameter of zone of inhibition (mm)			
			Bacillus paramycoides	*Pseudomonas aeruginosa*	*Staphylococcus epidermidis*	*Enterobacter hormaechei*
1	B	10	NA	NA	15.83+0.98	16+0.63
2	C	30	25.83+2.86	17.16+3.92	24.66+1.50	NA
3	P	10	NA	NA	NA	NA
4	Pb	300	NA	18.83+1.32	NA	19+1.26
5	G	10	31.83+1.60	27.83+1.83	29+1.09	32+1.67
6	N	30	27.83+2.04	21.16+1.32	24+1.26	30.33+1.36
7	Control	-	NA	NA	NA	NA

± Standard deviation; NA – No activity

Table 3. Antibacterial activity of the plant extracts against the oral isolates

Ethyl acetate plant extracts Zone of inhibition in mm at 100mg/ml concentration				
Plant Extracts	*Bacillus paramycoides*	*Pseudomonas aeruginosa*	*Staphylococcus epidermidis*	*Enterobacter hormaechei*
Sa	10.12± 0.26	9.9± 0.16	10.34± 0.16	10.26± 0.20
Ct	NA	NA	8.3± 0.15	NA
Rc	NA	10.5± 0.43	8.366± 0.25	10.43± 0.3
Cm	12.33± 0.12	NA	10.3± 0.28	14.3± 0.18
Positive control	16.12± 0.24	14.34± 0.14	14.55± 0.18	15.22± 0.22
Negative control	NA	NA	NA	NA
Methanol plant extracts Zone of inhibition in mm at 100mg/ml concentration				
Sa	8.23± 0.15	10.3± 0.58	NA	10.33± 0.18
Ct	8.46± 0.2	10.4± 0.36	NA	NA
Rc	10.26± 0.15	10.6 ± 0.26	12.23 ± 0.14	NA
Cm	12.26± 0.11	14.3± 0.34	14.36± 0.33	14.12± 0.11
Positive control	15.56± 0.24	14.4± 0.14	14.24± 0.18	15.36± 0.22
Negative control	NA	NA	NA	NA

± Standard deviation; NA – No activity

Table 4. Antibacterial activity of the potent plant extracts at 25mg/ml concentration against the oral isolates

Zone of inhibition in mm at 25mg/ml concentration				
Plant Extracts	*Bacillus paramycoides*	*Pseudomonas aeruginosa*	*Staphylococcus epidermidis*	*Enterobacter hormaechei*
Sa(EA)	6.8± 0.13	NA	8.4± 0.28	8.23± 0.20
Cm(M)	10.2± 0.15	12.12± 0.30	10.82± 0.23	10.43± 0.23
Positive control	16.2± 0.24	14.34± 0.14	14.44± 0.18	15.01± 0.22
Negative control	NA	NA	NA	NA

± Standard deviation; NA – No activity

Separation of the Phytoconstituents by TLC and Column chromatography

The methanolic extract of *Citrus maxima* was subjected to TLC using different solvent systems. Methanol: Chloroform (9:0.5) showed maximum separation (**Fig 1**). The TLC plates were observed under UV short, long-wavelength and under visible light. The solvent front was marked and the R_f value was calculated and tabulated in **Table 5**.

Table 5. R_f value of the methanolic leaf extract of *Citrus maxima*

Number of bands	R_f value
1.	0.478
2.	0.565
3.	0.575
4.	0.608
5.	0.652
6.	0.695
7.	0.782

Fig 1. TLC plate of *Citrus maxima* methanolic leaf extract under visible light

The column was run for 1g of *Citrus maxima* methanolic leaf extract to separate and identify the bioactive compounds responsible for its antimicrobial activity. The same solvent system used for the TLC was incorporated as the mobile phase. Five different fractions were eluted out and separated based on the colors. The fractions were further evaporated to remove the solvent in a watch glass and the dry weight of the elute was calculated which is depicted in **Fig 2**.

