

## The Role of Natural Products in Oral Health

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### Abstract

**Background:** Halitosis is the unusual bad odor of mouth which is mainly caused by bacteria in the oral cavity which produces volatile sulfur compounds (VSCs). The main origin of these bacteria is on a tongue. Halitosis causes serious problems for health and social relation of patients. The aim of this study was to the evaluation of some selected natural products in the treatment of halitosis through inhibition of enzymes responsible for VSCs production.

**Materials and Methods:** Samples were collected through scrapping the back of tongue of individuals and cultured on BHI medium. Number of bacteria in samples was calculated using serial dilution method along with optical density determination. Cell lysis of bacteria was obtained and used as enzyme source. Effect of natural products including chlorhexidine, zinc citrate, zinc acetate, zinc chloride, caffeine, gallic acid, nicotinic acid and trigonelline was assessed on L-cysteine desulphydrase

**Results:** However L-cysteine desulphydrase assay with bacterial lysate did not show significant changes in absorbance of samples especially control tube, assay of the enzyme using whole bacteria indicated remarkable differences between control and treatments. Accordingly, control tube showed the highest amount of absorbance (0.183) compared to samples containing natural products. Natural products decreased the absorbance in a relatively similar range. The most effective natural product with enzyme inhibitory effect was identified to be trigonelline with the absorbance of 0.103. Zinc derivatives including zinc citrate (0.12), zinc chloride (0.11), and zinc acetate (0.12), gallic acid (0.11), caffeine (0.12), and nicotine amide (0.11) indicated similar activities.

**Conclusion:** Natural products have an inhibitory effect on one of the main enzymes contributing in halitosis named L-cysteine desulphydrase. Although trigonelline showed the strongest inhibitory activity other natural products also inhibited the enzyme significantly. This study has introduced natural products as a promising source in the treatment of halitosis.

**Keywords:** Halitosis; Oral Malodor; Vscs; Periodontal Region; Mouth Malodor; Oral Cavity; Anaerobic Bacteria; Rhino Halitosis; Gram -ve Bacteria; Dentures; Saliva; Gas Chromatography; Microorganisms; Antibacterial Agent; Natural Product; Bacterial Enzyme; Enzyme Activity; Lysed Bacteria; Inhibitory Activity; Colony

### Abbreviations

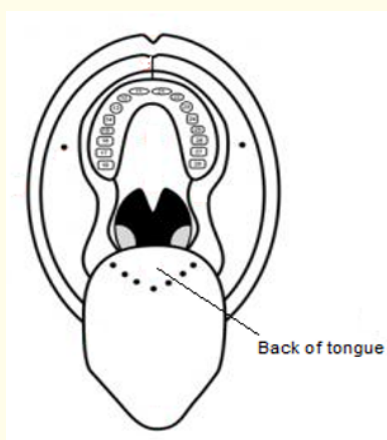
VSCs: Volatile Sulfur Compounds; PCR: Polymerase Chain Reaction; LPS: Lipopolysaccharide; BHA: Brain Heart Infusion Agar; BHI: Brain Heart Infusion; NA: Nutrient Agar; PBS: Phosphate Buffer Solution; OD: Optical Density; CFU: Colony Forming Unit

### Introduction

#### Background

Halitosis is defined as the unpleasant odor of mouth that can be originated from oral or non-oral source [1,2]. This issue causes serious social problems for people in society. The unusual bad odor of mouth that may be caused by some drinks (coffee, orange juice...) and foods (garlic, onions...) is different from halitosis. A number of bacteria are responsible for oral malodor such as *Treponema denticola*, *Porphyromonas gingivalis*, and *Bacteroides forsythus* that should be basically recognized and treated. Oral malodor occurs principally by volatile sulfur compounds (VSCs) such as hydrogen sulfide, methyl mercaptan, dimethyl sulphide, butyric acid, putrescine, ammonia, amines, and cadaverine [3-5] that are the products of protein degradation by microbial community.

The main source of VSC's producing bacteria is on the tongue [6]. The back of the tongue and periodontal region have been known as the important places for the formation of volatile compounds (Figure 1) [7]. Additionally, mouth malodor may have a non-oral origin, it has been indicated that some disorders such as upper respiratory disease are responsible for the bad odour of mouth or gastrointestinal disorders [8,9]. National Institute of Dental Research reports that 65 million Americans endure from malodor [10-12].



**Figure 1:** Schematic illustration of the oral cavity. Back of the tongue is an important part of oral cavity in halitosis [13].

## The origin of halitosis

### Oral cavity

Malodor mostly comes from the mouth as this part of the body is responsible for approximately 85% of bad breath [5,14]. People with restrict mouth hygiene and good teeth arrangement, the major reason of malodor is probably to be the dorsal area of a tongue [4]. Access to this part to study the microbial community is possible using a plastic spoon or tongue blade. In most cases, a mucoïd discharge is collected from the back of the tongue that its origin has not been determined but it is thought this discharge comes from the back of the nose [4].

The texture and anatomy of the tongue also are related directly to the accumulation amount of coating. Generally, grooved tongues have more risk for providing an appropriate environment for bacterial growth than smoother tongues. Accordingly, the amount of coating on the tongue and number of bacteria are related to each other [4]. In addition, with decreasing of a number of anaerobic bacteria in this area, the bad odor decreases. Studies have been shown that periodontal diseases increase the mouth malodor [15,16]. The most important bacteria responsible for mouth malodor was found to be *Treponema denticola*, *Porphyromonas gingivalis* and *Bacteroides forsythus* [17,18].

### Other sites of halitosis generation

In addition to mouth, bad breath also comes from non-oral parts of the body. Among these, a nasal area is important to study and bad odor can be detected from nose strongly. This problem may be related to nasal infection or mucous thickness and secretion amount. The bad odor comes from nose called Rhino halitosis that usually detected by a cheesy smell that is different from other types of bad odors [4]. Generally, bacteria use the nasal mucus as a food source so increasing the nasal secretion is related to the bacterial community. Bacteria use the mucus and produce sulfur compounds that are responsible for bad odor. Cysteine and methionine are widely used by anaerobic bacteria resulted from mucus proteins degradation.

## Bacteriology

Several studies have been shown that the major reason of malodor is bacteria, notably; various types of Gram negative bacteria are involved in this issue [4]. The most important genera of bad odor producing bacteria include *Eikenella corrodens*, *Fusobacterium nucleatum*, *Bacteroides loescheii*, *Treponema denticola*, *Porphyromonas gingivalis* and *Centipeda periodontii* [19]. Bacterial species living in the mouth of human are very diverse as about 100 different species of bacteria are present in this place [4]. Accordingly, any area of the mouth that bacteria can colonize and grow there can be considered as a source of malodor. Dentures are one the important source of oral bad odor especially if it has been kept in mouth overnight. Food impaction sites also can be other cause of malodor [20]. Notably, saliva has an important role in mouth malodor; interestingly, there is a reverse correlation between salivary flow and mouth bad breath [21]. At night, the salivary amount decreases because of fasting and lack of water intake that result in increasing of mouth malodor intensity. On the other hand, chewing raises saliva flow that can clean the mouth cavity and decrease malodor [22]. Conversely, two clinical investigations did not show the reasonable relation between saliva and bad odor intensity [23,24]. *Streptococcus salivarius* was the most prevalent bacteria detected in individuals with fresh breath. Only 16.6% of people with mouth malodor have this bacterium in their mouth [24].

