Advances in Bioresearch
Adv. Biores., Vol 7 (1) January 2016: 173-180
©2015 Society of Education, India
Print ISSN 0976-4585; Online ISSN 2277-1573
Journal's URL:http://www.soeagra.com/abr.html
CODEN: ABRDC3
ICV Value 8.21 [2014]

Advances in Bioresearch

ORIGINAL ARTICLE

Antioxidant and Antibacterial activity of Methanol Extract of *Malphigia glabra* against viridans Group of Streptococci involved in Dental Caries

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ABSTRACT
Viridans group streptococci (VGS) are the heterogenous group of organisms present in the oral cavity and known to cause dental infections, if untreated they gain entry to sterile sites causing bacterial endocarditis, septicaemia and other pyogenic infections. Now a day's several reports have been reported on bacterial resistance among this group to many synthetic drugs. On these facts, the present study is focused on the antioxidant and antibacterial activity of Malphigia glabra against viridians group of streptococci isolated from infected teeth samples. Methanol extract of Malphigia glabra was evaluated against clinically isolated Streptococcus mutans, Streptococcus sanguis, Streptococcus mitis and Streptococcus salivarius by agar well diffusion technique. Further the extract is evaluated for quantitative and qualitative invitro antioxidant activities. The methanol extract effectively inhibited all the clinical isolates. Total phenol and flavonoid content was found to be 49.62 ±0.815 and 46.78± 0.626 respectively. Results of DPPH activity showed a dose dependent activity with IC 50 value of 153.58 µg/ml. The IC 50 value for Fe^{++} reducing power of extract was found to be 450.45 µg/ml for methanol extract and also exhibited dose dependent activity. The results of methanol extract showed lesser activity when compared to standard. The present study reveals that the methanol extract of Malphigia glabra can be used as a potent antioxidant and antibacterial agent. In future this plant may be used for the treatment of dental caries infections.
Key words: Viridans group of Streptococci, Catalase, Optochin, Antioxidant and Antibacterial.

Received 24/07/2015 Accepted 29/11/2015

How to cite this article:
Venugopal. T.M, Mallikarjun. N, Somasundar. K, Nagaraj. K & Bhargavi Prabhuswamy. Antioxidant and Antibacterial activity of Methanol Extract of *Malphigia glabra* against viridans Group of Streptococci involved in Dental Caries. Adv. Biores., Vol 7 [1] January 2016: 173-180. DOI: 10.15515/abr.0976-4585.7.1.173180

INTRODUCTION

The dynamic environment of oral cavity involves changes in pH, nutrient availability, carbohydrate source and oxygen tension. Even though at these fluctuations in the oral environment, there are more than 750 bacterial species colonizes the oral cavity, co-existing as complex populations in biofilms. Among the most abundant microorganisms in the mouth, the oral streptococci are represented by species which are associated with oral health, as well as these species that are primarily associated with disease [12]. Streptococci present in the oral cavity consist of poorly defined heterologous group of organisms called viridians group of streptococci (VGS), which produces alpha hemolysis on blood agar containing sheep erythrocytes [10] [3].

Viridans group of streptococci (VGS) can behave as opportunistic pathogens causing diseases that range from mild infections to life threatening conditions. The most important clinical representatives of this group are *Streptococcus oralis (mitis), Streptococcus sanguis, Streptococcus mutans, Streptococcus milleri and Streptococcus salivarius*. [13]. *S. mutans* for instance, has a strong association with the development of dental caries, while *S. sanguinis*, *S. oralis* and *S. mitis* are responsible for 40-60% of native-valve infection of endocarditis. Members of the anginosus group are causative agents of pyogenic abscesses in a number of body sites. Other opportunistic infections caused by VGS include septicaemia in neutropenic patients, and occasionally causes meningitis [28].

