2.1. Oral Biofilm

The connection between oral infection and microbial activities by numerous species of microorganisms is well recognized (Jenkinson et al., 2005). The vast and different types of microorganisms are associated with oral cavities which are responsible for many chronic diseases one such disease is periodontitis (infection of the periodontal ligament which is attached with alveolar bone). Gingival inflammation in response to bacterial plaque accumulation is considered the key factor for the onset of periodontitis (Murakami et al., 2018). Foci of infection develop as a result of chronic periodontitis or abscess of tissue attached with the root apex of the tooth which may cause bacterial endocarditis and glomerulonephritis (Dietrich et al., 2017). Plaque induced gingivitis may exhibit various patterns of observable symptoms of inflammation localized to gingiva and initiated by accumulation of microbial biofilm on teeth (Murakami et al., 2018). Oral microflora can also contain Protozoal, fungal and viral species in addition to bacterial species. More than 750 bacterial species occupy the dental cavity out of which 50% are unknown and many of these species are involved in oral infection (Jenkinson et al., 2005). Different types of microorganisms are present in the microhabitat of the oral cavity attach to the teeth surface, gingival tissue, tongue, and buccal tissue. Each site uniquely allows the organisms to adhere to their surfaces. Oral biofilms are the population of microbes associated with the oral cavity which are encased in an extracellular polymeric material. These biofilms are root cause several diseases which can create problems for the host system. The biofilms formed by bacterial cells fixed in extracellular polymeric substance composed of proteins, DNA and polysaccharide material. The scientific researchers for the last few decades have tried to reveal the significance of the bacterial population and the complex properties related to these communities. It is very fascinating fact regarding these microbes that they can engineer their system accordingly to the ecosystem where they are restricted to as a result of a number of events of internal metabolism which amount to the contribution from their genetic makeup. This feature possessed by these microorganisms makes them a study of interest as it reveals viable measures of protecting the human society from adverse effects developed by the microbial communities (Marsh, 2016). A large number of bacterial species have been identified in oral biofilms (Zaura et al., 2009; Marsh, 2006). Dental plaque or dental biofilm is actually composed of mixed biofilm (Li et al., 2004). There must be a strong communication between bacteria for sequential development of biofilm. Bacteria which firmly attach to the tooth surface assemble as multispecies to form a dental biofilm. If the bacteria are not
firmly attached to the surface they get washed away with mouth fluid. The extent of formation of the biofilm also depends on the degree of similarity among the bacterial communities. The aggregation of the bacteria of same species arises because of the auto-aggregation process while co-aggregation process involves the aggregation of distinct species. Oral microorganisms get attached to the teeth enamel, epithelial tissue or other bacteria that are immobilized on the particular surface (adhesion). *Streptococci* and *Actinomyces* are the initial colonizers responsible for the development of biofilm (Dige et al., 2007). The receptors which are recognized by these colonizers include phosphate-rich protein(statherin) and an enzyme (alpha amylase). *Streptococci* identify receptors present in the salivary pellicle (covering the enamel). *Actinomyces* recognize proline-rich protein and statherin. Fusobacterium which is also responsible for oral infection binds to statherin protein (Dige et al., 2007).

2.2. Formation of oral biofilms

Several stages are involved in the formation of biofilm. In primary reversible stage, the adsorption of bacteria on the oral surface takes place followed by the passive addition of bacteria initiated by weak long-range force of attraction which is eventually supported by covalent and hydrogen bonds, as a result, the attachment becomes irreversible (Sutherland, 2001). The biofilm which is irreversibly attached to the surface can tolerate stronger chemical and mechanical forces (Sutherland, 2001). At a small distance from the oral surface, the negative charge on the bacteria are repelled by the negative charge on that particular surface (Palmer et al., 2007). This repulsive force can be neglected by attractive force like Vander Waals forces among bacteria and the oral surface moreover flagella and fimbriae give additional support for the attachment to the tooth surface (Palmer et al., 2007). The presence of glycoproteins in the saliva has an essential role because they bind to the teeth surface which is responsible for pellicle formation. The pellicle formed by the glycoprotein act as receptors which are recognized by different oral microorganisms. Many Gram-positive species like *Streptococcus, Actinomyces, Haemophilus* etc. which act as primary colonizers bind to the pellicle on the teeth enamel (Spratt et al., 2003).

