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# Antimicrobial Properties of Three Different Bioactive Compounds of Cassia Species Against *S. mutans* Serotype C (ATCC 25175). An Invitro Study

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## ABSTRACT

**Introduction:** Cassia species (Caesalpinaceae) is a medicinal plant used in traditional Indian medicine for various ailments. In this study, three different bioactive compounds of two medicinal plant species Cassia tora and Cassia fistula were obtained and their antimicrobial properties were compared and evaluated against *S.mutans* serotype C (ATCC25175) and were conducted as a triple-blind study to identify their effectiveness.

**Aim:** The study aims to evaluate and compare the antimicrobial properties of three different Bioactive Compounds of Cassia species and 0.2% chlorhexidine against *S. mutans* serotype C (ATCC 25175).

**Results:** All the Bioactive Compounds had good antimicrobial activities based on their zones of inhibition; the highest zone of inhibition in mm was formed by Extract 8 which was Cassia Flower extract having a mean inhibitory zone of 9.93mm +0.76. Statistical analysis of the results with Kruskal Wallis and Mann Whitney Post Hoc test proved that at any concentration of Extract 8 the inhibition results are comparable to that of 0.2% chlorhexidine with p=0.05. The results confirmed the antimicrobial potential of the Bioactive Compound of Cassia Flower and hence it can be used as a preventive means for dental caries.

**Conclusion:** The Bioactive Compound of Cassia Flower extract derived from cassia plants are only required in minute quantities as compared to their crude extracts. The study confirmed the antimicrobial potential of the plant at different concentrations can be used as preventive and therapeutic measures in preventive dentistry and due to its reduced potency can be used in children effectively.

**Keywords:** Bioactive compounds, Chlorhexidine, Cassia, Dental caries, Herbal medicines

## INTRODUCTION

The Global Burden of Disease Study in 2016 estimated that 2.4 billion people suffer due to dental caries of permanent teeth worldwide and an additional 486 million children suffer from caries of primary teeth.<sup>1</sup> Thus, prevention is important as it not only detrimentally impacts the quality of life but also becomes a financial drain due to the need for extensive treatments. Prevention can be achieved by maintaining the ecological balance of the naturally existing cell structures. Zaura et al. in 2014 reported that maintaining the stability of the oral biome is important to prevent ‘dysbiosis’ and ac-

ording to the author acute infections of the oral mucosa occur but are rare.<sup>2</sup>

The oral microbiota survives the daily chemo-mechanical insults from either food or oral hygiene practices which results in variations in temperature, pH or symbiotic microbes. By maintaining the state of dynamic equilibrium within a community of organisms subject to gradual changes ecological homeostasis can be achieved.

According to He et al., 2011 and Schlafer et al., plaque microflora have a symbiotic relationship with the host, acting as a barrier to opportunistic pathogens and carrying out metabolic

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processes that benefit the host.<sup>3,4</sup> The emerging need is to target pathogenic microorganisms thus shifts the focus towards maintaining the 'holobiont'.<sup>5</sup>

Bowden in 1996 reported that Mutans streptococci, particularly *Streptococcus mutans* as significant odontopathogens that are implicated to be highly associated with caries in humans.<sup>6</sup> *Streptococcus mutans* being acidogenic and aciduric drives the microbial ecological shift that leads to dental caries and thus appears in the primary teeth of children even under six years of age.<sup>7,8</sup>

Chemotherapeutic antimicrobial agents are known to be lethal for the normal commensals and cellular structures. Chlorhexidine is the commonly used potent agent with a proven efficiency that also targets the natural microflora.<sup>9,10</sup> It has a risk of developing skin injuries, such as skin erythema, burns, blisters discolouration of teeth and xerostomia in the mouth.<sup>11,12</sup> Naturally occurring organic substances have fewer disadvantages compared to synthetically obtained chemical plaque or caries inhibiting agents and are milder for use especially in children and infants.<sup>13</sup>