Currently, no recognized "gold standard" exists for the identification of viridians streptococci satisfactorily, which has led to an inadequate reporting of streptococcal infections, and potentially has serious implications for diagnosis and treatment [28]. More recently, a molecular approach has been used to define the taxonomy of the viridans streptococci on the basis of genetic relatedness [2]. Accurate identification of VGS to the species level is therefore important to characterize the pathogenic potential of individual species and monitor trends in antimicrobial susceptibility in emerging infections [28].

Antibacterial studies of standard drugs against VGS showed that these organisms are susceptible to penicillin uniformly. However, the emergence of strains intermediately resistant or highly resistant to penicillin is increasingly recognized worldwide. Erythromycin has been recommended as an alternative drug for treatment of patients who are allergic to penicillin and is widely used in the antibiotic prophylaxis of bacterial endocarditis associated with dental procedures. In the past, erythromycin and clindamycin showed good activity, however recent studies have shown that macrolide resistance may also be a problem in certain areas [14].

The patterns of indiscriminate use of antibiotic have led the development of resistance among this group of bacteria, and further it is shifted to gain multiple resistances which are referred as multi drug resistance (MDR).

The resistance problem demands a renewed effort be made to seek antibacterial agents effective against pathogenic bacteria resistant to current antibiotics. One of the possible strategies towards this objective is, rational localization of bioactive phytochemicals [26]. Natural products produced from plant extracts have great potential as antimicrobial compounds against drug resistant microorganisms and these phytoconstituents have a potential to revert the antimicrobial drug resistance.

Malpighia glabra, or Barbados cherry (Acerola) is a shrub or small tree growing 10 to 15 feet tall, native in southern Texas, West Indies, North Southern America, Central America and Mexico. It has leaves which are small oval with pointed ends, neither glossy nor leathery, very dark green. Branches are flexible, the flower is rose-pink and the fruit is edible, a rich source of Vitamin C. In Brazil, it is popularly called "acerola"or "Barbados cherry" and "West Indian cherry" in tropical America. The fruits of acerola are known to its nutritional capacity and vitamin contents. It is a rich source of Vitamin C, flavonoids, Carotenoids, precursors of vitamin A, lycopene and also contains traceable elements such as thiamin, riboflavin, niacin, pantothenic acid, calcium, iron and magnesium [24].

In recent years, many studies have been conducted on fruit extracts, their role of reactive oxygen species in the etiology of various diseases and scanty information had been reported on biological activities of leaves, bark and root. Thus, the present study was conducted to know antioxidant activity and inhibitory activities of leaf extract (methanol) of *M.glabra* against viridians streptococci involved in dental caries infection.

MATERIAL AND METHODS

Collection of clinical samples:

Infected teeth samples were collected from the patients suffering from severe tooth pain visiting to District Mc-Gann Hospital, Shivamogga, and Karnataka, India. Teeth samples from patients with dental leisons were collected upon uprooted by the dentists into a reduced transport media (0.4% agar, 0.15% thioglycollate). Further these, samples were brought to the laboratory for the isolation of viridians group of streptococci.

Isolation of viridans streptococci from infected teeth samples:

Clinical samples were vortexed in a vortex mixer and streak inoculated on blood agar media (Himedia, Mumbai) supplemented with 5% defibrinated sheep blood cells. The plates were kept for incubation at 37^{o}C for 24 hrs and observed for alpha hemolysis. Colonies showing greenish coloration were picked up, further subcultured and stored at 4^{o}C. Morphological and Biochemical tests like Gram's staining , KOH solubility test, Optochin sensitivity test , Catalase test (slide test), Arginine hydrolysis, Esculin hydrolysis, Urea hydrolysis, Voges-Proskauer (VP) test, Mannitol and Sorbitol fermentation were carried out [19].