2.3 Reason for biofilm formation

Many reasons can be responsible for biofilm state of bacteria (Jefferson et al., 2004). The first biofilm can elevate tolerance to unfavorable conditions of an environment. Bacteria which have a
capability to form a biofilm can also resist strong shear forces (Rasmussen et al., 2006). Second EPS matrix which encases the bacteria protects the bacteria against the antibacterial agents by restricting the diffusion of these agents. The bacterial mobility which gets restricted in the biofilm creates perfect favorable conditions for exchange of DNA by a process of conjugation, few of which encode for antibiotic resistance (Hausner et al., 1999).

2.4 Biofilm resistance to antibiotics

Biofilm-forming bacteria possess resistance against antibiotics. The resistance possessed by biofilm-forming bacteria is 1000 times higher than their planktonic (Costerton et al., 1995; Nickel et al., 1985). Various factors are involved to shield biofilm bacteria from antibiotics. Some of the mechanisms which are responsible for resistance against antibiotics are as under:

a) Limited Penetration of antibiotics

The Exopolymeric substance which encases the bacteria in the biofilm has a significant role to develop resistance against antibiotics by limiting the diffusion of these antibacterial agents so that they are unable to penetrate into the biofilm.

b) Persister cells

The small sub-population of cells in the biofilm known as persister cells possess an extremely slow growth rate or growth rate is zero (Lewis et al., 2007; Keren et al., 2004). A large number of antibiotics which are currently used target the processes pertinent for cell growth or division are not influential against persister cells.

c) Efflux pumps

Efflux pumps permit bacterial cells to remove intracellular toxins as well as antibiotic drugs. These are also present in the planktonic bacteria but few efflux pump genes are expressed more in a biofilm which signifies their contribution in antibiotic resistance. Efflux pump gene PA1874-1877 was reported in P. aeruginosa. The expression level of this efflux pump gene is much higher than in planktonic bacterial cells (Zhang et al., 2008). The gene which encodes this efflux pump increases its resistance to antibiotics like gentamicin and ciprofloxacin.

2.5. Signaling in the biofilm
Signaling in the biofilm mainly occurs due to Quorum sensing. Quorum sensing is the regulation of gene expression at a high density of cells during cell to cell communication process. The concentration of autoinducers at high cell density reaches a threshold which causes the expression of the gene and regulates different physiological processes (Nickel et al., 1985). The secretion of small molecules (autoinducers) by the bacteria helps bacteria to communicate with each other for the formation of biofilm. A universal intergeneric signaling molecule Auto-inducer(AI)-2 plays a significant role in the formation of biofilm (Hardie et al., 2008; Bassler et al.,1997). Less concentration of AI-2 is enough for oral bacteria to aggregate. As the cell density of bacteria raises the concentration of AI2 also gets increased. At a maximum concentration of AI2, the strong biofilm is formed.

2.6. *Streptococcus mutans* biofilms

*Streptococcus mutans* a gram-positive bacterium which is mainly found in dental biofilms. It is found in abundance than other Streptococcus species like *S. sanguinis*, and *S. mitis* because of its acid tolerant nature and thus have the capability to survive at low pH on oral caries. Expression of many exoenzymes (glucosyltransferase) in *S. mutans* make it a main producer of EPS in the oral cavity. *S. mutans* can immediately colonize on the surface of the tooth and develop biofilms which are responsible for tooth decay (Koo et al,2004). The biofilm dependent lifestyle is observed in *S. mutans* (Bowen et al,2011; Burne,1998; Marsh,1999). High diversity of the microbial population rapidly and randomly is contacting with the proteinaceous material (pellicle) present on the surface of the tooth. A particular group of microorganisms including *Streptococci* and *Actinomyces* species may attach to the pellicle in low number and after some time these species adhere with different bacterial species (Nobbs et al,2011). *S. mutans* recognize particular salivary proteins like (CSP-I) common salivary protein-1 which then helps the bacterium to interact with salivary coated surfaces containing pellicles (Oli et al,2012). The oral bacteria are capable to invade the blood circulation and thus can cause many systemic diseases. Pathogenic bacteria found in the oral cavity are classified into the groups as pathogen responsible for dental caries and pathogen responsible for periodontitis. *Streptococcus mutans* is considered as prime bacteria which is responsible for dental caries and can cause endocarditis.