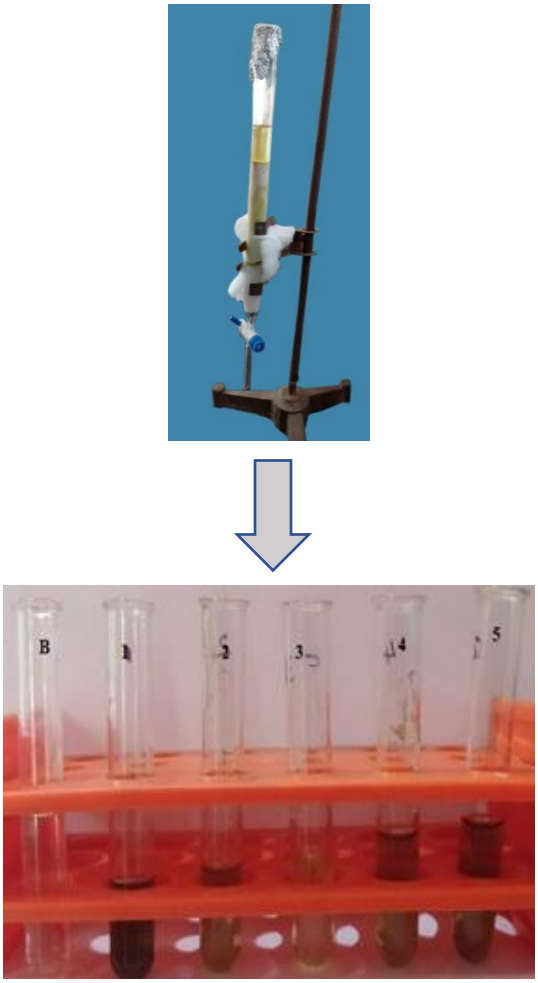


Fig 2. Column chromatography apparatus and the five fractions of *Citrus maxima* leaf extract separated by column chromatography.

Antibacterial assay of the fractions

10mg/ml of each fraction were screened for their antimicrobial activity against the oral isolates by well diffusion method to identify the bioactive compound responsible for the activity. The second fraction of the *Citrus maxima* methanolic leaf extract was found to inhibit the growth of all the oral isolates effectively in the range of 8-10mm zone of inhibition. The results are as tabulated in **Table 6**.

Table 6. Antimicrobial activity of column fractions of *Citrus maxima* methanolic leaf extract

Antimicrobial activity for fractions of *Citrus maxima* methanolic leaf extract (10mg/ml) Diameter of zone of inhibition (mm)						
Oral Isolate	**F1**	**F2**	**F3**	**F4**	**F5**	**CONTROL Gentamycin 10 ug/ml**
B.paramycoides	NA	8.4±0.3	8.3±0.28	NA	NA	15.5±0.264
P. aeruginosa	8.3±0.1	10.5±0.3	NA	7.56±0.351	8.63±0.305	20.6±0.251
S .epidermidis	9.3±0.21	8.4±0.251	8.2±0.20	NA	7.3±0.1	22.4±0.173
E. hormaechei	NA	8.3±0.208	NA	8.26±0.208	7.46±0.378	24.26±0208

± Standard deviation; NA – No activity

Identification of the bioactive compound by LCMS analysis

The second fraction (F-2) obtained from the column chromatography was subjected to LC-MS/MS analysis to identify the bioactive compound with its mass. The Total Ion Chromatogram (TIC) of the fractionated column, F2 are presented in **Figures 3a and 3b** respectively along with the respective standards. According to the chromatograms, a peak on Retention time 15.086 min was observed for F-2 in comparison with the standard Naringin whose Rt was 15.097.

Figure 4 and 5 show the experimental MS spectra of F-2 with Mass and MS/MS fragmentation, respectively. Positive radical ion species were observed with m/z for 580.53 for Naringin. The M/S experiments at m/z 580.53 showed two main fragments: 581.2 $(M+H)^+$ and 597.8 $(M+H_2O)$. The bioactive compound was identified as flavonoid Naringin ($C_{27}H_{32}O_{14}$) with a molecular weight of 580.53g/mol commonly found in citrus fruits and responsible for its bitter taste.

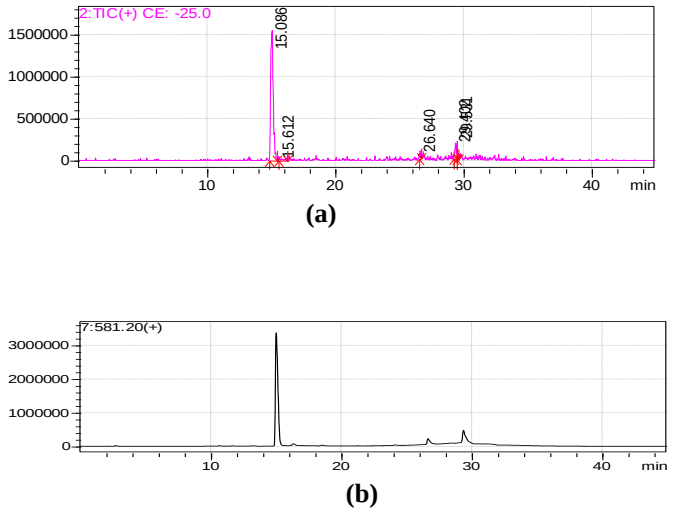
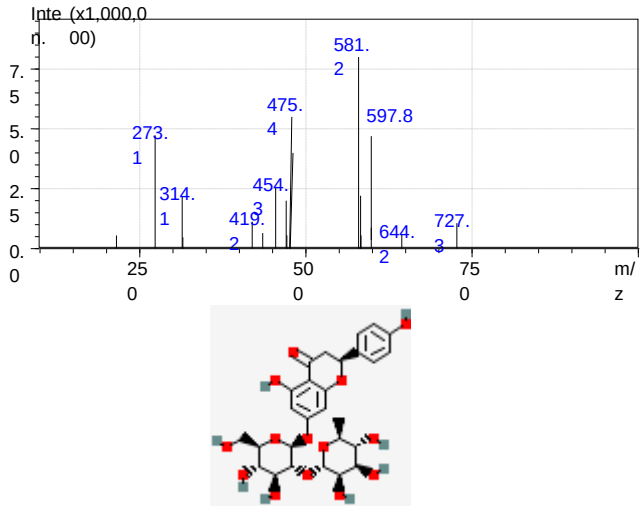


