QUALITY OF POVIDONE-IODINE AND CHLORHEXIDINE BASED MOUTHWASH/GARGLE PRODUCTS IN NAIROBI CITY COUNTY, KENYA

A thesis submitted in partial fulfillment of the requirements for the award of the degree of Master of Pharmacy in Pharmaceutical Analysis

BY

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This thesis is my original work and has not been presented in any other University for award of any degree.

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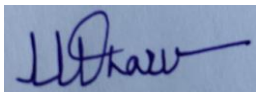

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This thesis is dedicated to my dear wife, Elizabeth Nyandieka and my daughter Kaylajune Gisairo for love and unselfish support during my research work. To my parents, Mr. Francis Pius Omweri, Mrs. Rebecca Nyaboke, my siblings, Tom Nyaberi, Wycliffe Matibe, Maureen Moraa, Asumpta Nyanchama, Euphem Kemunto, Enock Och'wangi, Silvia Bosibori, Sharon Kwamboka and Brian Kibagendi, for support and prayers in all my life.

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LIST OF ABBREVIATIONS/SYMBOLS AND ACRONYMS

µg/mL	Microgram per Milliliters
µL	Microliter
AAS	Atomic Absorption Spectroscopy
ADS	Anti-Discoloration Systems
APF	Acidulated Phosphate Fluoride
API	Active Pharmaceutical ingredient
AUC	Area Under the Curve
BP	British Pharmacopoeia
CAL	Clinical Attachment Level
cGMP	current Good Manufacturing Practices
CE	Capillary Electrophoresis
CHX	Chlorhexidine
CIP	Cahn-Ingold-Prelog
CPC	Cetylpyridinium Chloride
CRS	Chemical Reference Substance
FIA	Flow Injection Analysis
g	Gram
GLC	Gas Liquid Chromatography
HPLC	High Performance Liquid Chromatography
HSCT	Hematopoietic Stem Cell Transplantation
KEBS	Kenya Bureau of Standards
M	Molarity
mL	Milliliters
MRSA	Methicillin Resistant Staphylococcus Aureus
MS	Mass Spectroscopy
mV	Millivolt
NMR	Nuclear Magnetic Resonance
OM	Oral Mucositis
PAT	Process Analytical Technology
QA	Quality Assurance
pH	Potential of Hydrogen
PPB	Pharmacy and Poisons Board
PVC	Polyvinyl Chloride
PV-I	Povidone-iodine
RSD	Relative Standard Deviation
S-mark	Standardization Mark
TFT	Thin Film Transistor
UPLC	Ultra-Performance Liquid Chromatography
USP	United States Pharmacopoeia
VGA	Video Graphic Array
Λ	Lambda

ABSTRACT

Introduction

Povidone-iodine (PV-I) and chlorhexidine (CHX) are broad spectrum antimicrobial agents that are active against a number of gram-negative and gram-positive bacteria. Povidone-iodine and CHX are listed as essential drugs by the Ministry of Health (MoH) Kenya and World Health Organization (WHO) for the management of sores, gum swelling and bad breath as gargles and mouth rinses/mouthwashes.

Most gargle and mouth rinse products are readily available and have been used as over-the-counter medication. Despite the increase in the number of PV-I and CHX based mouthwash and gargle brands in the Kenyan market in recent years, only a few have been registered with the Pharmacy and Poison Board (PPB). The increased influx of unregistered products from multiple sources in the Kenyan market poses challenges on the regulatory body when it comes to the evaluation and monitoring of their quality in the market. Furthermore, a comprehensive database containing all products that are meant to be used as gargles and mouthwashes is not available at PPB. Therefore, there is need to survey, sample and analyze the different brands of PV-I and CHX mouthwashes and gargle products in the market to establish their quality.

Study objective

The objective of this study was to determine the quality of mouthwash and gargles products containing CHX and PV-I in Nairobi County

Methodology

In this study, a convenience sampling was used to select 15 brands (34 samples) of PV-I and 9 brands (15 samples) of CHX mouthwashes and gargles from retail pharmacies in Nairobi County. Povidone-iodine samples were analyzed using potentiometric titration for iodine content while chlorhexidine samples were assayed using High Performance Liquid Chromatography (HPLC). Both analytical methods were as per BP (2017) pharmacopoeia (BP, 2017a) specifications. The values for the assay were compared with the limits set in the BP (2017) (85% to 120 %, chlorhexidine – 95% to 105%). Analysis of Variance (ANOVA) was conducted on the assay values for both products to detect inter-batch variations.