2.7. *Staphylococcus aureus* biofilm
Staphylococcus aureus is a gram-positive bacteria is usually present in nasal passages and ears of patients (Smith et al., 2011). Earlier studies indicated that S. aureus not only causes many infections like Osteomyelitis and chronic wound infections (Hansson et al., 1995) but also has an association to infections caused during dental implant. The biofilms formed by S. aureus particularly the methicillin-resistant S. aureus are tolerant to antimicrobial agents (Jones et al., 2001). The facial space infection caused by Staphylococcus aureus is an infection which can spread to the spaces in the head and neck regions from the infected tooth. This dentoalveolar infection if not treated immediately can cause serious problems in patients. The oral pathogens isolated from patients with this type of infection include Staphylococcus aureus and Streptococcus viridians. Other Gram-positive oral bacteria which have been isolated from the site of infection include Fusobacterium nucleatum and Spirochetes. Gram-negative bacteria like Pseudomonas species and Klebsiella species have been isolated from the site of infection.

2.8. Pseudomonas aeruginosa biofilm

Oral cavity can act as a source for respiratory pathogens due to poor oral hygiene. The existence of P. aeruginosa and Acinetobacter in the biofilm formed on subgingival surfaces and saliva of patients with oral diseases. The number of P. aeruginosa and Acinetobacter species in saliva and biofilm samples were found to be more in chronic periodontitis (CP) patients (Souto et al., 2014). The EPS matrix of P. aeruginosa play an important role in cystic fibrosis (formation of thick and sticky mucus in the lungs). Alginate, Pel and Psl are examples of some exopolysaccharides formed by P. aeruginosa (Rabin et al., 2015).

2.9. Medicinal plants with antibiofilm property

2.9.1. Juglans regia

The medicinal plants have been acknowledged to cure diseases generated by several oral pathogens one such commonly used medicinal plant is Juglans regia, commonly known as walnut is a big deciduous tree which is found in Iran, Baluchistan, Himalayan regions of India, Armenia and several temperate regions (Chopra et al., 1956). Almost all the parts of this large tree-like stem, bark, root, seeds, leaves and oil are used to cure several diseases (Taha et al., 2011). Efficiency of acetone and aqueous extracts of walnut has been determined by testing these extracts on saliva samples obtained from patients which have problems related to dental cavities, acetone extracts were depicted to be more efficient as antipathogenic medicine (Deshpande et al., 2011). The walnut
tree has antimicrobial property because of various phytochemical constituents present in it such as phenolic compounds, alkaloids, tannins, ellagic acid, flavonoids and steroids (Bandow et al., 2003). In India, Kashmir occupies the largest position in the total production of walnut. It is highly beneficial and common plant of Kashmir where it is known as Doon. People from this region use different parts of this tree to treat many diseases. Many bacteria possess resistance against various antibiotics and because of the high rate of chemical antibiotics side effects, plants with medicinal value can be included as alternatives to chemical antibiotics. Phenolic compounds of the root and stem are thought to be beneficial for human health, limiting the threat of infection by minimizing the oxidative stress (Silva et al., 2004). In addition to their anti-carcinogenic activity, the walnut bark has antioxidant and metal chelating activity (Middleton et al., 1998). The bark of walnut tree contains flavonoids, phenolic compounds, alkaloids and steroids (Bandow et al., 2003). The bark from *Juglans regia* is used as a tooth cleaner. In dentistry, walnut stem bark is recognized to treat infections initiated by oral pathogens.