## Diagnosis of halitosis

Halitosis may be detected by different methods such as measurement of volatile sulfide compounds, bacterial enzymes responsible for the production of malodor compounds, salivary test, chemical sensors, and study the organoleptic intensity [4]. Among them, the quantitating organoleptic intensity has been introduced as the gold standard for diagnosis of malodor. The organoleptic test is a simple and old method that includes smelling the nose and mouth [25].

The other way for detection of halitosis is the measurement of VSCs using gas chromatography. Gas chromatography is very reliable and sensitive method, although it is expensive and not economic for all experimental sections. Recently, portable devices in this area have been introduced [26]. Measurement of VSCs using portable devices that monitor the electrochemical reaction has been developed that is equivalent to the sulfur compounds levels [26]. Among them, the Halimeter® is a well-known device for detecting and measurement of VSCs. This method has some limitations. For instance, it is not able to measure the sulphide compounds level accurately. Additionally, its sensitivity for all sulphide compounds is not similar as it is sensitive hydrogen sulfide more than methyl mercaptan [26].

## Bacteriological studies

For studying the causative agents of malodor a combination of nasal endoscopy, laryngoscopy and sample culture directly is required. Collecting of specimens from the tongue and other oral areas and studying the biofilm and scraped samples reveal the bacterial species responsible for VSCs production. Among the bacterial isolates from tongue, *Prevotella melaninogenica*, *Porphyromonas gingivalis*, *Fusobacterium nucleatum* and *Actinobacillus actinomycetemcomitans* are the most prevalent bacteria. *Treponema denticola*, *Porphyromonas gingivalis* and *Bacterioides forsythus* are the most important odor producing bacteria isolated from teeth. Detection of bacteria by traditional culture method usually has some limitations such as this method is time-consuming and has an inherent tendency to the isolation of microaerophilic and anaerobic bacteria. Molecular techniques such as polymerase chain reaction (PCR) have gained more attentions to the identification of VSCs producing microorganisms recently. PCR methods are sensitive, reliable and accurate. A large number of samples are analyzed simultaneously even with the low amounts of DNA. Additionally, real-time PCR has been supplied quantitative analysis of oral malodor-producing bacteria as by this method five common VSCs producing bacteria has been identified called *Porphyromonas gingivalis*, *Fusobacterium nucleatum*, *Prevotella intermedia*, *Tannerella forsythia* and *Treponema denticola* [27,28].

## Effect of VSCs in periodontal disease

There is a direct relation between the periodontitis with the halitosis [29]. Hydrogen sulfide and methyl mercaptan are the most important volatile sulfide compounds in halitosis. Lipopolysaccharide (LPS) of bacteria induces inflammation in gingiva but it is not sufficient alone for gingivitis. Studies have been shown the presence of VSCs facilitate the passing of bacterial LPS to gingival tissue [30].

## Treatment methods

The effective way to eliminate oral malodor is hygiene performances; one good example of dental hygiene practices is using dental floss. There is a direct relation between using dental floss and fresh breath. In addition, toothpicks can be effective in cleaning dental pores and sites that have the role in odor production. Another way to decreasing of bad odor is the mechanical removal of biofilm by disrupting using tongue scrapers, chewing gum, and brushing. This action leads to decreasing the VSCs production in the mouth. Applying tongue brushing should be performed regularly to reduce the bacterial community in the oral cavity. Cleaning the back of tongue can be effective in patients with the gag reflex. Increasing mouth hydration also has a significant role in reducing malodor [20].

On the other hand, there are patients with applying oral hygiene that suffer from halitosis [4]. In such cases, rinsing with mouthwash solutions is effective to the reduction of malodor. Notably, mouthwash solutions contain chemical ingredients such as alcohol that have side effects (such as taste disturbance) of soft tissues of the mouth including gums. Usually, using mouthwashes at night before sleeping has the greater effect because bacterial activity increases at this time [5].

Moreover, using antibacterial chemicals such as triclosan, sodium fluoride, zinc chloride, chlorine dioxide, and chlorhexidine (causes discoloration of teeth) is effective to reduce the bacterial number in the mouth. These medicines act as antibacterial agent and decrease the bacteria on the tongue so leads to the reduction of VSCs production. An antibacterial agent should have some criteria such as without effect of over growing of pathogenic bacteria, without a negative effect on the soft tissue of mouth (lips), and reduction of malodor according to the Rosenberg intensity scale which is a standard of organoleptic scale that extensively used in breath research [4].

## Natural products

Natural products have been applied for treatment of oral diseases, using plants extracts in medicine field dates back to near 5000 years ago [31]. Natural products have a wide range of diverse chemical structures which has been interested in drug discovery. Among the origins of the natural products, plants are considered as a rich source for drug discovery.

Piper betle leaves have been used mostly in Asia for reducing halitosis through reduction of methyl mercaptan and hydrogen sulfide, the presence of allyl pyrocatechol in this plant has a major role in decreasing malodor [32]. Green tea has been used widely for treatment of oral diseases, Curcuma zedoaria and Camellia sinensis are two types of green tea that have been tested as the mouthwash to reduce the bad odor of halitosis [33]. Garlic extract also has been used against oral bacteria to treat mouth malodor. Accordingly, garlic extract showed an antibacterial effect against *Streptococcus mutans*, *Streptococcus sanguis*, *Streptococcus salivarius*, *Pseudomonas aeruginosa* and *Lactobacillus* spp [34].

## Aims and Hypothesis

The aim of this study is to find the ability of natural products including chlorhexidine, zinc citrate, zinc chloride, zinc acetate, gallic acid, caffeine, nicotinic acid and trigonelline to inhibit the bacterial enzymes (involved in volatile sulphuric compounds) with focusing on the reduction of VSCs through the enzyme activity.

The hypothesis is similar to the aim but it is just that the natural products will inhibit the production of the VSCs and the whole project is to test that.

## Materials and Methods

### Tongue scrapings

Twenty volunteers including ten females and ten men were subjected to the tongue scraping. The information of individuals was recorded completely. All of the subjects did not have any specific disease. The sterile Whatman Omni swabs (Sigma-Aldrich) were used to scraping the back of the tongue. All samples were put in preservation media containing 4% w/v BHI, 0.4% w/v peptone, 0.002% w/v DL-dithiothreitol, 10% w/v glycerol, and 0.00012% w/v haemin. The scrapers were pooled and mixed using a vortex to transfer all bacteria to the broth. Thereafter, 1 ml of this broth was transferred to cryo vials and preserved at -20°C for 18h and then transferred to and -80°C. Samples were taken out the -80°C freezer and mixed with 10 ml preservation media broth and use immediately.

### Preparation of media

Culture media including Brain Heart Infusion (BHI) broth, Brain Heart Infusion agar (BHA) and nutrient agar (NA) were prepared according to the manufacturer instruction.

### Preparation of samples

The samples were cultured on BHI agar using swab from stocks and plates were incubated for 24h at 37°C. After incubation, the colonies (2 - 3 colonies) were transferred to 10 ml BHI broth medium and incubated for 24 h at 37°C. Thereafter, 1 ml of medium containing bacteria were transferred to an Eppendorf vial (1.5 ml) and centrifuged for 10 minutes at 1400g. The supernatant was discarded and the pellet resuspended in phosphate buffer solution (PBS) and vortexed for 2 minutes. One ml of sample was added to the cuvette and the absorbance of the sample was recorded using a spectrophotometer at 620 nm. PBS was used as a blank.