Cassia species (Caesalpinaceae) is a medicinal plant used in traditional Indian medicine for various ailments and grows annually in all tropical areas.<sup>14</sup> Over 5000 species of cassia flowering plants exist and possesses significant anti-inflammatory, antimicrobial antifungal properties and antioxidant properties.<sup>15,16</sup>

Bioactive Compounds are the main active ingredients or chemical substances which may be found in all parts of the plant and have biologically beneficial effects.<sup>17</sup> Thus, the study aimed to compare and evaluate the antimicrobial properties of three different Bioactive Compounds of Cassia species and 0.2% chlorhexidine against *S. mutans* serotype C (ATCC 25175).

## METHODS

This study was approved by the Institutional ethics committee of Dr DY Patil Vidyapeeth Pune, Maharashtra, India (ref: DPU/R&R(D)/32(21)/19) and was conducted as a triple-blind study. The aim was to evaluate and compare the antimicrobial properties of 3 different bioactive compounds at three concentrations (200mcg, 400mcg, 600mcg) and 0.2% chlorhexidine gluconate against *S. mutans* serotype C (ATCC 25175).

### Extract Preparation<sup>18</sup>

The extracts of cassia species were obtained from the scientist which was from the two medicinal plant species *Cassia tora* and *Cassia fistula* from Western Pune Maharashtra, India, shade dried authentication was done by comparing with herbarium specimens preserved in the Botanical Survey

of India, Pune (Maharashtra). Authentication no of Cassia tora BSI/WC/Cert/2015/SG01, Cassia fistula is BSI/WC/Cert/2015/SG02.<sup>18</sup> The extracts were then finely pulverized and exactly weighed plant material was utilized to prepare extracts with measured volumes of solvents like ethyl acetate, acetone, ethanol, methanol and distilled water then removed under pressure. Weighed amounts of the extracts marked at Extract 6, Extract 7 and Extract 8 were then collected from the scientist and utilized for the study.

### Microbiological assay laboratory processing<sup>19</sup>

Well, the diffusion method was used to determine the zone of inhibition of 3 bioactive extracts in the concentration of (200µg, 400µg, 600µg) against mutans serotype C (ATCC 25175). All the tests were performed under sterile conditions in triplicate by standard norms and protocols. 0.2% chlorhexidine against *S. mutans* serotype C (ATCC 25175) was used as the positive control. An adequate amount of Mueller Hinton Agar was evenly distributed over the surface of 15 cm diameter petri-dish to a thickness of 5 mm and allowed to solidify under aseptic conditions. *Streptococcus mutans* serotype C (ATCC 25175) was inoculated with a sterile spreader on the agar medium. Standard wells were made with a cupbearer (9.0mm)

Then 0.2% chlorhexidine gluconate, bioactive Compounds of cassia species with concentrations of 200µg, 400µg, 600µg in 0.5ml DMSO solvent was inserted in separate wells of agar plates inoculated with *Streptococcus mutans* serotype C (ATCC 25175). The plates were incubated at  $37 \pm 0.1^\circ \text{C}$  for 24 hours. The same procedure was followed for different concentrations of bioactive compounds. After incubation, the plates were observed for the zone of inhibition and measured in millimetres.

## RESULTS

The zones of inhibition in mm were obtained. The results were compared statistically with chlorhexidine in the form of mean and standard deviation. The p values were obtained. Kruskal Wallis and Mann Whitney Post Hoc test was conducted to analyze the data.

The tables and graphs below project the mean and standard deviation of inhibition zones at 200µg, 400 µg, 600µg for the three Bioactive compound extracts- Extract 6, Extract 7, Extract 8 and 0.2%chlorhexidine gluconate against *S. mutans* serotype C (ATCC 25175) and their comparison.