Results

The results obtained showed that about 16 samples (47.1 %) from 8 brands of PV-I products complied with BP (2017) specifications for the assay of PV-I while 10 samples (66.7 %) from 5 brands of CHX complied with the assay of chlorhexidine mouthwash and gargle products as per BP (2017) specifications (BP, 2017a).

Conclusion

From the assay results, not all PV-I and CHX mouth wash and gargle products met the required regulatory standards. Therefore, PPB should conduct continuous market surveillance on all the PV-I and CHX containing mouth wash and gargle products to ensure quality assured products circulate in Kenya.

CHAPTER 1
GENERAL INTRODUCTION

1.1 Background

Oral cavity harbors many microbial agents that may causes diseases (Oyanagi and Tagami, 2012). Some of the bacteria present in oral cavity include *Streptococcus sp, Prevotella sp* and *Veillonellab sp* (Nasidze et al., 2009). These microbes can cause tooth decay (dental caries) and periodontal diseases. Dental caries appears when bacterial processes convert sugars, for instance, sucrose, fructose and glucose in food residues on teeth to acids that dematerialize hard tooth structure such as enamel, dentine and cementum. *Streptococcus mutans* and *Lactobacillus* have also been associated with dental caries (Kidd and Fejerskov, 2016). Plaque causes periodontal diseases as a result of the associated inflammation which may lead to gum detachment from the teeth. Likewise, anaerobes like *Porphyromonas gingivalis and Treponema denticola* have been associated with periodontal infections (Loesche, and Grossman, 2001).

Physical removal of the plaque is the main prevention strategy for periodontal diseases and dental caries although antimicrobial agents have also been recommended. However, it has been found that only few antimicrobial agents have clinical efficacy against plaque. This is attributed to the fact that most antimicrobial agents are not effective against oral microorganisms. Nevertheless, a number of antimicrobial agents such as essential oils, surfactants, metal salts, phenols, chlorhexidine, plant extracts and enzymes are in use (Pitten, Splieth and Kramer., 2000). Chlorhexidine, a surfactant has superior antiplaque properties and therefore it is regarded as the gold standard. It has superior and persistent antibacterial effect when compared to other antiplaque agents which have only immediate effect and once removed from the mouth, plaque buildup again (Jones, 2000; Balagopal and Arjunkumar, 2013).

Antiplaque agents are formulated as mouthwashes and gargles. According to Farah *et al* and Parashar, a mouthwash is a medicated solution for gargling and rinsing the mouth for the management of bad odor (halitosis), periodontal diseases, and treatment of secondary infections such as oral mucositis (OM). They are suitable for use as antimicrobial, anti-inflammatories, analgesics and for prevention of caries (Farah S Camile, McIntosh Lidija, 2009; Parashar, 2015). On the other hand, gargles are non-swallowable aqueous solutions for the management of throat conditions (Bachenheimer, 2010).

For improved efficacy, mouthwash and gargles are supposed to adsorb onto soft and hard tissues in the throat and mouth for prolonged antiplaque effect. This property is referred to as substantivity. Factors such as pH, temperature, concentration and the time period a product remains in the mouth to elicit therapeutic effect promote substantivity of the associated products (Parashar, 2015). For instance, increase in the concentration of the active pharmaceutical ingredient (API) in the mouthwashes and gargles significantly reduces chances of halitosis and leaves an appealing taste while products with a higher pH than water causes a neutralizing effect in the mouth reducing likelihood of acidic tooth erosion. The antibacterial effect of chlorhexidine is temperature dependent whereby, storage at appropriate temperature ensures therapeutic efficacy (Jain, 2012). An increase in mouthwashes and gargles resident time leads to an increase in efficacy due to the increase in contact time (Van Zyl and Van Heerden, 2010).

1.2 Antiplaque agents

The common antiplaque agents include phenols, essential oils, surfactants, natural/herbal products, metal salts, enzymes, alcohols, halogens, and oxidizing agents. Table 1.1 shows some of the commonly used antiplaque agents.