### 2.9.2. *Acacia nilotica*

*Acacia nilotica* generally called as babul belonging to family Mimosaceae is a crucial multipurpose plant. Several parts of this plant are used to eliminate various types of infections. The stem bark of this plant possesses antioxidant, antibacterial and antioxidant properties (Ali et al., 2012). The stem bark of *Acacia nilotica* is used to treat many diseases like wound ulcers, leprosy, skin diseases, dental caries and gingivitis (Ali et al., 2012). Roots of this plant possess anticancerous properties and used for the treatment of tuberculosis and liver diseases (Kalaivan et al., 2010). The leaves and gum of this plant are used to relieve the pain caused by a sore throat. *Acacia nilotica* inhibited the growth of several oral microorganisms like *Streptococcus mutans* and *Pseudomonas aeruginosa* which are responsible for biofilm formation on tooth surface (Okoro et al., 2014).

### 2.9.3. *Terminalia arjuna*

*Terminalia arjuna* generally known as Arjuna family is considered as an important cardioprotective since ancient times. The essential phytocomponents present in the stem bark of Arjuna are alkaloids, flavonoids, phenolic compounds, terpenoids and tannins (Mandal et al., 2013). Arjuna extract showed effective antimicrobial activity against several oral pathogens. Arjuna is becoming successful as a substituent of chemical drugs for their effectiveness in preventing many oral diseases like periodontitis and dental caries. The bark of Arjuna has the capability to eliminate the oral microbes and thus preventing the onset of many oral infections. The bark of this plant...
showed antimicrobial activity against *Pseudomonas aeruginosa* and *Staphylococcus aureus* which are also responsible for the formation of oral biofilm.

### 2.9.4. *Azadirachta indica*

*Azadirachta indica* is generally known as Neem. *Streptococcus sanguis* subjected to pre-treatment with extract of neem showed a considerable reduction of the bacterial union to saliva conditioned hydroxyapatite, which is a complex of enamel and bone (Vanka *et al*., 2001). Insoluble glucan synthesis was also repressed with neem extracts, indicating that neem can reduce the attachment of *Streptococcus* on the tooth surface (Wolinsky *et al*., 1996). Neem extract formed the maximum inhibition zone on *Streptococcus mutans* at 50% concentration (Prashanth *et al*., 2007). The effect neem mouth rinse towards saliva levels of *Streptococcus mutans* has been studied. It was detected that the growth of *Streptococcus mutans* was repressed with or without the introduction of alcohol. The effectiveness of neem has been seen against *Candida albicans* and *Enterococcus faecalis*. Because of antimicrobial activities of neem plant makes it a probable mediator substituent for root canal irrigation (Vanka *et al*., 2001).

### 2.9.5 *Allium sativum:*

*Allium sativum* is generally called as garlic belongs to a genus, Allium. Aqueous extract of garlic possesses antibacterial property towards a broad variety of Gram-negative, Gram-positive bacteria and enterotoxin strains of *E. coli* which are resistant to different types of chemical drugs for this reason used to correct oral diseases (Ankri *et al*., 1999). Garlic extract remarkably inhibited *Streptococcus mutans* (Xavier *et al*., 2007) derived from dental caries of human this microorganism shows resistance to antimicrobial mediators like tetracycline, erythromycin, amoxicillin, and erythromycin (Fani *et al*., 2007). Fresh garlic extract displayed efficient effect than old one (Sasaki *et al*., 1999). Adverse effects were reported like the undesirable taste, foul breath, and vomiting (Groppo *et al*., 2007) but the effectiveness of garlic extract was greater than chlorhexidine towards the particular bacteria, therefore, can be considered as an efficient mouth rinse. Mouth rinse having 10% of garlic in a quarter Ringer solution showed an immense decrease of oral pathogenic bacteria (Elnima *et al*., 1983).

### 2.9.6. *Piper betle*

*Piper betel* is a vine leaf included in a family of Piperaceae. Aqueous extract from leaves in crude form showed the decreased effect on the growth, attachment capability and glucosyltransferase
action against the *Streptococcus mutans* (Nalina *et al.*, 2007). Another study concluded that on exposure of bacterial species to *Piper betle* leaf extract indicated immense ultrastructural changes. Relating to dental biofilm the repressed growth of bacteria can hinder the biofilm on the surface of the tooth. Consequently, the introduction of this extract in mouthwash can control oral plaque (Nalina *et al.*, 2007).