**Table 1, Figure 1** showed the extracts with inhibition zones at 200µg, 400 µg, 600 µg for the three Bioactive compound extracts- Extract 6, Extract 7, and Extract 8 were compared and evaluated against *S. mutans* serotype C (ATCC 25175). At 200µg concentration, the highest zone inhibition in mm

was formed by Extract 8 concentration having a mean inhibitory zone of  $2.03\text{mm} \pm 0.86$ . At  $400\mu\text{g}$  concentration, the highest zone inhibition in mm was formed by Extract 8 concentration having a mean inhibitory zone of  $6.57\text{mm} \pm 0.81$ . At  $600\mu\text{g}$  concentration, the highest zone inhibition in mm was formed by Extract 8 concentration having a mean inhibitory zone of  $9.93\text{mm} \pm 0.76$ . At all concentrations of extract, 8 showed the highest zone of inhibition. For all concentrations of Extracts 6, 7, and 8 at  $200\mu\text{g}$ ,  $400\mu\text{g}$  and  $600\mu\text{g}$  there was a significance of  $p = 0.027$ . The mean inhibition zone against *S. mutans* serotype C (ATCC 25175) was formed by 0.2% chlorhexidine which was  $16.07 \pm 0.30$  (Graph 2).

**Table 2** depicts the Post hoc test or Kruskal Wallis and Mann Whitney presenting differences between the extracts 6, 7 and 8 at a concentration of  $200\mu\text{g}$ ,  $400\mu\text{g}$ ,  $600\mu\text{g}$  revealed that a significant difference was present between  $200\mu\text{g}$  and  $600\mu\text{g}$  concentrations with  $p = 0.021$ . A significantly large inhibition zone was formed against *S. mutans* serotype C by  $600\mu\text{g}$  concentration as compared to  $200\mu\text{g}$  concentration. No significant difference was found between  $200\mu\text{g}$  and  $400\mu\text{g}$  concentration as well as  $400\mu\text{g}$  and  $600\mu\text{g}$  concentration.

For Extract 7 at  $200\mu\text{g}$  Post hoc test revealed that a significant difference was present in the inhibition zone when compared to  $600\mu\text{g}$  extract the significance was  $p = 0.022$ . No significant difference was found between  $200\mu\text{g}$  and  $400\mu\text{g}$  concentration as well as  $400\mu\text{g}$  and  $600\mu\text{g}$  concentration.

For Extract 8 at  $200\mu\text{g}$  extract,  $400\mu\text{g}$  extract and  $600\mu\text{g}$  Post hoc test revealed that a significant difference was present between  $200\mu\text{g}$  and  $600\mu\text{g}$  with  $p = 0.022$ . A significantly large inhibition zone was formed against *S. mutans* serotype C by  $600\mu\text{g}$  concentration. No significant difference was found between  $200\mu\text{g}$  and  $400\mu\text{g}$  concentration as well as  $400\mu\text{g}$  and  $600\mu\text{g}$  concentration.

**Table 3** showed the difference between the inhibition zones of Extract 6, 7 and 8 at different concentrations of  $200\mu\text{g}$ ,  $400\mu\text{g}$ ,  $600\mu\text{g}$  and 0.2% chlorhexidine. The mean inhibition zone against *S. mutans* serotype C (ATCC 25175) was formed by 0.2% chlorhexidine which was  $16.07 \pm 0.30$  (Figure 2).

For Extract 6 a significant difference was found between  $200\mu\text{g}$  extract concentration and 0.2% chlorhexidine with  $p = 0.046$ . No significant difference was found between  $400\mu\text{g}$  and chlorhexidine,  $600\mu\text{g}$  and chlorhexidine indicating that the higher concentration of bioactive compounds showed a comparable action as that of chlorhexidine. The difference between  $600\mu\text{g}$  of Extract 6 and 0.2% chlorhexidine was  $9.53 \pm 0.050$  with  $p = 0.05$ .

For Extract 7 at  $200\mu\text{g}$ ,  $400\mu\text{g}$ ,  $600\mu\text{g}$  and 0.2% chlorhexidine no significant difference was present between any of the extracts and chlorhexidine indicating that at any concentration of Extract 7 the inhibition results are comparable to that of 0.2% chlorhexidine with  $p = 0.05$ . The difference

between  $600\mu\text{g}$  of Extract 7 and 0.2% chlorhexidine was  $9.43 \pm 0.050$ .