Table 1.1: Chemical classification of antiplaque agents used in mouthwashes and gargles

Antiplaque agents' class	Examples
Phenols	● Triclosan
Essential oils	● Thymol, eucalyptol and menthol
Surfactants a) Cationic surfactants ● Biguanides ● Quaternary ammonium compounds ● Pyrimidine derivatives ● Bispyridine derivatives b) Anionic surfactants	● Chlorhexidine digluconate ● Cetylpyridinium chloride ● Hexetidine ● Octenidine hydrochloride ● Amino alcohols ● Sodium lauryl sulphate ● Delmopinol
Natural products	● Extracts from plants such as *tt*-farnesol, apigenin ● Sanguinarine, aloe vera and calendula
Metal salts	● Zinc citrate and zinc chloride ● Salts of copper such as copper gluconate ● Stannous fluoride
Enzymes	● Lactoperoxidase ● Lactoferrin ● Lysozyme
Alcohol	● Ethyl alcohol and Isopropyl alcohol
Halogens	● Povidone-iodine ● Fluorides such as sodium fluoride and acidulated phosphate fluoride
Oxygenating agents	● Sodium peroxycarbonate, sodium peroxyborate, and Hydrogen peroxide.

Adopted from (Marsh, 2012) and (Jafer *et al.*, 2016)

1.2.1 Phenols

Phenolic compound such as triclosan have been used as mouthwashes in medicine for centuries (Srinivasan et al., 2013). Triclosan is a non-ionic anti-inflammatory as well as antiseptic compound commonly incorporated in mouthwashes to treat plaques (Blinkhorn et al., 2009). The antibacterial property of triclosan results from disruption of plasma membrane by increasing the permeability, inactivating enzymes, disruption of cellular transport, inhibition of reproduction and metabolism of bacterial cells (Nascimento *et al.*, 2008). However, triclosan is known to have a low substantivity and therefore, it is compounded with

copolymers to enhance substantivity (Pires et al., 2007). Figure 1.1 below illustrates the chemical structure of triclosan.

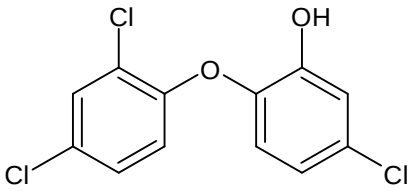


Figure 1.1: Chemical structure of triclosan

1.2.2 Essential oils

Essential oils commonly found in mouthwashes and gargles include eucalyptol, menthol and thymol (Ouhayoun 2003; Filoche, Soma and Sissons, 2005). Essential oils elicit antibacterial effect through endotoxin extraction from Gram-negative bacteria, inhibition of bacterial enzyme and cell destruction. Additionally, these oils are known to possess anti-inflammatory and antioxidant (free oxygen radical scavengers) activities as well as the ability to inhibit prostaglandin synthetase activity. Studies have shown that their antibacterial and plaque-permeating abilities promote their efficiency in reducing gingivitis, halitosis and plaque (Pan, Leung and Rubin, 2002; Sharma *et al.*, 2004; Parashar, 2015). Ulkur, Arun and Ozdemir showed that dental cleanliness is fundamentally improved in the wake of washing the mouth utilizing basic oils, for example; thymol, eucalyptol and menthol in Listerine® and xylitol in Concentrate® (Ulkur, Feyza and Arun, 2013; Tulin and Ozdemir, 2013). Figure 1.2 shows the structures of the commonly used essential oils in mouthwash and gargles.

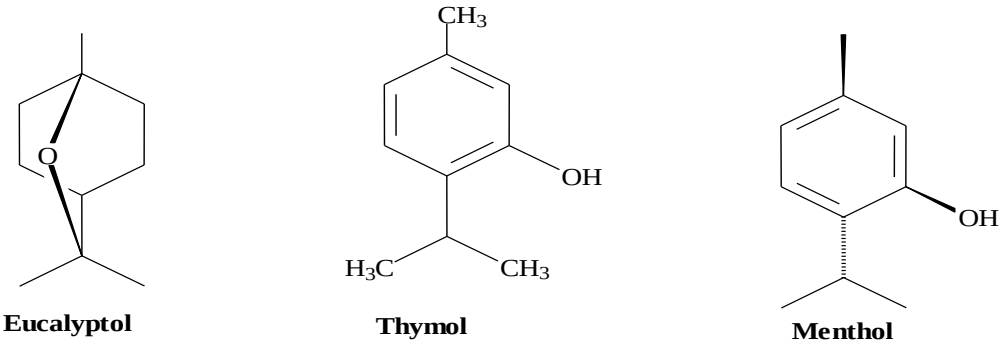


Figure 1.2: Chemical structures of some constituents of essential oil

1.2.3 Surfactants

Surfactants are compounds which help to lower the surface tension between fluids as they have a wetting ability property. Reduction of surface tension enables spreading of liquids by

