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# Susceptibility of multi-drug resistant oral pathogen and its biofilm formation to *Rosa damascena* mill

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**Abstract**
The Rosa Damascena is one of the most important species of Rosaceae family mainly known for its perfuming. Its major products are perfume and volatile oil. This plant contains several components like terpenes, glycosides, flavonoids, and anthocyanins that have beneficial effects on human health. This study was carried out to evaluate the effect of the flowers and leaves of Rosa damascena of various solvent extract on the oral pathogen using invitro antibacterial study and specific biofilm formation assay. The flowers and leaves of R. damascene were subjected to extraction using ethanol, methanol and distilled water by hot percolation method. All the extracts showed inhibition at minimum concentration of 25µg/ml. The aqueous extract exhibited significant anti-microbial activity and inhibition of biofilm formed by the oral pathogen. In contrast the flower extracts showed potent inhibitory effect on both oral pathogen and its biofilm formation. On a long run history in herbal remedies for tooth and gum problems, this study may add a proof in eradicating dental caries that is caused by the multi drug resistant oral pathogen.

**Keywords:** *Rosa ddamascena*, multi drug resistant oral pathogen, streptococcus mutans, specific formation biofilm assay

## Introduction
In India plant wealth is greatly exploited for its therapeutic potential and medicinal efficacy to cure cavity. The human oral cavity is inhabited by more than 500 species of bacteria at $10^8$-$10^9$microbes per ml of saliva or mg dental plaque (Rosan B., *et al* 2000) [1]. Caries is a disease caused by plaque bacteria such as Streptococcus mutans and Strep. Sobrinus (Loesche WJ, 1986) [2, 6] Mutansare gram positive, facultative anaerobic bacteria commonly found in human oral cavity and is a significant contributor to tooth decay (Ryan KJ *et al*, 2004) [3] (Loesche WJ, 1996) [4]. They are biofilm bacteria and are considered to be the primary etiologic agents of human dental caries and hence it is also called as cariogenic mutansStreptococci (Hamada S., *et al*, 2008) (Loesche WJ, 1986). [5, 6] They produce glycosyl transferase (GTF) enzymes that synthesize glucan from the glucose moiety of sucrose that causes the cariogenicity of the dental microbes. Due tomutansplaying role in tooth decay, there have been many attempts to make a vaccine against that particular microbe. As of now, such vaccines have not been so successful in humans (Klein JP, *et al*, 2007) [7]. In many traditional cultures, there are no plastic bristle brushes, rather, the use of herbal "chewing sticks" for relieving dental issues were followed. Over a few decades, extensive research has been carried out on cariogenic Streptococc is pecies and was found that Strep. mutansis gradually acquiring resistance to commonly used antibiotics and hence are called as drug resistant S, mutan. [9] The main target in the study was to determine anti-bacterial study of the flowers and leaf extracts of oil bearingrose (Rosa damascene Mill.) against multi drug resistant oral pathogen (Streptococcus mutans). After shade drying and powdering the flowers and leaves, hot extraction with 80% ethanol, methanol and distilled water were carried out respectively. The extraction yielded more with distilled water. Based on earlier studies and phytochemical tests, the polyphenols and essential oil present in the flower petals and leaves of the plant gave a lead for the study. The effect of the plant was evaluated using *in vitro* methods such as anti-bacterial effect by Paper disc agar diffusion method and inhibition of biofilm formation by Specific biofilm formation assay method against Streptococcus mutans.

## Materials and Methods
### Plant Material
The plant parts such as fresh flowers and leaves were collected from local market. The plant parts were authenticated by the Botanist Prof. Dr. P. Jayaraman Sir (M.Sc., Ph.D.), Director, Plant Anatomy Research Centre (PARC), Medicinal Plant Research Unit, Tambaram, Tamil Nadu.