For Extract 8 at  $200\mu\text{g}$ ,  $400\mu\text{g}$ ,  $600\mu\text{g}$  and 0.2% chlorhexidine no significant difference was present between any of the extracts and chlorhexidine indicating that at any concentration of Extract 8 the inhibition results are comparable to that of 0.2% chlorhexidine with  $p = 0.05$ . The difference between  $600\mu\text{g}$  of Extract 8 and 0.2% chlorhexidine was only  $6.13 \pm 0.050$ .

## DISCUSSION

Current evidence suggests that the cariogenic factors that drive streptococcus mutans to be virulent are its potential to thrive in acidic environment's, its ability to lower the pH, ferment simple sugars, synthesize sucrose, promote its adhesion and buildup the plaque-ecology especially for those having a low socioeconomic status with limited access to healthcare.<sup>20</sup> Milsom et al. described that children with an already existing caries lesion have a 5–6 times higher incidence of developing new caries lesions compared to previously caries-free children.<sup>20</sup>

Peterson et al (2014) concluded with next-generation sequencing (NGS) that Streptococcus species was found to be the most abundant genus (>50% of the microorganisms) in the microbial composition of the dental plaque.<sup>21</sup> Damle et al (2016) concluded that the number of *S. mutans* colonies increases with increasing age and it is more in children with lesions that can be seen clinically and the amount of *S. mutans* in the saliva is directly proportional to the colonized surfaces.<sup>22</sup>

In this study chlorhexidine (CHX) was used as the control as it is the most extensively used anti-plaque and anti-bacterial agent and it is highly active against the *S. mutans* strain. Among the available chemotherapeutic agents, chlorhexidine mouthwash is considered the "gold standard" due to its proven efficiency, but it exhibits cytotoxic activity on ectodermal cells causing altered taste sensation and extended use causes xerostomia, brown-staining of teeth and fillings.<sup>23</sup>

Therefore, the nature-based alternatives with fewer side effects would be beneficial than the chemically prepared agents. They also have a two-fold advantage of minimal side-effects and being alcohol-sugar free. Nature-based plant products like Aloe vera, Bloodroot Garlic and Propolis have several advantageous properties anti-microbial, wound healing, anti-helminthic, anti-inflammatory, anti-fungal properties of medicinal plants.<sup>24</sup>

In this study, Cassia plant species were selected for their anti-inflammatory, hypoglycaemic, antiplasmodial properties.<sup>25</sup> Thus can be used as a broad-spectrum antibacterial agent that is nature-based. It is easily obtainable, in Asia, South

Africa, Mexico, China, East Africa and Brazil thus making it cost-effective.<sup>26</sup>

Bioactive compounds are substances are present within all part of the plant such as flavonoids which includes a huge group of naturally occurring organic compounds. It is found in a large variety of plants including fruits, seeds, grains, sea vegetables, nuts, and wine.<sup>26</sup>

The Microbiological assay of the good diffusion method was used to determine the zone of inhibition and after incubation at 37°C for 24 hours as per standard protocols. The diameters of inhibition zones produced by the plant extract were measured in mm.

Statistical analysis of the results with Kruskal Wallis and PostHoc Mann Whitney Test and concluded that the mean and standard deviation of inhibition zones at 600µg for the three Bioactive compounds extracts- against *S. mutans* serotype C (ATCC 25175) identified that the highest zone inhibition in mm was formed by Extract 8 concentration having a mean inhibitory zone of 9.93mm ±0.76 followed by Extract 7 was 6.63 mm±0.30 inhibition zone. The least effective concentration of Bioactive compound of cassia species was found with Extract 6 which formed a small zone of 6.53 mm±0.55.