Collection of plant materials and preparation of methanol extract:

Leaf material of *Malphigia glabra* were collected in and arround Shivamogga district, Karnataka, India. Leaf material was washed in running water and was shade dried. The dried samples were powdered mechanically and exhaustively subjected for soxhalation using methanol (Himedia, Mumbai) as a solvent [29]. The extract was then filtered using a cotton plug and finally through filter paper. The crude samples were rotary evaporated in a dessicator and stored at 4^{o}C in a refrigerator for further use.

Antibacterial activity of crude extract against viridians group of streptococci

Antimicrobial activity was carried out using agar well diffusion method [29]. Briefly, Petri plates were poured with 20mL of sterile Mueller-Hinton agar media (MHA) (Himedia, Mumbai). The test cultures

(100 µL of suspension containing 108 CFU/mL bacteria) were swabbed on the top of the solidified media and allowed to dry for 10 min. The tests were conducted at three different concentrations using crude extract (10mg/ml, 25 mg/ml, 50 mg/ml of crude extract dissolved in 10 % dimethyl sulfoxide, DMSO). The wells of 6mm were done on agar surface using a sterile cork borer. The crude extracts were loaded to wells by using a micropippette and left for 30 min at room temperature for compound diffusion. Wells were also loaded with negative control (10% DMSO) and positive control (Tetracycline 1mg/ml). These plates were incubated for 24 hrs at 37°C. Average zone of inhibition were recorded in mm and the experiment was carried out in triplicates.

Estimation of total phenol content:

The total phenolic content (TPC) was determined by the spectrophotometric method [1]. In brief, 1 ml of sample (1 mg/ml) was mixed with 1 ml of Folin-Ciocalteu's phenol reagent. After 5 min, 10 ml of a 20% Na_2Co_3 solution was added to the mixture followed by the addition of 13 ml of deionized distilled water and thoroughly mixed. The mixture was kept in the dark for 90 min at 23°C, after that the absorbance was recorded at 760 nm. The TPC was determined from extrapolation of calibration curve which was made by preparing gallic acid solution. The TPC were expressed as milligrams of gallic acid equivalents (GAE) /gm of dried sample. The experiment was designed in triplicate and control was taken without plant extract.

Estimation of total flavonoid content:

Total flavonoid content was estimated colorimetrically using aluminum chloride [21]. An aliquot (1ml) of extract and standard solution of catechol (10mg/100ml) was added to 10ml flask containing 4ml of distilled water. To this 0.3 ml 5% NaNo2 was added. After 5min of incubation, 0.3 ml of 10% $AlCl_3$ was added. After 6min, 2 ml of 1M NaOH was added and the total volume was made up to 10 ml with distilled water. Absorbance was read at 510nm with a spectrophotometer.

DPPH radical scavenging assay:

The free radical scavenging activity of methanol fraction was measured *in-vitro* by 2,2^{I}- diphenyl-1-picrylhydrazyl (DPPH) assay [18]. The stock solution was prepared by dissolving 24 mg DPPH in 100 ml methanol and stored at 20°C until used. The working solution was prepared by diluting DPPH solution with methanol to attain an absorbance of about 0.98±0.02 at 517 nm using the spectrophotometer. Different concentrations of methanolic extracts and ascorbic acid (200,400,600,800 and 1000 µg/ml of methanol) were mixed in separate tubes with 2ml of DPPH. The reaction mixture was shaken well and incubated in the dark for 30 mins at room temperature. Absorbance was recorded at 517 nm. The control without plant extract was prepared. The scavenging activity was estimated based on the percentage of DPPH free radical scavenged was calculated using following equation:

Scavenging activity = Control absorbance – Sample absorbance/Control absorbance x 100

Ferric reducing assay:

Ferric reducing assay was determined by adopting the previously described method [7]. Different concentrations of methanolic extracts and ascorbic acid (200,400,600,800 and 1000 µg/ml of methanol) were mixed in separate tubes with 2.5ml of phosphate buffer (200mM, pH 6.6) and 2.5ml of 1% potassium ferricyanide. The mixture was placed in a water bath for 20 min at 50°C, cooled rapidly and mixed with 2.5ml of 10% trichloroacetic acid and centrifuged at 3000 rpm for 10 mins. After this, 2.5 ml of supernatant was mixed with 2.5 ml of distilled water and 1ml of 0.1% Ferric chloride. The amount of iron (II)- ferricyanide complex formed was determined by measuring the formation of Perl's Prussian blue at 700nm after 10min. The higher absorbance of the reaction mixture indicates increased reducing power. The results were expressed as ascorbic acid equivalent antioxidant activity.