### Serial dilution preparation and optical density measurement

Series dilution was prepared by adding twenty microliters of the sample to 180 microliters of PBS in micro-tube and vortexed (10-1). Then 20 µl of suspension transferred to the second tube to making 10-2 dilution. The procedure was extended to achieve the solution with 10-8 concentration. Thereafter, 10 microliter of each dilution was cultured on nutrient agar medium and spread through a sterile swab. The plates were incubated for 18hr at 37°C. The colony forming unit (CFU) was calculated according to the below equation:

$$\text{Colony forming unit (CFU)} = \frac{\text{Number of colonies} \times \text{dilution factor}}{\text{Amount of sample on plate}}$$

To optimization of correlation between a number of colonies and absorbance, dilution of samples was repeated three times and finally, two dilutions were selected for determination of colony forming unit.

### Preparation of cell extracts (crude enzyme extracts)

Cell lysis was performed using Cell lytic B (Sigma-Aldrich) according to the manufacturer instruction. Briefly, bacterial isolates were inoculated in 10 ml BHI broth medium and incubated in the shaker incubator (180 rpm) at 37°C for 24h. Then, 1.5 ml of the bacterial culture was adjusted to an OD of 0.8 - 1.0 at 620 nm, was added to the micro tube and centrifuged at 1400g for 10 minutes. The supernatant was removed and the cell was resuspended in 0.4 ml of Cell lytic B. The solution was vortexed for 15 minutes and put in the shaker at 100 rpm, at room temperature for 15 minutes to ensure full extraction of the soluble proteins. Thereafter, the cell lysate was centrifuged at 4500 rpm for 10 minutes to pellet any insoluble material. The soluble protein fraction was removed from the cell debris. In order to ensure about the complete lysis of bacteria, 10 µl of bacterial lysate was cultured on nutrient agar and incubated at 37°C for 18h.

### L-methionine γ-lyase assay

To access the L-methionine γ-lyase activity, 0.4 ml of 0.5M potassium phosphate buffer at pH 8, 0.5 ml of 0.1 M L-methionine, 0.2 ml 0.1 mM pyridoxal 5'-phosphate, and cell lysate in a final volume of 2.0 ml were mixed. Water was used instead of cell lysate as blank. The mixture was incubated for 10 min at 37°C. Thereafter, 250 µl of 50% trichloroacetic acid was added as the terminator of reaction. The mixture was centrifuged at 4500 rpm for 20 minutes and 1ml of the supernatant was mixed with 2 ml of the acetate buffer and 0.8 ml of 3-methyl-2-benzothiazolone hydrazone hydrochloride. The solution was incubated for 30 minutes at 50°C and then the absorbance was recorded at 320 nm [35].

### Optimization of L-methionine γ-lyase assay

The assay of the enzyme was conducted with some modifications (to develop the assay):

- 1) 1 ml of bacterial lysate was mixed with 100 µl of lysozyme, then the process was followed according to the mentioned above.
- 2) 2 ml of bacterial lysate and 100 µl of lysozyme were mixed and placed in a shaker for 30 minutes. After adding of chlorhexidine (CHX) and incubation, the test tubes were centrifuged for 20 minutes.
- 3) In this case, 500 µl of bacterial lysate and product control were added to test tubes and incubated for 15 minutes. Thereafter, CHX was added and centrifuged at 4500 rpm for 10 minutes. In the other set of experiments, pH of potassium phosphate buffer and sodium acetate was checked to be 8 and 5 respectively. The solution of potassium dihydrogen phosphate ( $\text{KH}_2\text{PO}_4$ ) and dipotassium hydrogen

phosphate ( $K_2HPO_4$ ) was used for adjusting the pH of potassium phosphate buffer. In addition, acetic acid was used for reaching to the pH5 in sodium acetate. (but with these modifications to develop the assay we could not change the tubes colour to yellow which shows that alpha ketobutyrate reacts with acetate buffer and 3 methyl-2-benzothiozalone).

### L-cysteine desulphydrase assay

L-cysteine desulphydrase activity was assessed by formation of methylene blue in reaction. 0.2 ml of Tris-HCl (pH 7.5), 0.2 ml of 2.5 mM dithioerythritol, 0.2 ml of 10  $\mu$ M pyridoxal-5'-phosphate, lysed bacteria solution and 1% cysteine were mixed and incubated for 15 minutes at 37°C. In order to terminate the reaction, 0.1 ml of 20 mM N', N'-dimethyl-p-phenylenediamine dihydrochloride dissolved in 7.2 N hydrochloric acid (HCl) and 0.1 ml of 30 mM ferric chloride (FeCl<sub>3</sub>) dissolved in 1.2N HCl was added. The mixture was incubated for 30 min at room temperature and formation of methylene blue was detected spectrophotometrically at 670 nm and the graph was made using a molar extinction coefficient of  $28.5 \times 10^6$  (Fukamachi, *et al.* 2002).

### Natural products

Natural products including chlorhexidine, zinc citrate, zinc acetate, zinc chloride, caffeine, gallic acid, nicotinic acid and trigonelline were obtained from Sigma-Aldrich and dissolved in deionized water before using.

### Effect of natural products on L-cysteine desulphydrase activity

To assess the effect of selective natural products on the activity of the enzyme, 200  $\mu$ l each of Tris-HCl (pH 7.5), 2.5 mM dithioerythritol, and 1% cysteine were added to the tube (5 ml capacity). Moreover, 200  $\mu$ l of lysed bacteria solution was added to test tubes. Tube 1 without bacterial lysate and tube 2 without natural products considered as controls. Natural products including chlorhexidine, zinc citrate, zinc acetate, zinc chloride, caffeine, gallic acid, nicotinic acid and trigonelline were added to other tubes at concentrations of 0.25, 0.5, and 1 % and incubated for 15 minutes at 37°C. Solution of 20 mM N',N'-dimethyl-p-phenylenediamine dihydrochloride dissolved in 7.2M HCl and 0.1 ml of 30 mM FeCl<sub>3</sub> dissolved in 1.2M HCl was used as stop solution. The tubes were incubated for 30 minutes at room temperature and formation of methylene blue was detected spectrophotometrically at 670 nm. Replications of experiments were typically conducted to verify the results.

### Optimization of L-cysteine desulphydrase activity

In order to optimization of L-cysteine desulphydrase activity and figuring out of  $V_{max}$  of the enzyme, the assay was performed at higher concentrations of cysteine as substrate and different incubation times (to check the control and rate of reaction). Bacteria were inoculated into BHI broth and incubated for 24h at 37°C. Lysis of bacteria was performed according to the method mentioned in section 2.6. To detection of enzyme activity, 200  $\mu$ l of Tris-HCl (pH 7.5), 2.5 mM dithioerythritol, and 200 $\mu$ l of lysed bacteria solution were mixed in all tubes. Two concentrations of 2% and 4% of L-cysteine were added to tube 1 and 2 respectively to assess the effect of substrate concentration on enzyme activity. Moreover, L-cysteine at the concentration of 1% was added to tubes 3 to 7 and incubated at 37°C for different times including 15, 25, 35, 45, and 55 minutes. Thereafter, 0.1 ml of 20 mM N', N'-dimethyl-p-phenylenediamine dihydrochloride dissolved in 7.2 M HCl and 0.1 ml of 30 mM FeCl<sub>3</sub> dissolved in 1.2M HCl was added to all tubes as Terminator and absorbance was recorded at 670 nm. In the second attempt, the activity of the enzyme was assessed by applying the more concentrations of cysteine. The assay was conducted as mentioned above but L-cysteine was used at concentrations of 1, 10 and 20%. Moreover, 3 tubes were considered without bacterial lysate at the same concentrations of cysteine (to check if the enzyme present in cell lysis solution).