The three bioactive compounds provided by the scientist were Quercetin (Extract 6), Gallic acid (Extract 7), and Cassia flower (Extract 8). All three bioactive compounds have antimicrobial properties based on their zones of inhibition, but the extract labelled as number 8 which is Cassia flower (Tora and Fistula) showed the highest antimicrobial zone when compared to the three extracts.

Quercetin (Extract 6) was isolated and found from both Cassia tora L as well as Cassia Fistula.<sup>26</sup> Quercetin is the main bioflavonoid known for its anti-inflammatory, anti-hypertensive, vasodilator effects, anti-obesity, anti-hypercholesterolemic and anti-atherosclerotic activities.<sup>26</sup> It is a naturally occurring flavonoid pigment present in many fruits and vegetables. It is antioxidant-rich and plays an important role in helping the body. It is not only an antioxidant but also has anti-inflammatory and anticancer activities.<sup>27</sup> Quercetin induces apoptosis by inducing the dissociation of Bax from Bcl-xL, activating caspases, and inhibiting phosphorylation.<sup>27</sup> It is metabolized immediately by enzymes in the epithelial cells and further metabolized by the liver. Thus quercetin has proven effects that benefit oral health.<sup>27</sup> This study proved that Quercetin has an antibacterial effect with a zone of inhibition of 6.53 mm±0.55. This was compared to the zone of inhibition of 0.2% chlorhexidine which was 16.07mm. The zone of inhibition was also compared to the other extracts and was found to be the least.

Gallic acid (GA) is in the group of hydroxybenzoic acids.<sup>28</sup> Many plant extracts have been found to inhibit the growth of

oral bacteria, particularly *S. mutans* and thus may prevent the formation of dental caries. It is the most popular trihydroxy benzoic acid. An important source of gallic acid is also tea containing up to 4.5 g/kg fresh weight in tea leaves it also occurs mostly in certain red fruits, black radish, and onions. Apart from its phytochemical role, gallic acid is also used in tanning, ink dyes, and the manufacture of paper.<sup>28</sup> Effects include antihyperglycemic, anti-lipid peroxidative, antioxidant antimicrobial, anti-inflammatory, anticancer, cardioprotective, gastroprotective, and neuroprotective effects.<sup>29</sup> Gallic acid can inhibit motility, adherence and biofilm formation of *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Streptococcus mutans*, *Chromobacterium violaceum*, and *Listeria monocytogene*.<sup>29</sup> In this study the zone of inhibition of Gallic acid on streptococcus mutants serotype C (ATCC 25175) was evaluated at the 600µg concentration the zone was 6.63 and compared with 0.2% chlorhexidine which was 16.07. The zone of inhibition in mm for Extract 7 (Gallic Acid) was more when compared to the bioactive compound of cassia species Extract 6 (Quercetin).

Cassia flower is also known as the ‘Golden Shower’ is widely grown as a decorative tree for its beautiful bunches of yellow flowers. Cassia plants have bright yellow flowers of characteristic- bell shape drop.<sup>30</sup> Cassia provides dyes and is widely used for its medicinal properties. The plant has antipyretic and analgesic effects.<sup>31</sup> It was found that extracts of Cassia fistula flowers contained tannins, flavonoids, and anthraquinones compounds in higher amounts.<sup>32</sup> Cassia flowers have known antibacterial and antifungal activity. In the present work, the extracts are obtained from Cassia fistula flowers. All the Bioactive Compounds had good antimicrobial activities based on their zones of inhibition but of all the three; the extract of cassia flower showed the highest zone inhibition in mm was formed by Extract 8 (Cassia Flower) concentration having a mean inhibitory zone of 9.93mm ±0.76. Mann Whitney test revealed that at any concentration of Extract 8 the inhibition results are comparable to that of 0.2% chlorhexidine with p=0.05. The difference between 600 µg of Extract 8 and 0.2 % chlorhexidine was only 6.13±0.050.