Statistical Analysis

All the result experiments were conducted in triplicates and the data Data are expressed as mean ± SD were analyzed by SPSS statistical package (version 12.0).

RESULTS

Isolation of viridans streptococci:

A total of 160 clinical isolates showing alpha hemolysis (viridians group) were recovered from 100 infected teeth samples. Results of biochemical characters were represented in table 1. On the basis of morphological and biochemical tests the organisms such as *Streptococcus mutans*, *Streptococcus sanguis*, *Streptococcus mitis* and *Streptococus salivarius* were isolated from the clinical sample. Among the isolated clinical samples *S.mutans* (58%) were recovered in all the clinical samples followed by *S.mitis* (15%), *S.sanguis* (14%) and *S.salivarius* (13%) respectively. (Figure 1).

Antibacterial activity of crude extract against viridians group of streptococci.

The antibacterial activity of methanol extract from *M.glabra* against each viridians streptococci species were graphically represented (figure, 2). The extract exhibited inhibitory activity against each bacterial

species screened. The extract exhibited a dose dependent antibacterial activity, ranging from 5.4±0.843 to 15.8±1.87 against all the isolated species. The highest activity was observed against *S.mutans* with zone of inhibition 15.8±1.87 followed by *S.mitis* with zone of inhibition 15.4±1.25 mm at 50mg/ml. The extract showed lowest activity against *S.sanguis* with zone of inhibition 5.4±0.843 mm followed by *S.mitis* with inhibition zone 5.8±0.91mm at 10 mg/ml. No inhibition zone was observed in case of DMSO. Tetracycline showed inhibition zone against all the isolated organisms ranging from 25.6±0.51 to 27.3±1.63 mm, the results revealed that, all the bacteria was found to be susceptible towards methanol extract and standard. Inhibition zone exhibited by methanol extract was less when compared to standard.

Total Phenol content

The total phenolic content was examined in the plant extract using the Folin-Ciocalteu's reagent is expressed in terms of gallic acid equivalent. The values obtained for the concentration of total phenols are expressed as mg of GA/µg of extract 49.62 ±0.815 µg /ml (Figure 3)

Total Flavonoid content

The concentration of flavonoids in methanol extract of *M.glabra* was determined using spectrophotometric method with aluminum chloride. The content of flavonoids was expressed in terms of catechol equivalent µg /ml of extract. The concentration of flavonoids in methanol extract of *M.glabra* is 46.78± 0.626 µg/ml. (Figure 4)

DPPH radical scavenginig activity:

The results of conversion DPPH+ (free radical) into DPPHH by methanol and ascorbic acid are graphically represented (figure 5). The methanol extract exhibited a pre eminent antioxidant activity in a dose dependent manner. The scavenging activity of standard (ascorbic acid) was greater than that that of solvent. The IC 50 value was found to be 1090 µg/ml.for ascorbic acid and 153.58 µg/ml for methanol extract.

Ferric reducing assay:

Ferric reducing assay was employed to estimate the antioxidant capacity of the samples *in vitro*. In this test, the OD ranges from 0.465 to 1.256 for ascorbic acid and 0.256 to 0.818 for methanol extract at 700 nm, which revealed that the methanol extract showed good reducing activity. When compared to standard methanol extract showed least activity (figure 6). The IC 50 value for ascorbic acid was found to be 187.6 µg/ml and 450.45µg/ml for methanol extract. In this study, the absorbance was found to increase with the dose of methanolic extract and standard which is suggestive of reducing power.