weakening the bonds between two liquids or between a liquid and a solid (Mundhada and Chandewar, 2015). Surfactants that are generally incorporated in mouthwash products are classified as anionic, cationic and non-ionic compounds (Wicks, McConville and Walsh, 2000). When incorporated in mouthwashes, anionic surfactants such as sodium lauryl sulphate, amino alcohol and delmopinol help to solubilize flavors and provide foam which assist in the removal of debris. Cationic surfactants such as CHX, cetylpyridinium chloride, hexetidine and octenidine hydrochloride have antibacterial effects. Lastly, non-ionic surfactants such as polyoxyethylene and polyoxypropylene are preferred because they are less irritant when compared with other surfactants with similar foaming ability (David and Paul, 2006a; Van, 2013). Figure 1.3 shows the structures of some of the commonly used surfactants.

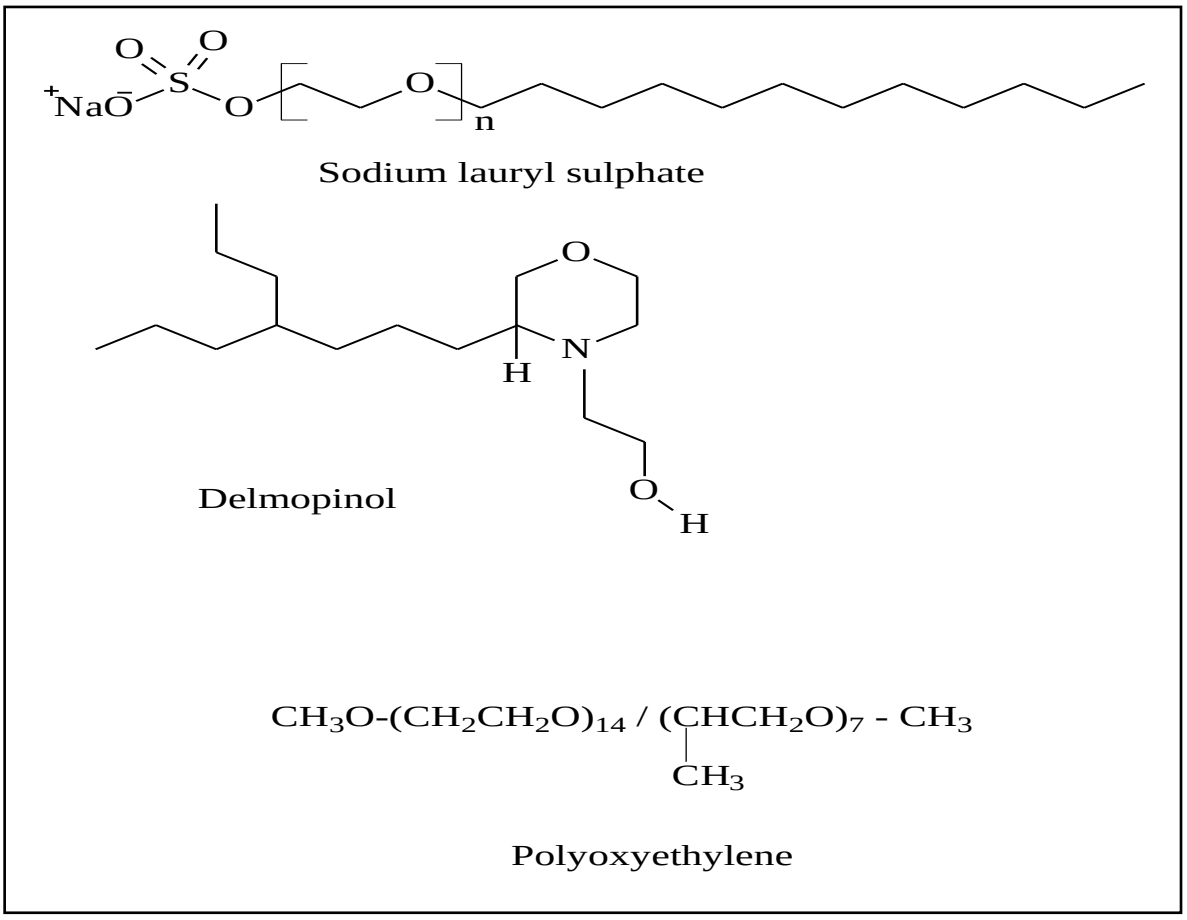


Figure 1.3: Chemical structures of the anionic and non-ionic surfactants

1.2.4 Oxygenating agents

Oxygenating agents used as mouthwashes and gargles include sodium peroxyborate/perborate, hydrogen peroxide and sodium peroxycarbonate/percarbonate.