The Cassia flower extract can be an excellent substitute for chlorhexidine which can be prepared in various forms like mouthwashes, gels, sustained-release forms, intracanal medicaments, lollipops etc. The bioactive compound extract of cassia flower can be used as new antimicrobials for children and adults as well as for the prevention of dental caries. Nowadays, patients are more aware of the harmful effects of synthetic formulations and prefer a natural herbal alternative that is well tolerated by the body. Thus, these natural herbal formulations with their bioactive compound extract can offer a non- potent resolution with minimal adverse effects to improve the oral health of children.

## CONCLUSION

Nature-based cassia plant species are available in all seasons and easily obtainable in many countries and hence provides a novel therapeutic future for combating oral diseases like caries in especially in children. Based on the findings of the study, it was concluded that: (1) The bioactive components of cassia flower extracts appears promising against *Streptococcus mutans* serotype c (ATCC 25175) and can be used effectively as a novel compound especially in children due to the nature-based non-synthetic properties for combating dental caries. (2) Future studies are needed to determine the antimicrobial activity of Cassia flower plant extract against *Streptococcus mutans* on the whole saliva to understand its efficacy against polymicrobial or mixed culture. (3) Lastly toxicological investigations of Cassia plant extract also need to be found out to confirm its safety for human trials.

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**Table 1: Descriptive data regarding inhibition zones at 200µg, 400µg and 600µg for the three Bioactive compounds**

Groups	N	200 µg Mean± SD	400 µg Mean± SD	600µg Mean± SD	Test statistics	Significance (p)
Extract 6	3	0.13± .06	3.50± .10	6.53± .55	7.26	<b>0.027*</b>
Extract 7	3	0.23±.15	4.07± .51	6.63± .30	7.20	<b>0.027*</b>
Extract 8	3	2.03±.86	6.57± .81	9.93± .76	7.20	<b>0.027*</b>

\*Significance at  $p < 0.05$

A significant difference between the extracts and different concentrations was present for Extract 6, 7 and 8.

**Table 2: Post hoc test presenting the difference between the concentrations of Extract 6, 7 and 8**

Extract	Concentration	Mean Difference	Significance(p)
Extract 6	200µg	400µg	3.37
		600µg	6.40
	400µg	200µg	3.37
		600µg	3.03
	600µg	200µg	6.40
		400µg	3.03
Extract 7	200µg	400µg	3.83
		600µg	6.40
	400µg	200µg	3.83
		600µg	2.57
	600µg	200µg	6.40
		400µg	2.57
Extract 8	200µg	400µg	4.53
		600µg	7.90
	400µg	200µg	4.53
		600µg	3.37
	600µg	200µg	7.90
		400µg	3.37

\*Significance at  $p < 0.05$

A significant difference is present between 200 and 600µg, with Extract 6

A significant difference is present between 200 and 600µg with Extract 7

A significant difference is present between 200 and 600µg with Extract 8

**Table 3: Difference between Extract 6, 7 and 8 versus 0.2% chlorhexidine concerning inhibition zones at 200,400 and 600µg**