2.9.7. **Rosemary officinalis and Cinnamon verum**

*Rosemary officinalis* is a woody herb with sweet smelling indigenous to Mediterranean area. Rosemary showed great effects against *Streptococcus mutans* (Dalirsani *et al.*, 2011). Its antimicrobial activity was greater than chlorhexidine mouthwash (Bozin *et al.*, 2007). More studies are recommended for making of herbal mouth rinse. Essential oils obtained from it indicated antimicrobial properties towards several bacteria (Bozin *et al.*, 2007). The higher antimicrobial property was seen against the strains *Arcobacter butzleri* using extracts of cinnamon, sage, barberry, chamomile and rosemary (Vanka *et al.*, 2001). Aqueous extract from cinnamon acquires great antimicrobial property towards *Pseudomonas aeruginosa* (Ibrahim *et al.*, 1991). When a mixture of Chinese chive, *cornifructus*, and the cinnamon extract was formed it possessed antimicrobial property towards *Escherichia coli*. These extracts showed high stability towards pH and heat (Mau *et al.*, 2001). Chamomile reduced the number of *Helicobacter pylori* (Weseler *et al.*, 2005). The extracts of chamomile have reasonable antimicrobial properties (Mckay *et al.*, 2006).

2.9.8. **Syzygium aromaticum**

*Syzygium aromaticum* generally known as clove is indigenous to Indonesia which is used as a spice throughout the world. The clove oil is commonly used to cure a toothache. Methanolic extracts in crude form possessed great effect towards Gram-negative pathogen which causes gum diseases (Cai *et al.*, 1996). Clove and its bud oil have a strong effect against five dental cavity causing microorganisms such as *Candida albicans*, *Staphylococcus aureus*, *Lactobacillus acidophilus* and *Saccharomyces cerevisiae* (Cai *et al.*, 1996).

2.9.9. **Mimusops elengi**

Is an average sized tree which is evergreen and concentrated to tropical forests of Asia and Australia. It is generally called as Spanish Cherry, Maulsari in Hindi, Bokul in Assamese and
Bakul in Bengali. The extract from the bark was examined to check its effect against the oral microbes taken from the children 6-12 years of age. The outcome verified the effect of this plant and showed that acetone extracts can be applied to cure diseases caused by oral pathogens (Desphande et al., 2010).

2.9.10. *Punica granatum*

*Punica granatum* is indigenous to Asia. The effect of extract towards numerous oral bacteria is because of phytoconstituents like Tannins and Alkaloids in leaves, fruits, root and stem (Silva et al., 2008). There is growing curiosity in using tannins as antimicrobial mediators to eliminate oral pathogens responsible for many oral diseases (Scalbert, 1991). The extracts from plant usually ethanolic extract, Aqueous extract, methanolic extract, and acetone extract showed a high effect in several studies on numerous bacteria (Reddy et al., 2007). In another study, the gel obtained from this plant showed the positive effect by hindering the biofilm formation (Pereira et al., 2006). A study of hydroalcoholic extracts of fruits of this plant showed an efficient antimicrobial property towards dental plaque pathogens (Menzes et al., 2006). Because of its anti-inflammatory properties, it is used to correct many throat infections, cough, and fever (Salgado et al., 2006). The most important constituents present in pomegranate are flavonoids, anthocyanidins, ellagic acid, ellagitannins, flavones, estrogenic flavonols and punicic acid (Cesar et al., 2003). Juice, seed oil and peel extract of pomegranate possess reduced effect on prostate cancer (Jurinka et al., 2008). A study reported that extract from pomegranate was more efficient to decrease the biofilm than chemical drugs when three or more organisms were involved in biofilm formation (Cesar et al., 2003).