In another experimental set, enzyme activity was assayed by using of filtration of bacterial suspension and lysed bacteria. First, bacteria were cultured in BHI broth for 24h at 37°C. Whole bacteria and lysed bacteria were filtered using Millipore filter 0.45  $\mu$ m. In addition, cysteine was used at concentrations of 1, 10, and 20%. Tube 1, 2, and 3 contained 1, 10, and 20% of cysteine respectively without bacteria, tube 4, 5, and 6 contained 1, 10, and 20% of cysteine with filtered whole bacteria and tube 9 and 10 contained 10% cysteine with filtered lysed bacteria and without bacteria as control respectively.

### Effect of natural products on enzyme activity of whole bacteria

Bacteria were cultured in BHI broth for 24h at 37°C. Then the bacterial suspension was centrifuged for 10 minutes at 4500 rpm and the supernatant was discarded. The pellet was dissolved in phosphate buffer solution and mixed through a vortex. Thereafter, optical density (OD) was measured to adjust on the value of 0.6. L-cysteine desulphydrase activity was assayed using 1% cysteine and 5% of natural products including zinc citrate, zinc acetate, zinc chloride, caffeine, gallic acid, nicotinic acid and trigonelline. Suspension of whole bacteria was added to the tubes and absorbance was recorded as mentioned above.

## Results

### Colony forming unit determination

In order to the determination of viable bacteria in samples, colony forming unit (CFU/ml) was measured by plating and optical density. Serial dilution is as the sample (three times to provide the best correlation between absorbance and number of colonies). Figure 2 shows the CFU/mL versus optical density curve shows the CFU/mL versus optical density curve. As shown, there is a relatively linear correlation ( $R^2 = 0.913$ ) between optical measurement and colony forming units. It has been determined that  $OD_{620}$  of 1 is equivalent to  $2.39 \times 10^8$  CFU/mL.

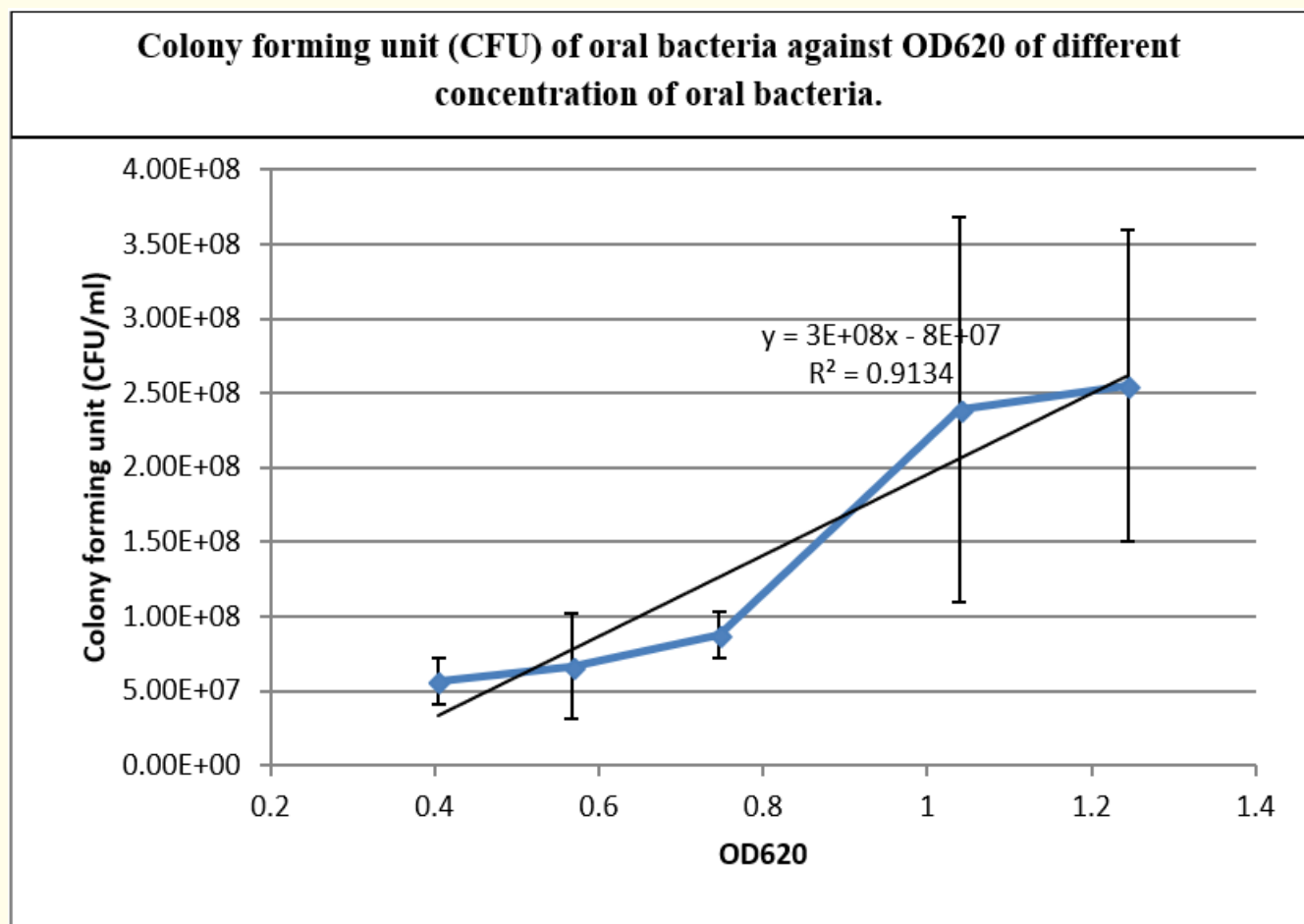


Figure 2: There is a linear correlation between OD and colony forming units. The error bars show the error of the absorbance reading.

### Effect of natural products on L-cysteine desulphydrase activity

The effect of natural products on L-cysteine desulphydrase activity is shown in figure 3. Accordingly, the control which contained no natural products did not show remarkable enzyme activity. Among the natural products, gallic acid and chlorhexidine showed the most absorbance values. Other natural products indicated no change in absorbance compared to control.

### Optimization of L-cysteine desulphydrase activity

L-cysteine desulphydrase activity at different incubation times has been shown in figure 4. The results show that the time of incubation has no remarkable effect on absorbance. Therefore, the activity of the enzyme was not remarkable even after 55 minutes of incubation with lysed bacteria as a source of enzyme.