Table 1: Biochemical properties of viridians streptococci isolated from infected teeth Samples.

Clinical isolates	Catalase	Optochin sensitivity	Arginine hydrolysis	Esculin hydrolysis	VP	Mannitol	Sorbitol
S.mutans	-	-	+	+	+	+	+
S.sanguis	-	-	+	+	-	+	+
S.mitis	-	-	-	-	-	-	-
S.salivarius	-	-	-	+	+	-	-

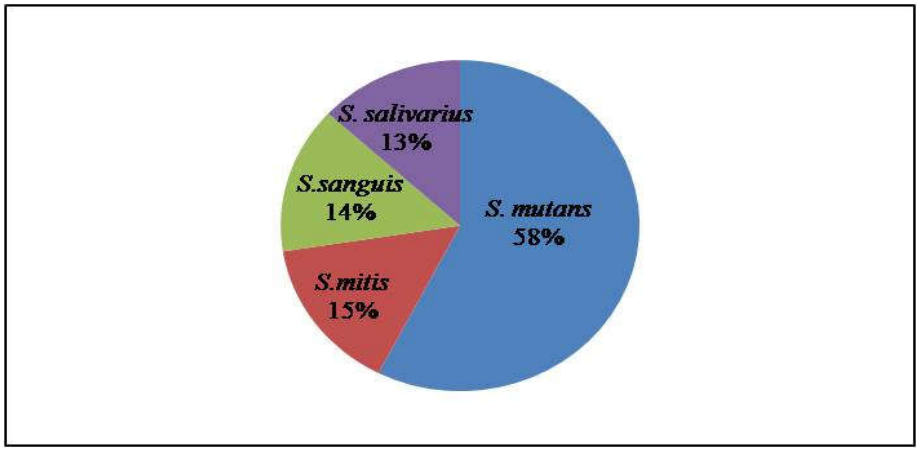

Figure 1: Isolation percentage of Streptococcus species from infected teeth samples.

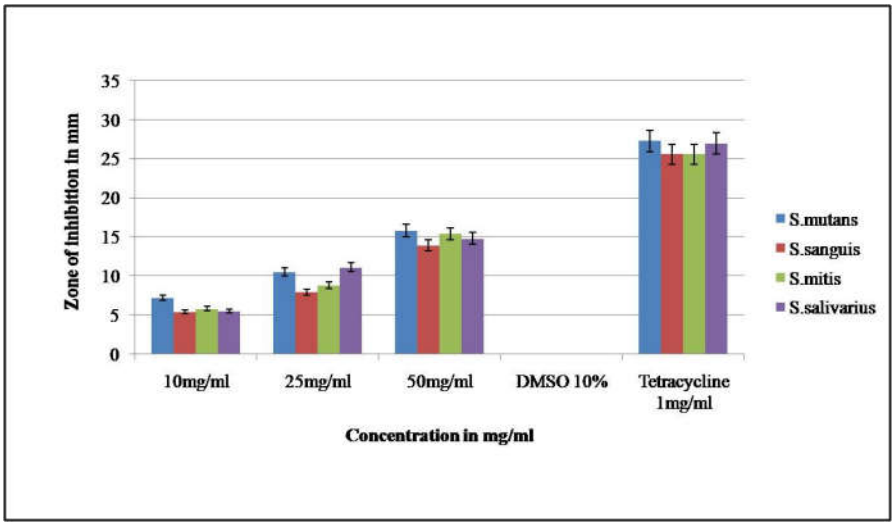

Figure 2: Antibacterial activity of methanol extract of *Malphigia glabra* against viridians streptococci.