### Extraction from Plant
The plant parts of the Rosa damascena such as flowers and leaves were properly washed in tap water and then rinsed in distilled water. They were well dried in shade and the dried parts were ground into coarse powder. About 250gms of the coarse powder of the leaf and flowers were extracted by hot percolation using 300ml of 80% ethanol, methanol and distilled water respectively for three hours. The extracts were then subjected to cotton filtration followed whatmann filter paper. The extracts were then concentrated by evaporation and dried by lyophilization technique. The lyophilized extracts were stored in refrigerator prior to use.

**Pathogen Collection**

The oral pathogen namely Streptococcus mutans available at MRD Life Sciences (P) Ltd. Lucknow, collected from Institute of Microbial Technology, (IMTECH), Chandigarh was subcultured and used throughout the study.

**Anti-Bacterial Study**

The anti-bacterial study was carried out using Afar diffusion- Paper disc method with three trials. The sterile plates with Tryptone glucose yeast extract agar medium were prepared. The sterile paper discs of 4mm diameter were used for the study. The sterile discs were charged with 0.05ml of 25µg/ml, 50µg/ml, 75µg/ml and 100µg/ml concentrations of leaf and flowers extracts respectively. The charged discs were placed in the petri plates which were pre inoculated with the bacterial strain. Chlorhexidine was used as the reference drug. All the petri plates were incubated at 35ºC overnight and the degree of sensitivity was determined by measuring the inhibition of bacterial growth in terms of clear zone in diameter in mm around the discs. (Bauer. AW, *et al*, 1966.) [12].

**Specific Biofilm Formation Assay**

**Biofilm preparation**

Prepare 5mL LB liquid cultures (supplemented with appropriate antibiotics and inducers) from stock culture. Allow the cultures to grow for 18-20 hours at 37 °C in a shaking incubator. Prepare 1:100 dilutions, with a total volume of 1 mL, with desired media for each liquid culture. About 100 µL of each dilution in sets of 4 wells in a round-bottom 96 well plate were prepared. Cover the 96-well plate and incubate at 37ºC for 48 hour. (Loo.CY, *et al*., 2000, Hirulkar. NB., 2010) [13, 14].

**Evaluation of the biofilm formation using Crystal Violet Assay method**

After incubation period shake the plate out over a tray to remove all bacteria. Rinse the 96-well plate in a large beaker of water and shake the water out over the tray. Lay a paper towel out on the bench top. Hit the 96-well plate against the covered bench top until no liquid remains in the wells. Stain all wells used in the assay with 125 µL of 0.1% crystal violet for 10 minutes. Shake the 96-well plate over the tray again and rinse out the crystal violet in a large beaker of water. Cover the bench top with more paper towels and hit the plate against the bench top until all wells are free of liquid crystal violet.

Note: Make sure that the only crystal violet remaining is bound to a biofilm at the bottom of a well. Rings of crystal violet around a well are not indicative of biofilm formation and should be rinsed again, as excess stain will skew the results of the assay. Leave the plate face up on the bench top overnight to dry. Add 200 µL of 30% acetic acid to all wells that were stained to solubilize the crystal violet. Allow the acetic acid to sit for 10 minutes (E.G. Di Domenico, et al).

**Measurement**

Six tubes were used for each drug extract. Two were used to measure Biofilm growth (B tubes), two were used to measure growth in suspended culture (G tubes) and two served as control for abiotic factors (NC tubes). The absorbance at 570 nm of each resultant solution after staining was measured spectro photometrically. Finally the biofilm formation was measured by the following formula:

$$SBF = \frac{(B\text{-}NC)}{G}$$

The result of the extracts was expressed as percentage inhibition of the biofilm formed. (Eqn 1).

$$\text{Percentage Inhibition} = \frac{\text{OD Negative control} - \text{OD Experimental}}{\text{OD Negative control}} \times 100$$