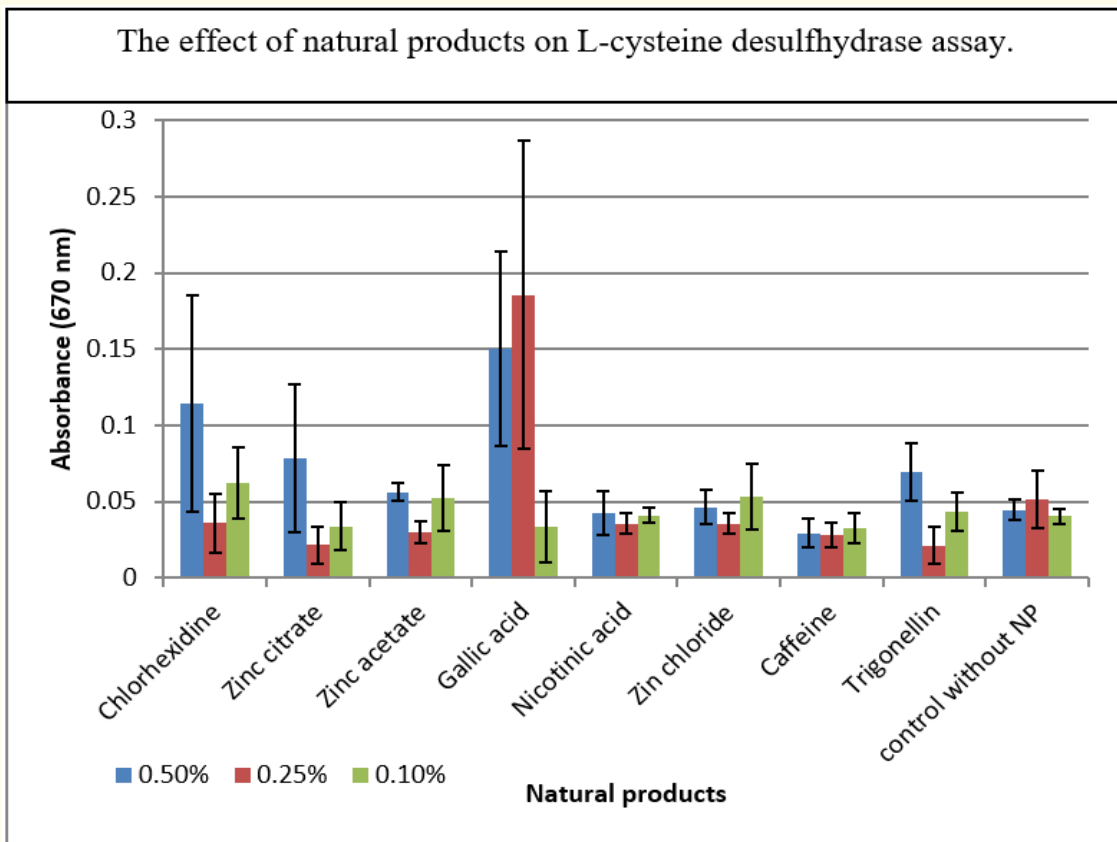


Figure 3: Effect of natural products at three different concentrations of 0.25, 0.5, and 1% on the activity of L-cysteine desulphydrase in presence of lysed bacteria as a source of enzyme, the error bars show the error of absorbance reading.

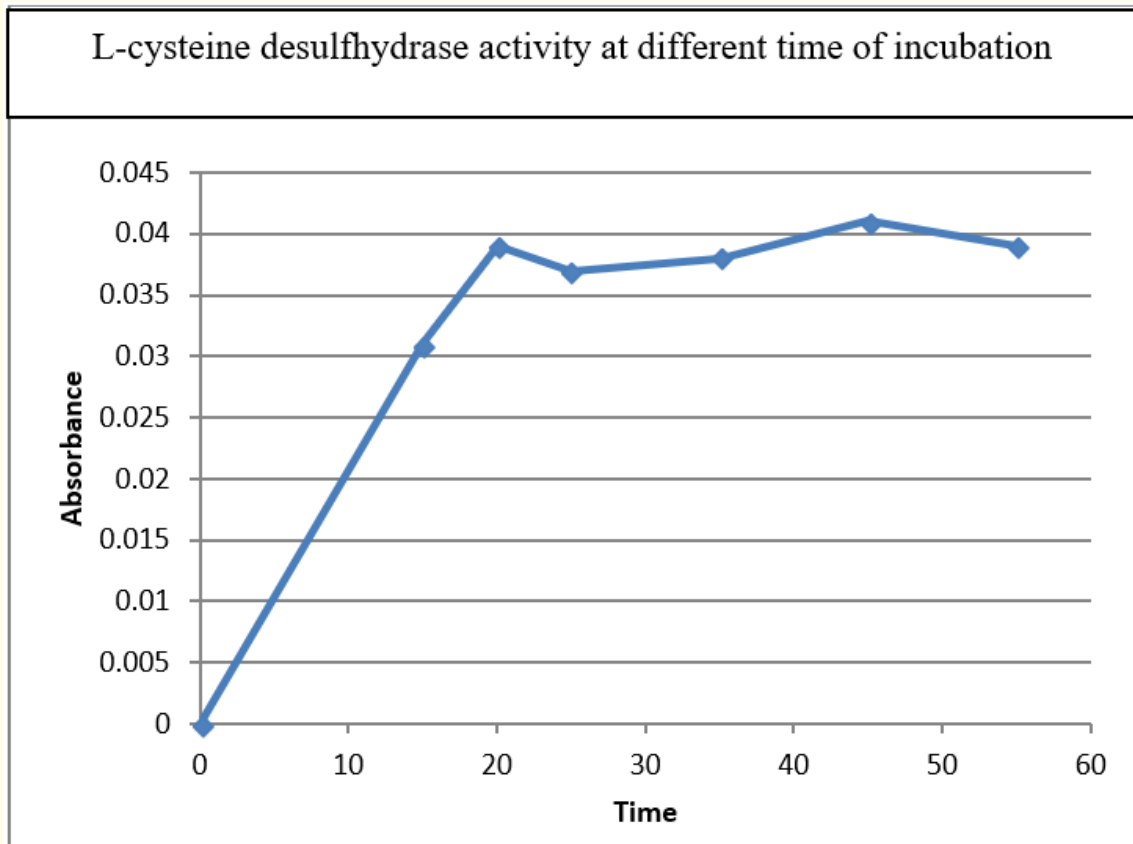
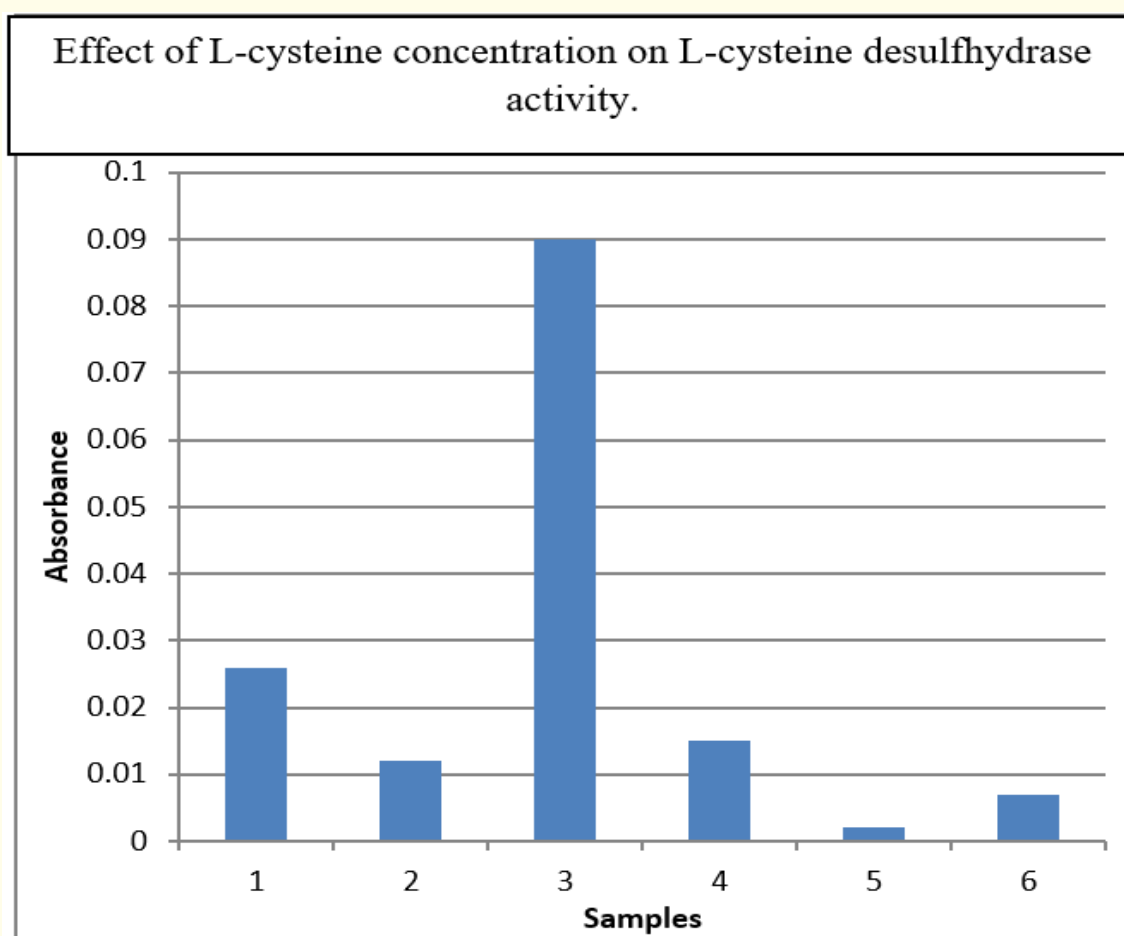