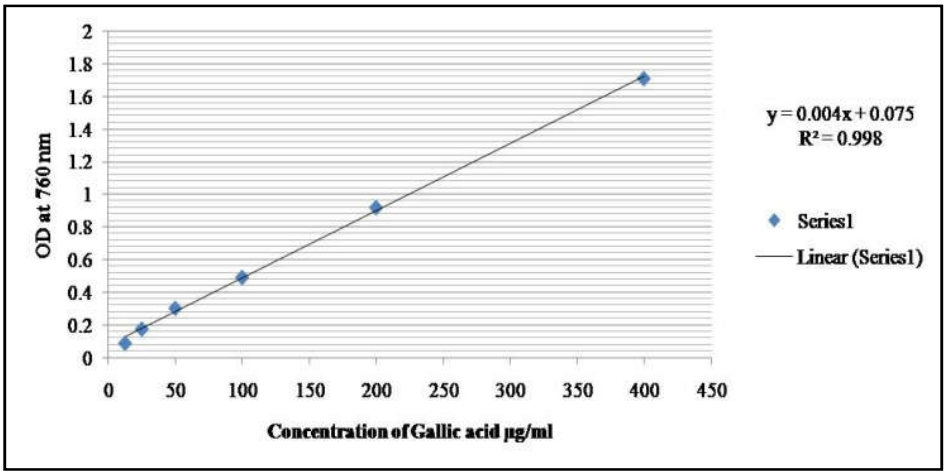

Figure 3: Standard curve for phenolic compounds using gallic acid.

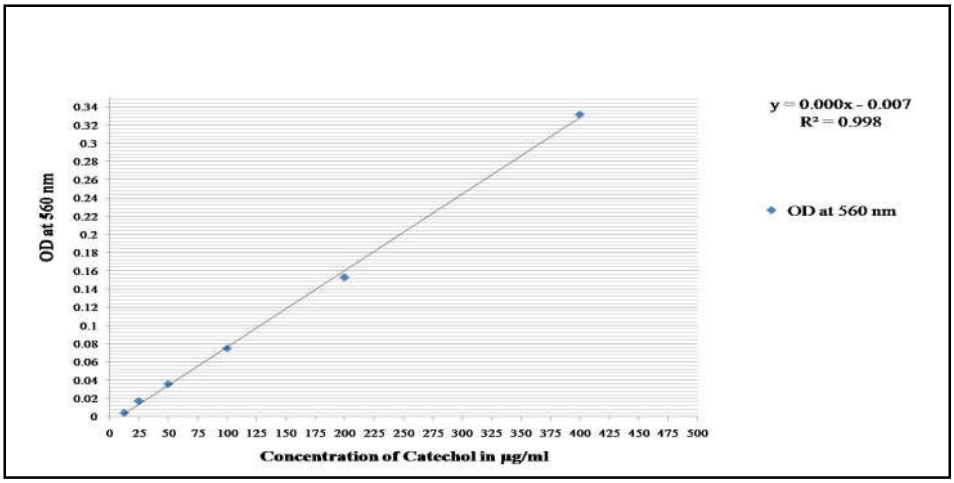

Figure 4: Standard curve flavonoid compound using catechol.