**Results**

In the study, the flowers and leaves of R. damascena were extracted with 80% ethanol, methanol and distilled water. The extracts were tested for anti-bacterial effects and Specific biofilm formation assay against the multidrug resistant oral pathogen S. mutans [Table no: 1 and Table: 2] respectively. Data were expressed as Mean ± Standard deviation. The extracts possessed significant effect on inhibiting bacterial growth at four different concentrations such as 25µg/ml, 50µg/ml, 75µg/ml & 100µg/ml. It was found that the water extract of the flower showed 30.01±0.09mm and leaf showed23.51±1.13mm of clear zone of inhibition at 100µg/ml concentration. The biofilm assay was carried out with 100µg/ml concentration of all the extracts using 96 well micro plate techniques. The results exhibited inhibitory effect of about 84.66±1.528% with flower water extract and 67.08±1.0% with leaf water extract on the biofilm formation. The results of the water extract of both leaf and flower were found significant when compared to the other extracts. This study exhibits the lead role of the plant having significant antibacterial activity against the oral pathogen and its biofilm formation and thus can be a noteworthy evident that the oral pathogen Streptococcus mutansis more susceptible with Rosa damascena plant.

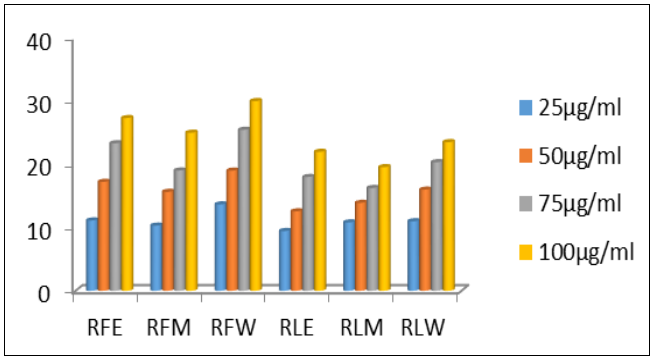


**Fig 1**

**Table 1:** Anti-bacterial activity (diameter of zone of inhibition in mm)

| Name of sample | 25µg/ml | 50µg/ml | 75µg/ml | 100µg/ml |
|---|---|---|---|---|
| $RF_E$ | 11.12±0.34 | 17.23±0.78 | 23.33 ± 0.57 | 27.32±0.21 |
| $RF_M$ | 10.30±0.98 | 15.65±1.0 | 19.02 ± 1.0 | 25.0±0.98 |
| $RF_W$ | 13.67±0.6 | 19.0±0.22 | 25.46 ± 1.0 | 30.01±0.09 |
| $RL_E$ | 9.45±0.43 | 12.56±0.18 | 18.0 ± 1.0 | 21.98±0.34 |
| $RL_M$ | 10.82±0.32 | 13.90±0.21 | 16.26 ± 1.15 | 19.56±1.19 |
| $RL_W$ | 11.0±0.87 | 16.0±0.9 | 20.34 ±1.09 | 23.51±1.13 |

$RF_E$ – R. damascena flower ethanolic extract; $RF_M$ – R. damascena flower methanolic extract; $RF_W$– R. damascena flower water extract; $RL_E$ – R. damascena leaves ethanolic extract; $RL_M$– R. damascena leaves methanolic extract; $RL_W$– R. damascena leaves water extract