2.9.11. *Emblica officinalis*

Generally, known by different names like amala, Indian gooseberry, oval and amlaki. The various phytoconstituents like phenols, flavonoids, tannins, polyphenols, ellagic acid, and gallic acid are present in this plant (Nair et al., 2007). They have a capability to prevent oral infections by reducing the negative factors of pathogenic microbes such as *Streptococcus mutans* and *Lactobacillus* (Hasan et al., 2007). The effect of amala was greater than chlorhexidine and can serve as an effective mouth rinse (Hasan et al., 2007).
2.9.12. Sanguinaria Canadensis

*Sanguinaria Canadensis* generally called as bloodroot is a flowering perennial plant found in North America and Canada. A mouthwash and toothpaste with extract of *Sanguinaria Canadensis* given for at least six months during the medical treatment decreased the dental biofilm by 57% and inflammation of gum by 60% than the control group where the effect was 27% and 21% respectively (Eley et al., 1999). At a concentration of 16 microgram/liter, this plant reduced 98% of the microbial population from the dental biofilm of human and combining effect of extract from *Sanguinaria Canadensis* and zinc suppressed the number of different streptococci found in the mouth (Eley et al., 1999).

2.9.13. Cratoxylum formosum

Is generally called as Mempat. The gum obtained from Mempat is a natural mediator that has been used broadly by tribal people in Thailand to reduce dental cavities (Suddhasthira, 2006). A gum obtained from Mempat showed a great effect towards *Streptococcus mutans* and can act as a good herbal product against dental cavities (Suddhasthira, 2006).

2.9.14. Acacia catechu

*Acacia catechu* belonging to family Fabaceae and subfamily Mimosoideae is broadly used Ayurveda for treatment of many diseases. The heartwood extract from *Acacia catechu* possesses an efficient effect against various microbes which cause dental cavities (Geetha et al., 2011). A research on this plant concluded that about 80-95% of reduction was seen in biofilm till 15 days (Kumar et al., 2009). Effective and broadest antimicrobial effect was found with ethanolic extract against several oral pathogens *Staphylococcus aureus* and *Pseudomonas aeruginosa* (Gulzar et al., 2010). Hence Acacia proved to be great antimicrobial mediator towards oral infections which are caused mainly by *Streptococcus mutans*.

2.9.15. Curcuma longa (Turmeric)

Turmeric requires a temperature of 20-30°C. Turmeric consists of tubers whose length is 2-3 inches and having a diameter of one inch. Because of its antibacterial properties, it is used to eliminate dental diseases (Ramirez et al., 1995). In Ayurveda, it is extensively used from a long time having no side effects and has different medicinal properties which include analgesic, anti-inflammatory, antioxidant, antiseptic and anti-carcinogenic activity (Kiso et al., 1983). In another study, it was detected that curcumin can cure different pre-cancerous situations such as oral submucous fibrosis,
lichen planus, and leukoplakia. The oil from turmeric possessed oncopreventive activity (Das et al., 2010) and cure numerous oral diseases. Application of roasted, ground turmeric reduces the pain and swelling. Another study concluded that turmeric solution can greatly reduce the gum inflammation than chlorhexidine (Suhag et al., 2007) As compared to chlorhexidine the usage of herbal oral rinses like neem, turmeric and Triphala showed higher decrease in biofilm, inflammation of gums and gum bleeding. Mixture of various herbal components helps in maintaining good oral hygiene as a result reduces dental biofilm and decreases inflammation of gums (Al-Tae et al., 2012; Atul et al., 2011)

Table-2.1: List of Plants used in the study to inhibit biofilm formation

<table>
<thead>
<tr>
<th>Plant</th>
<th>Classification</th>
<th>Part used</th>
</tr>
</thead>
</table>
| **Juglans regia** | Kingdom-Plantae
Division- Magnoliophyta
Class- Magnoliopsida
Family- Juglandaceae
Genus- Juglans
Species- regia | Stem bark |
| **Acacia nelotica** | Kingdom-Plantae
Division- Magnoliophyta
Class- Magnoliopsida
Family- Mimosaceae
Genus- Acacia
Species- nelotica | Stem bark |
| **Terminalia arjuna** | Kingdom- Plantae
Division- Magnoliophyta
Class- Magnoliopsida
Family- Combretaceae
Genus- Terminalia
Species- arjuna | Stem bark |