Figure 4: In addition to studying the effect of time on enzyme activity, we investigated the role of cysteine concentrations as well. As shown in figure, there is no difference in absorbance between samples with different concentrations of cysteine.





**Figure 5:** 1) 1% cysteine with lysed bacteria 2) 10% cysteine with lysed bacteria 3) 20% cysteine with lysed bacteria 4) 1% cysteine without bacteria 5) 10% cysteine without bacteria 6) 20% cysteine without bacteria.

### Enzyme activity with whole bacteria

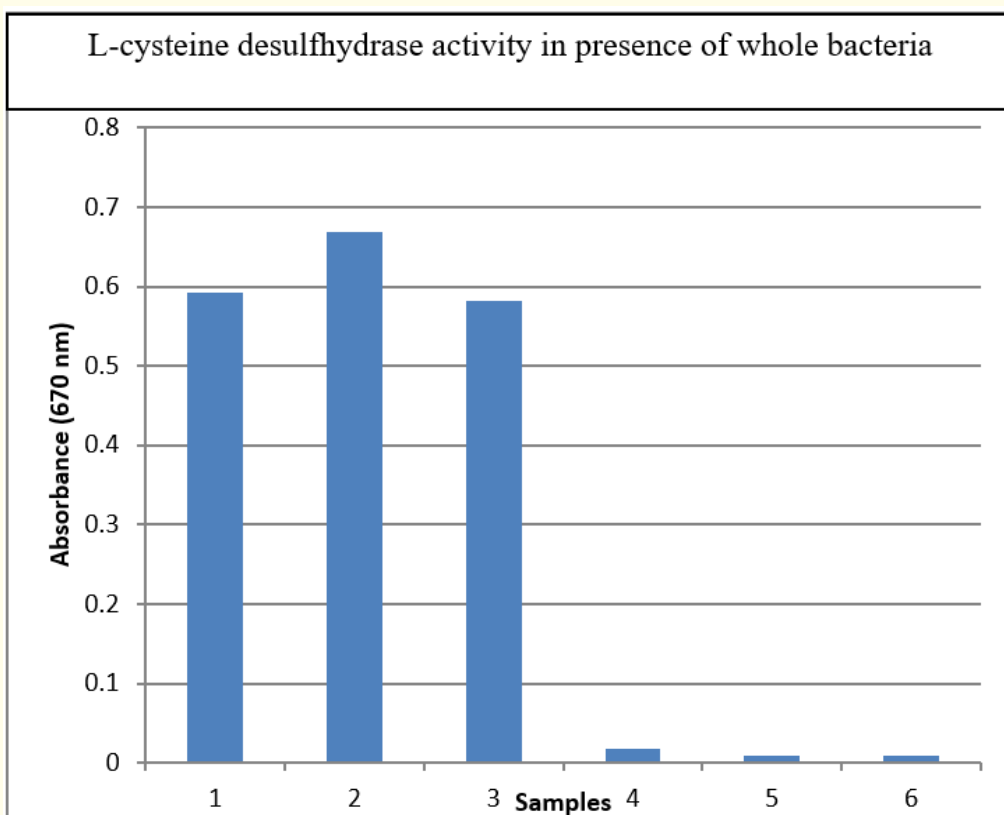
The results of enzyme assay in presence of whole bacteria have been shown in figure 6. Clearly, the enzyme used the substrate efficiently in presence of whole bacteria and the sample containing cysteine 10% showed the highest absorbance (OD 670 nm= 0.668). Samples 1 and 3 which contained 1% and 20 % cysteine respectively showed relatively similar absorbance. In addition, the tubes without bacteria (sample 4, 5, and 6) used as controls and showed very low absorbance according to our hypothesis.

### Enzyme activity assay using filtered bacteria

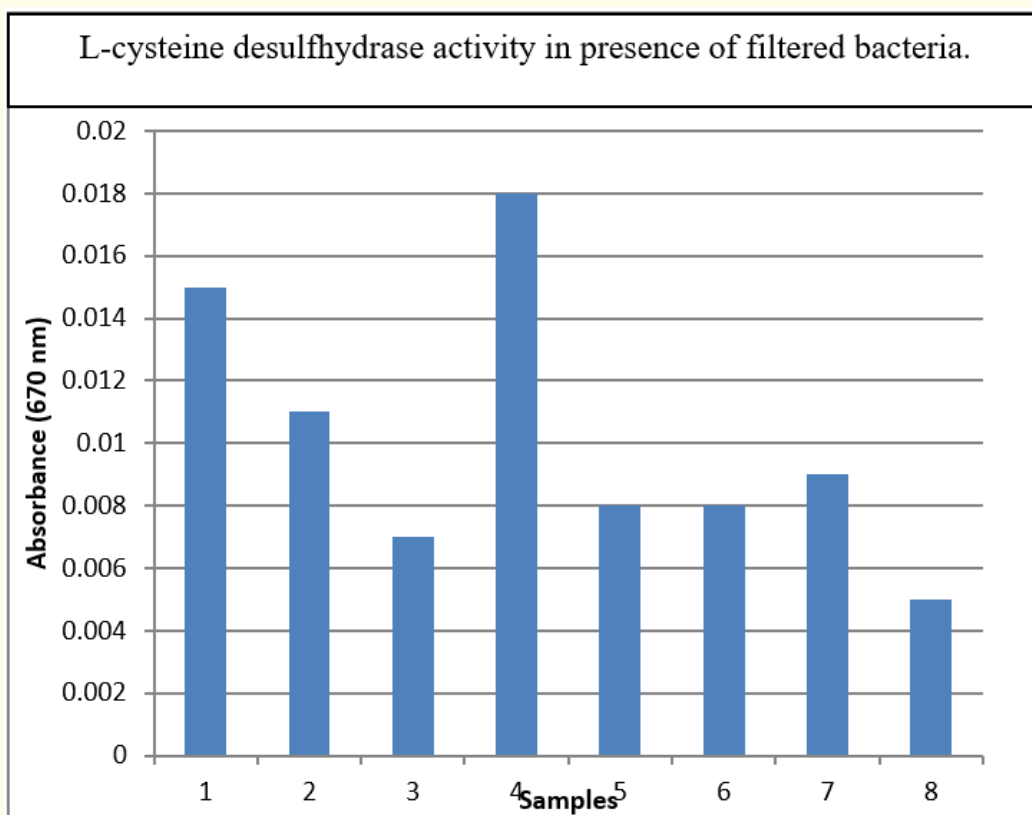
As shown in figure 7, enzyme assay with filtered whole bacteria (samples 4, 5, and 6) did not show high absorbance compared to samples with no bacteria (samples 1, 2, and 3). We also examined the concentration of cysteine that was not contributing to enzyme activity. Moreover, filtered lysed bacteria (sample 7) showed low absorbance and revealed the filtration of lysed bacteria was not appropriate to approach.