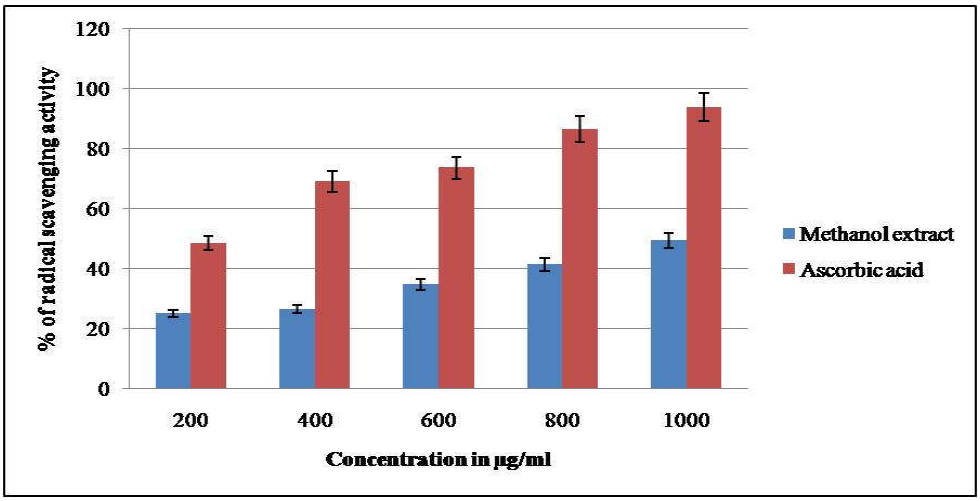

Figure 5: DPPH radical scavenging assay of Ascorbic acid and methanol extract of *M.glabra*.

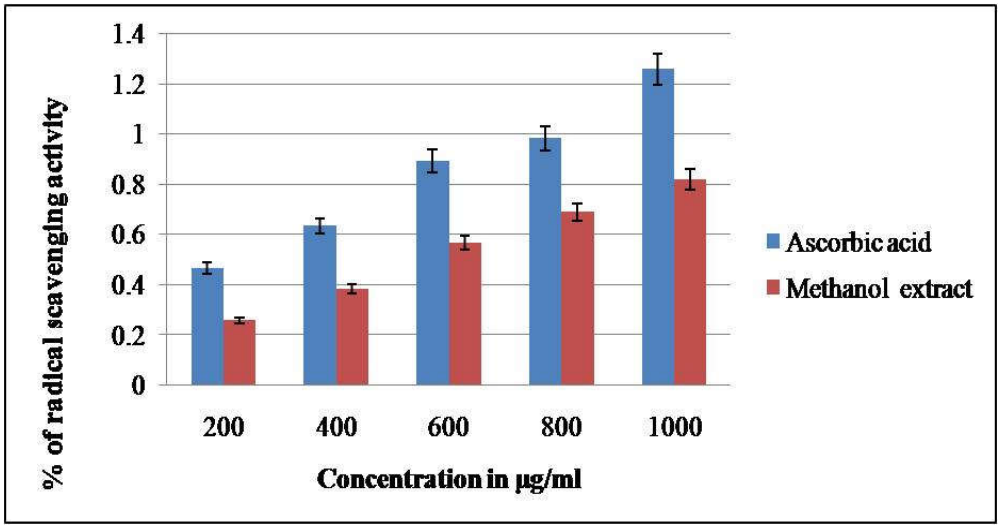

Figure 6: Ferric reducing assay of Ascorbic acid and methanol extract of *M.glabra*.

DISCUSSION

The present study on microorganisms of the genus Streptococci is of great clinical interest due to their pathogenic potential. In Oral microbiology, there is a significant concern with regards to the renowned group i.e. "viridans streptococci" [22].This group includes *Streptococcus salivarius*, *Streptococcus sanguis, Streptococus mitis,* and *Streptococcus mutans*. [13]. VGS are docile tenants of mouth and gut, they can cause disease and deaths among their hosts when they gain entrance to sterile sites like bacterial endocarditis, acute respiratory distress syndrome (ARDS) and shock [25]. Identification of VGS was done, based on the colony morphology, Gram's stain, Optochin sensitivity test and catalase reaction [14]. Biochemical characters play an important role in the identification of viridians streptococci as the organisms of this group are less reactive to Lancefield group antigens. Several studies have been conducted on isolation of Viridans streptococci by performing biochemical test like Hippurate Hydrolysis(HH), Esculin Hydrolysis (EH), Voges - Proskauer (V.P.), Arginine - Dihydrolase (AD), and Carbohydrate test (CH) [19] [23] [25].