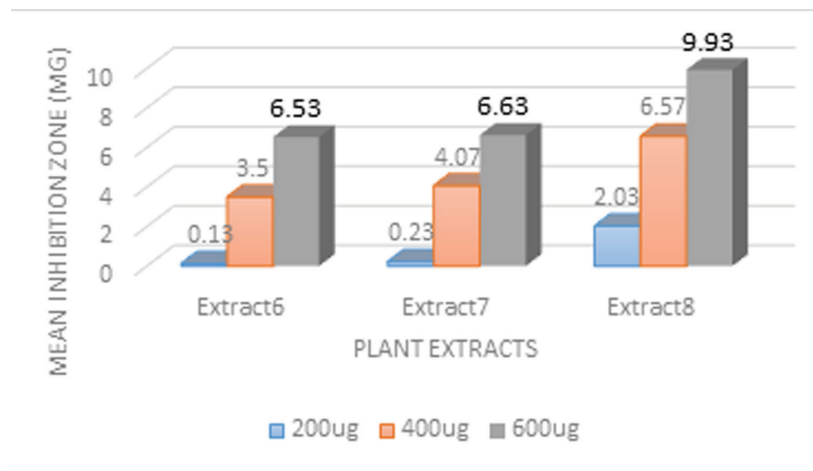
Extract	Concentration	N	Mean	Difference	Mann-Whitney U	Significance (p)
Extract 6	200 µg	3	0.13			
	0.2% Chlorhexidine	3	16.07	15.93	0.00	<b>0.046*</b>
	400 µg	3	3.50			
	0.2% Chlorhexidine	3	16.07	12.57	0.00	0.050
	600 µg	3	6.53			
	0.2% Chlorhexidine	3	16.07	9.53	0.00	0.050
Extract 7	200 µg	3	0.23			
	0.2% Chlorhexidine	3	16.07	15.83	0.00	0.050
	400 µg	3	4.07			
	0.2% Chlorhexidine	3	16.07	12.00	0.00	0.050
	600 µg	3	6.63			
	0.2% Chlorhexidine	3	16.07	9.43	0.00	0.050
Extract 8	200 µg	3	2.03			
	0.2% Chlorhexidine	3	16.07	14.03	0.00	0.050
	400 µg	3	6.57			
	0.2% Chlorhexidine	3	16.07	9.50	0.00	0.050
	600 µg	3	9.93			
	0.2% Chlorhexidine	3	16.07	6.13	0.00	0.050

\*Significance at  $p < 0.05$

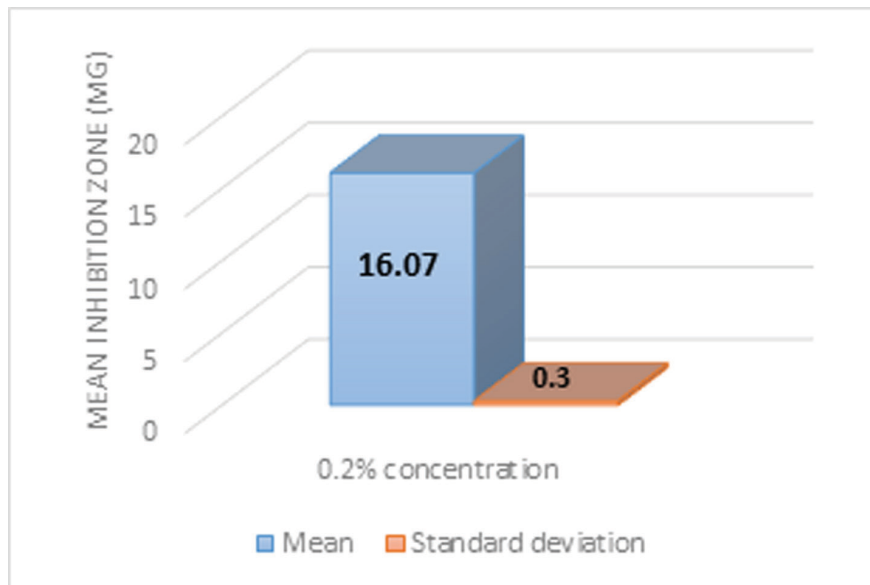
For Extract 6- A significant difference is present in the inhibition zone between 200µg and 0.2% Chlorhexidine

For Extract 7- No significant difference is present in inhibition zone between 200µg and 0.2% Chlorhexidine, 400µg and 0.2% Chlorhexidine, 600µg and 0.2% Chlorhexidine

No significant difference is present in inhibition zone between 200µg and 0.2% Chlorhexidine, 400µg and 0.2% Chlorhexidine, 600µg and 0.2% Chlorhexidine



**Graph 1:** Bar diagram representing mean inhibition zone of three plant extracts at 200, 400, 600 µg



**Graph 2:** Bar diagram representing mean inhibition zone of 0.2% chlorhexidine