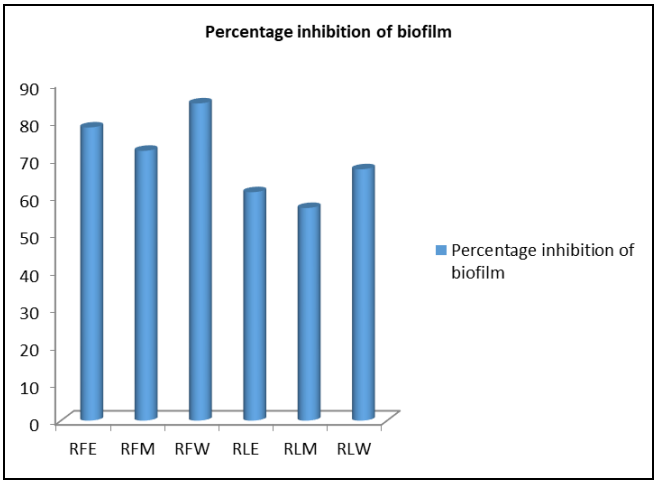


**Fig 2**

**Table 2:** Specific biofilm formation assay

| Name of sample | Percentage inhibition of biofilm |
|---|---|
| $RF_E$ | 78.24 ± 0.57 |
| $RF_M$ | 72.01 ± 1.0 |
| $RF_W$ | 84.66 ± 1.528 |
| $RL_E$ | 60.97±1.02 |
| $RL_M$ | 56.75± 0.57 |
| $RL_W$ | 67.08 ± 1.0 |

$RF_E$ – R. damascena flower ethanolic extract; $RF_M$ – R. damascena flower methanolic extract; $RF_W$– R. damascena flower water extract; $RL_E$ – R. damascena leaves ethanolic extract; $RL_M$– R. damascena leaves methanolic extract; $RL_W$– R. damascena leaves water extract

## Discussion

R. Damascena Mill. is one of the most holy ancient plant with extended historical use. It belongs to the family Rosaceae. The majority of the application of R. damascena includes production of Rose water or rose essential oil for its application in religious ceremonies, cooking or baking and in high grade perfumes. Although people in folklore, use rose water and or dried rose petals, the modern investigations have been confirmed for its antiviral, antibacterial, anticancer, antidepressant, antioxidant, analgesic, anti-inflammatory, anti-convulsant, hypnotic and relaxant effects. Alcoholic and aqueous extracts of Rose petals showed higher anti-bacterial activity than that of petroleum ether extract against E.coli. The bacteria was resistant to ethanolic extract of the petal while its aqueous extract showed more sensitivity. (Hirulkar NB, 2010) [14]. Furthermore, the rose oil confirmed acceptable anti-bacterial activity against Xanthomonasaxonopodis spp. Vesicatoria, (Basim E, *et al*, 2003) [16] Chromobacteriumviolaceum and Erwiniacarotovora strains, Staphylococcus aureus (Ulusoy S., etal, 2009) [17]. This paper carried out the anti-bacterial effect of the rose petals and leaves against Streptococcus mutans. This pathogen is a primary cause for dental caries having the ability to adhere to tooth surfaces while producing acid and surviving in acid conditions. (Harmada S, *et al*, 1980). Untreated dental caries will gradually lead to tooth loss, with ensuing chewing difficulties and ultimately a variety of health problems. (Allen PF, 2003) [18]. This condition has raised alarm in most of the developed countries and the scientists are forced to search an alternative medicine often from natural resources such as plants to control the growth of multi drug resistant oral pathogen. Mutansppare the biofilm forming bacteria and are considered to be the primary etiologic agents of human dental caries. They possess a variety of abilities to colonize tooth surfaces and under certain conditions are present in large quantities in cariogenic biofilms and also form biofilms with other organisms, including other streptococci and bacteria.(Loesche WJ, 1986) [2, 6]. Hence the use of herbal extracts for the control of the oral pathogen or dental diseases are considered interesting alternative to synthetic antimicrobials due to their lower negative impact and to overcome intrinsic or secondary resistance to the drug during therapy.The work of this paper evidences suggesting that all the extracts of the Rosa plant has anti-bacterial sensitivity on the dental pathogen and also significant effect on its biofilm formation

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## Conclusion

There is widespread emergence of resistance among microbial pathogens against the commercially available anti microbials. The research on traditional plants has proven to be bettersource in search for novel anti microbial compounds. Among the plants that are known for their medicinal value Rose flowers and leaves are safe, economical, effective and easily available. The authors have recommended further research before considering them for clinical use. Authors also recommend the use of the plant Rosa damascena against dental caries and periodontal pathogens. As a need of the hour the research will aid in the development of a novel, innovative method that simultaneously inhibit the common dental diseases of mankind, besides lowering the development of drug resistance.

## Conflicts of Interest

There are no conflicts of interest.

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