Fig 3. TIC of (a) F-2 with Retention time at15.086 min and (b) standard Naringin with Retention time at 15.097 min



Naringin

Fig 4. MS spectra of F-2 with the fragments 581.2 $(M+H)^+$ and 597.8 $(M+H_2O)$

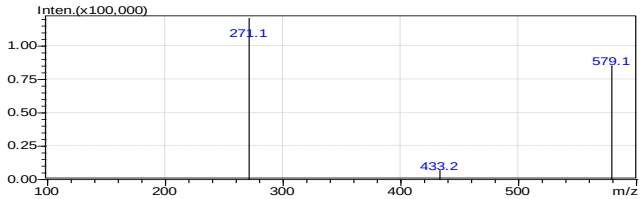


Fig 5. Ionization of the compound Naringin by MS/MS analysis

Plants are the most abundant source of therapeutic agents as they produce a diverse range of bioactive molecules and hence play a vital role in human health and well-being [22]. Of the several compounds produced by the plants, its antimicrobial activity is mainly attributed to the secondary metabolites like flavonoids, tannins, phenolic compounds, alkaloids and terpenoids produced as a means of defense against the biotic and abiotic stress [23]. One of the common traditional practices includes chewing neem and meswak sticks, which has now found its place in the composition of various herbal-based oral products [24, 25]. In our study, we have used four medicinal plants which claim their traditional use in oral health and hygiene but are not a component of any existing

herbal-based oral products. The antibacterial activity of these four plants extracted in two solvents ethyl acetate and methanol was tested against the four plaque-associated isolates. Ethyl acetate flower extract of *Saraca asoca and* methanol leaf extract of *Citrus maxima* was found to inhibit all the four isolates at 100mg/ml concentration with an average zone of inhibition of 10.15mm and 13.76mm respectively while at 25mg/ml concentration only the methanolic leaf extract of *Citrus maxima* was found be effectively inhibiting the growth of all the isolates. Vijayalashmi and Radha in 2016 have reported the leaf extract of *Citrus maxima* for analgesic, anti-inflammatory, anti-tumor, hepatoprotective activity and antioxidant activity with the presence of a wide range of secondary metabolites like amino acids, carotenoids, coumarins, carbohydrates, flavonoids, steroids and terpenoids, thus proving its potency in the development of novel drugs. In order to identify the bioactive compound responsible for the antibacterial activity of *Citrus maxima*, its methanolic leaf extract was subjected to TLC, column chromatography separation techniques to separate the wide range of molecules the plant possessed. Five fractions eluted through column were screened for their antibacterial potential by well diffusion method against the four isolates at 10mg/ml concentration. Kwaja *et al*, (2016) screened the antibacterial activity of the column fractions of *Acacia nilotica* leaf extract, in their outcome they discussed the reason for the total loss of activity in all the daughter fractions or very low activity in comparison with the parent extract would be due to the unsuitable separation method adopted or due to the instability of the active compound. In the current study, the second (F2) column chromatographic fraction showed susceptibility against all the four isolates with an average inhibition of 8-10mm zone of inhibition, indicating the presence of the bioactive compound responsible for the activity in it. The compound was identified to be a flavonoid called Naringin by LCMS MS technique. Naringin is usually present in all the citrus fruits and is responsible for its bitter taste, its significant *in-vitro* antimicrobial activity against periodontal pathogens was reported by Tsui *et al.,* in 2008. Naringin has also reported its biological importance particularly on lipid metabolism in obesity, oxidative stress, and inflammation in the context of metabolic syndrome [28]. Naringin can be further screened for its Minimum inhibitory concentration, Minimum bactericidal concentration and for its toxicity leading to its application as an oral hygiene product.

CONCLUSION

From the above investigation it can be concluded that Naringin obtained from the leaf extract of *Citrus maxima* can be used to treat bacterial infections of oral origin after suitable *in vitro* and *in vivo* studies.

CONFLICTS OF INTEREST

The authors declare that there is no conflict of interest regarding the publication of this paper.

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None

DATA AVAILABILITY

Not declared.

FUNDING SOURCE

Not declared

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