### Effect of natural products on enzyme activity of whole bacteria

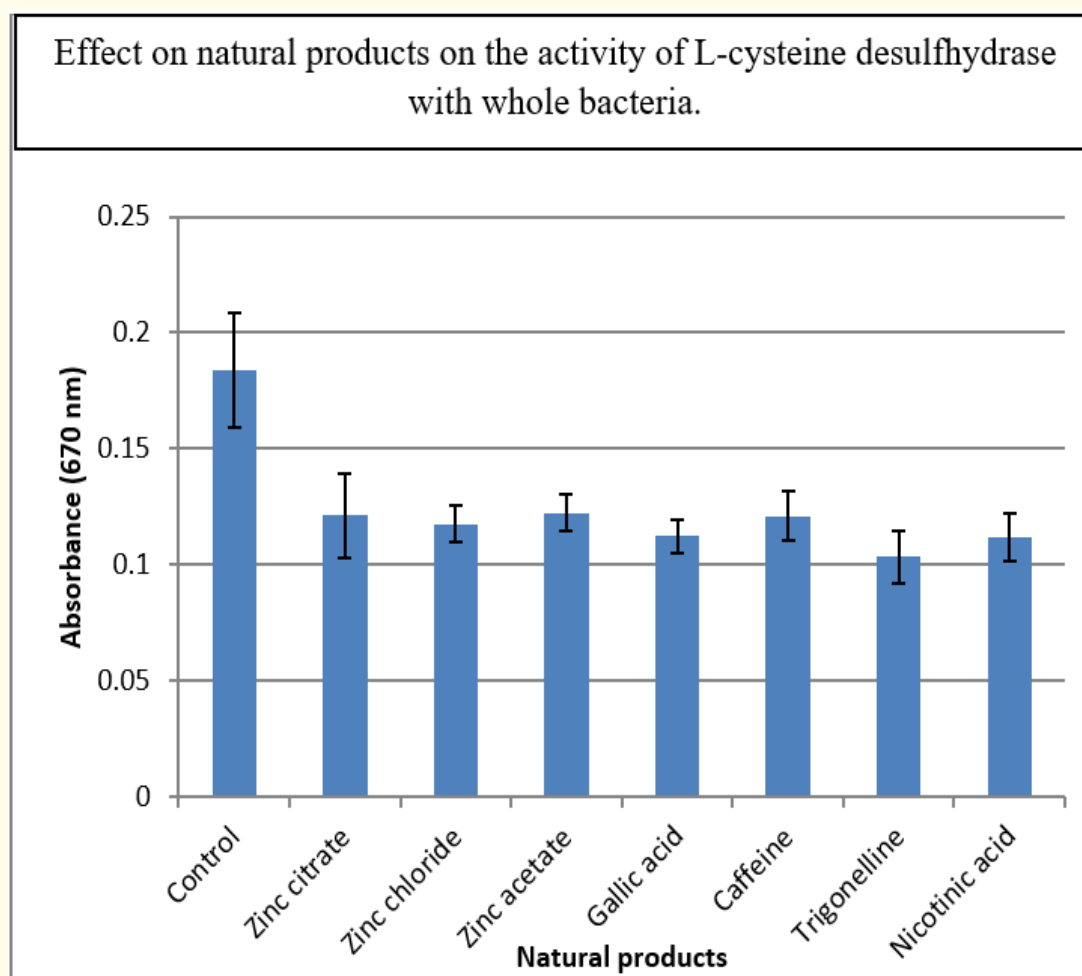
Inhibitory effect of natural products on the activity of L-cysteine desulphydrase has been shown in figure 8. As shown, control tube showed the highest amount of absorbance ( $0.183 \pm 0.024$ ) at 670 nm compared to treatments. Natural products decreased the absorbance in a relatively similar range. The most effective natural product with inhibitory activity was identified to be trigonelline with the absorbance of  $0.103 \pm 0.011$ . Zinc derivatives including zinc citrate, zinc chloride, and zinc acetate indicated similar activities.



**Figure 6:** 1) 1% cysteine with whole bacteria 2) 10% cysteine with whole bacteria 3) 20% cysteine with whole bacteria 4) 1% cysteine without bacteria 5) 10% cysteine without bacteria 6) 20% cysteine without bacteria.



**Figure 7:** 1) 1% cysteine without bacteria 2) 10% cysteine without bacteria 3) 20% cysteine without bacteria 4) 1% cysteine with filtered whole bacteria 5) 10% cysteine with filtered whole bacteria 6) 20% cysteine with filtered whole bacteria 7) 10% cysteine with filtered lysed bacteria 8) 10% cysteine with no bacteria.



**Figure 8:** Control tube showed highest absorbance and natural products decreased the absorbance reading. (The error bars show error in absorbance reading).

## Discussion

The bad odor of oral cavity is mainly produced by microbial community in a mouth. Volatile sulfide compounds are the most important reason of malodor that is produced by bacteria in the mouth [36]. The most common treatment methods of halitosis include mechanical reduction and chemotherapy such as using toothpaste and mouth washes [37]. In this study, the efficacy of natural products in reducing the activity of enzymes responsible for malodor production. Zinc citrate, zinc acetate, zinc chloride, caffeine, chlorhexidine, gallic acid, nicotinic acid, and trigonelline were selected as natural substances to inhibit the activity of L-cysteine desulfhydrase and L-methionine  $\gamma$ -lyase. Chlorhexidine and zinc compounds are used extensively in mouthwashes, as recently a mouthwash containing chlorhexidine and zinc lactate has been introduced to the market to the treatment of halitosis (Halita®, Dentaid SL, Spain) [38]. Gallic acid is a phenolic compound which is found in tea leaves and other plants, the antibacterial activity of this compound has been studied on oral bacteria especially the formation of *Streptococcus mutans* biofilms [39]. Caffeine is mainly found in tea leaves, beans, coffee, guarana, and nuts [40]. Gov and colleagues [41] showed that coffee has the inhibitory effect on microbial volatile sulfur compound production as the results indicated that 2% coffee reduced VSCs production and malodor levels by 85%. Trigonelline is an alkaloid compound that is produced by plants and has therapeutic potential especially for diabetes [42]. Trigonelline also is found in coffee and has shown inhibitory activity against bacteria such as *Serratia marcescens* and *Enterobacter cloaca* [43].