Several studies conducted by different researchers had shown that VGS are isolated from oral cavity. In a study, based on morphological, cultural and biochemical characteristics 53 isolates belonging to viridians streptococci were isolated and identified from 39 samples**.** *S. mutans* was frequently isolated and followed by *S.salivarious, S.mitis, S.milleri* and *S.sanguis* [13]. In another study, 65 isolates were characterized based on the hemolytic and biochemical properties. [8]. Similar type of prevalence results was observed in our laboratory study in which, we have isolated *S.mutans* (58%), *S.mitis* (15%), *S.sanguis* (14%) and *S.salivarius* (13%) from infected teeth samples.

Antibiotics such as ampicillin, chlorhexidine, erythromycin, penicillin, tetracycline and vancomycin have been very effective in inhibiting the pathogens involved in dental caries [9]. However, indiscriminate use of these antibiotics results in dearrangements of the oral and intestinal microflora causing undesirable side effects [5]. Other studies also concentrated on drug resistance among VGS. To reduce this risk and side effects, several researchers have advocated the plant based product to combat drug resistance menace [6]. In our study we have screened leaf extract of *Malphigia glabra* against all isolated strains; our results indicated dose dependent inhibitory activity in extract.

To substantiate our findings we have carried out qualitative and quantitative antioxidant properties of the plant extract. Our study reveals that phenolic and flavonoid compounds are present in a prominent amount. Phenolic compounds present in the plants are rich in hydroxyl groups and is responsible for scavenging ability [30]. The chemical structure of phenolic compounds had ability of phenoxide ion delocalize. The phenoxide ion can lose a further electron to form the corresponding radical which can also delocalize [15]. Flavonoids are the group of phenolic compounds which are the main resources to scavenge oxidizing molecules including singlet oxygen, and various free radicals implicated in several diseases [17]. There are several methods that are employed to screen *in-vitro* antioxidant activity of herbal drugs. An easy, rapid and sensitive method for the antioxidant screening of plant extracts is free radical scavenging assay using 1,1, diphenyl-2-picryl hydrazyl (DPPH) and Fe^{3+} reducing assay [1]. In DPPH assay, the reactive rate and the ability of the radical scavenger depend on the rate and the peak value of disappearance of the DPPH [20]. In reducing power assay, the yellow colour of the test solution changes to green depending on the reducing power of the test specimen. The presence of the reductants in the solution causes the reduction of the Fe3+/ ferricyanide complex to the ferrous form. Higher absorbance of the reaction mixture indicates a higher reducing power. Indirectly Fe2+ can be monitored by measuring the absorbance at 700 nm [16]. The plant used in this study belongs to Malphiginaceae family and a several studies have been carried using fruit extracts which is known to have high vitamin C content. Similar studies were concentrated on the fruits extract of different species of Malphiginaceae family, *M. glabra*, *M. emarginata* and *M. punicifolia* [11]. Related work was carried on this fruit and highlighted the presence of Vitamin C along with additional nutrients likes flavonols and anthocyanins. Other studies have also concentrated on antioxidant properties of fruit extract [4]. Two types of anthocyanin compounds, Cyanidin-3-α–O- rhamnoside and pelargonidin-3-α–O-rhamnoside were isolated from *M.emarginata*. These compounds exhibited antidiabetic activity [27]. However the new compounds tetranorditerpenes acerolanins A–C (1–3) with a rare 2H-benz [e]inden-2-one substructure were isolated from the aerial parts of *M. emarginata* and these compounds were studied for their cytotoxic activity [11]. Our study also reveals that, the crude leaf extract of *M.glabra* showed the presence of both the phenolic and flavonoids contents in appreciable quantity and these may contribute to antioxidant and antibacterial properties against VGS.

CONCLUSION

Even though, free radical scavenging ability and antibacterial activity of methanol extract of *M.glabra* against VGS is low when compared to standard. The extract has the proton-donating ability and could serve as free radical inhibitors or scavenger and also proves that this extract can be used to treat several dental caries infections. Further there is a need to explore the ability of this plant, which can be utilized to treat several physiological disorders and other microbial infection.

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