Lysis of bacteria was performed using Cell lytic B. Lysis process was confirmed by the cultivation of bacterial lysate on the nutrient agar plate. If bacteria have been lysed completely, no colonies would be observed on nutrient agar. In this step, some colonies of bacteria were observed after 24h incubation that showed the lysis procedure was not completely done so the lysis step was carried out for the second time. It is possible that the concentration of lysis solution and bacterial masses was not in a proper ratio. For complete lysis, lysozyme was added to the lysis solution of the kit. Thereafter, lysis process was approved by the cultivation of cell lysate on nutrient agar.

Two enzymes, L-methionine  $\gamma$ -lyase and L-cysteine desulphydrase, were selected for investigation of inhibitory activity of natural products. L-methionine  $\gamma$ -lyase converts L-methionine to methane thiol, 2-Ketobutyrate, and ammonia (NH<sub>3</sub>). Thereafter, 2-Ketobutyrate reacts with 3-Methyl-2-Benzothiazolinone Hydrazone (MBTH) and forms azine derivative. The first attempt to calculate the activity of the enzyme was not successful (the yellow colour in the tubes was not shown) therefore the procedure was optimized through changing some steps. In this step, it is assumed that bacteria did not lyse properly or enzyme did not extract or was inactive. For instance, lysozyme was added to bacterial lysate or time of incubation was changed and samples were incubated for more times to ensure the complete lysis of cells. Moreover, we used different times of centrifugation to study the effect of several parameters in the enzyme assay. In addition, the pH of potassium phosphate buffer and sodium acetate was monitored to be 8 and 5 respectively. The results of L-methionine  $\gamma$ -lyase assay were not reasonable because we did not observe more absorbance in control. The tube which contained bacterial lysate and substrate expected to have the highest absorbance compared to treatments but the results did not show this hypothesis. Changing the various factors did not show promising outputs. It is assumed that enzyme concentration in the bacterial lysate was too low that was not detected by this method. The second enzyme, L-cysteine desulphydrase, was assayed in a bacterial lysate. Effect of natural products was assessed on L-cysteine desulphydrase activity. The results showed no differences between absorbance of control tube which contained bacterial lysate and substrate so optimization of enzyme assay was carried out through changing the time of incubation, cysteine concentration, and bacterial lysate volume. These changings were carried out because the absorbance of control tube (without natural products) was low. Therefore, we decided to study the effect of some variations such as time, substrate concentration and volume of bacterial lysate to optimize the absorbance of samples. Chlorhexidine, zinc citrate, and gallic acid increased absorbance which was against our hypothesis (They could have reacted with the substrate, something in assay or colour density of products). Control tube which contained no natural products did not show different absorbance compared to other samples.

To optimization of the assay, different concentrations of cysteine including 1, 2, 4, 10 and 20% were used (to check if the enzyme present in cell lysis solution). In addition, incubation times increased to 55 minutes. In tubes containing high concentrations of cysteine, it was expected the absorbance to be high due to the bacterial enzyme would be reacting with cysteine but we did not observe high absorbance and absorbance was similar to the tubes without bacterial lysate. This observation shows that bacterial lysate has not provided the proper source of enzyme because the absorbance of sample contained bacterial lysate and substrate showed low absorbance even when we increased the cysteine concentration and bacterial lysate volume. In another set of experiments we used whole filtered bacteria and recorded the absorbance of samples but the tubes containing whole filtered bacteria did not show desirable results. As similar above, the absorbance of samples was low and it did not show the difference in absorbance compared to tube contained no bacterial lysate. It can be explained that bacterial enzyme might stick to the cell membrane and has not been extracted properly or during centrifugation it has been accumulated in the bottom of a micro tube. Variation of pH of buffers also can affect the enzyme extraction efficiency. In addition, it is possible that target protein has been degraded during lysis procedure. Because protein extraction is a very sensitive procedure and is affected by various factors such as pH of buffers, temperature, and lysis solution components. Notably, expression of proteins in bacteria is affected by various parameters so it can be assumed that expression level of the enzyme might be too low. Consequently, we used whole bacteria instead of bacterial lysate. A control tube containing whole bacteria showed OD 670 nm = 0.183 compared to bacterial lysate which revealed that lysate suspension did not have the adequate amount of enzyme. Natural products also showed inhibitory activity against L-cysteine desulphydrase activity. As described in the results section, trigonelline showed the most activity OD 670 nm = 0.103 compared to other natural products and other compounds indicated similar inhibitory activity.

In several studies the effect of chlorhexidine in the reduction of VSC levels and organoleptic score have been shown [23,38,44,45]. De Boever and Loesche showed rinsing of mouth with 0.12% chlorhexidine decreased mouth malodor and VSCs level (73.3%) significantly [45]. Effect of chlorhexidine on oral bacteria has been investigated by Winkel, et al [38]. They studied the effect of a mouth rinse containing chlorhexidine (0.05%) and zinc-lactate (0.14%) as the main ingredients in the treatment of halitosis. Treatment with mouthwash indicated a significant reduction in organoleptic score from 2.8 to 1.5 indicating the effective way to controlling of malodor [38]. This stronger effect compared to chlorhexidine could be explained by synergetic interactions between chlorhexidine and Zn<sup>+2</sup>. Our results also

have confirmed the efficacy of zinc in the treatment of halitosis. We showed that zinc citrate, zinc chloride, and zinc acetate inhibited the L-cysteine desulphhydrase enzyme. Reduction of an organoleptic score by using the mouthwash containing zinc can be concluded by inhibiting the VSCs producing enzymes such as L-cysteine desulphhydrase.

Green tea is one of the most prominent natural substances to control of a number of microorganisms in the oral cavity. Lodhia, *et al.* showed the effect of green tea on reducing of VSCs such as CH<sub>3</sub>SH and H<sub>2</sub>S which are contributing in malodor. Their findings indicated that green tea reduced VSCs level in mouth air. This inhibitory effect was stronger on CH<sub>3</sub>SH than H<sub>2</sub>S [46]. Zeng, *et al.* reported that green tea extract was able to reduce the odorant sulphurs. Correspondingly, some malodor treatment products such as chewing gum and mouth washes and sprays contain tea polyphenols [47].

Most studies on controlling of oral malodor focus on inhibition of bacteria which are VSCs producers. This is the first time that inhibitory effect of natural products on enzyme responsible in halitosis has been studied. As we shown, natural products can affect this particular enzyme, other studies have shown this inhibition to anti-microbial level but nobody is looking specifically for this setup [48-50].

## Conclusions

To conclude, natural products are a rich source in the treatment of human diseases and our findings confirmed that these compounds have the positive role in oral health. In the current study, we have introduced trigonelline, caffeine, zinc derivatives and nicotine amide as the promising agents for inhibiting of L-cysteine desulphhydrase enzyme. The more studies need to discover the mechanisms of action of these compounds.

## Warranty Statement

This is a student project. Therefore, neither the student nor Kingston University makes any warranty, express or implied, as to the accuracy of the data or conclusion of the work performed in the project and will not be held responsible for any consequences arising out of any inaccuracies or omissions therein.

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