1.2.4.1 Sodium peroxyborate

Sodium peroxyborate produces hydrogen peroxide and borate by hydrolysis when in contact with water (McKillop and Sanderson, 2000; Schubert, 2011). Sodium peroxyborate is prepared by reacting sodium hydroxide and borax leading to the production of sodium metaborate. In presence of a surfactant (to control crystal size), sodium metaborate is subsequently reacted with hydrogen peroxide to produce hydrated sodium peroxyborate (Schubert, 2011). Sodium peroxyborate produces oxygen radicals when incorporated in detergents, laundry bleaches, cleaning products and tooth bleaching formulas (Inc, 2015). It has some antiseptic as well as disinfectant properties. In eye drops, sodium peroxyborate has been used as a preservative while during organic synthesis (such as production of sulfones and sulfoxides from thioethers), it provides oxidizing activity (McKillop, Kabalka and Reddy, 2008).

1.2.4.2 Hydrogen peroxide

Hydrogen peroxide is a pale blue clear liquid in its purest form. Its viscosity is slightly higher than that of water. Its highly unstable peroxide bond dictates its chemistry. It is unstable in presence of light and therefore it is stored with a stabilizer in a weakly acidic solution (Budi, 2008)**.** Hydrogen peroxide is prepared using a four-step anthraquinone process. Hydrogen is reacted with anthraquinone (Q) to produce anthrahydroquinone (HQ) in a reaction catalyzed by palladium catalyst. The resultant solution is then rendered free of the catalyst by filtration. Bubbling compressed air through the solution produces hydrogen peroxide through oxidation. The final product is then extracted using a liquid-liquid extraction column and concentrated by vacuum distillation (Campos, Blanco and Fierro, 2006). Hydrogen peroxide is used as bleaching agent for pulp and paper (Hage and Lienke, 2006). It is also extensively utilized in the manufacture of laundry detergents (Jones, 1999). Various organic peroxides such as peroxy acids, dibenzoyl peroxide (floor whitening agent and in management of acne) and organic peroxide-based explosives such as acetone peroxide have been produced using hydrogen peroxide. Hydrogen peroxide has disinfectant and antiseptic properties (Block, 2001; Russell, 2001; Rutala and Weber, 2008; Falagas *et al.*, 2011). In cosmetic industry, hydrogen peroxide has been used as bleach for the human hair and hence the name ‘peroxide blonde’ when blended with aqueous ammonia (Lane, 2003) as well as a tooth whitener and management of acne (Capizzi *et al.*, 2004). In alternative medicine, it has been used to manage conditions such as emphysema, HIV-AIDs, cancer and influenza (Douglass II, 2003), although there is no evidence of its effectiveness.

1.2.4.3 Sodium percarbonate

In presence of water, sodium percarbonate produces hydrogen peroxide (dissociate to oxygen and water), sodium ions and carbonate ions. Sodium percarbonate is manufactured through crystallization of hydrogen and sodium carbonate solution at appropriate concentration and pH (Pritchard and Islam, 2003). Sodium percarbonate is an oxygenating agent which works by providing oxygen radicals that loosens debris, kill obligate anaerobes, and get rid of light stains (Hasturk *et al.*, 2004). Mouthwashes containing sodium percarbonate are therefore recommended for stain removal, management of acute ulcers in patients, pre-prosthodontic treatment in patients with intellectual/physical impairment and in reduction of gum inflammation (Parashar, 2015).

1.2.5 Halogens

Halogen-based mouth gargles and rinse products contain either fluorine or iodine such as sodium fluoride and PV-I.

1.2.5.1 Povidone-iodine

Povidone-iodine is chemically constituted by hydrogen iodide, povidone and elemental iodine. It is soluble in water, isopropyl alcohol, ethanol, propanol, glycerol and polyoxyethylene (Kumar, 2009). Povidone-iodine is produced from the reaction between polymer povidone and iodine (Reddy, 2012). It is a wide antimicrobial agent that has effect against protozoa, bacteria, virus, and fungi. As a mouth, PV-I has been effective in the reduction of gingivitis and plaque. When used frequently, it helps to sustain oral hygiene as well as reducing duration, severity, and incidence of mucositis (Parashar, 2015).

1.2.5.2 Sodium fluoride

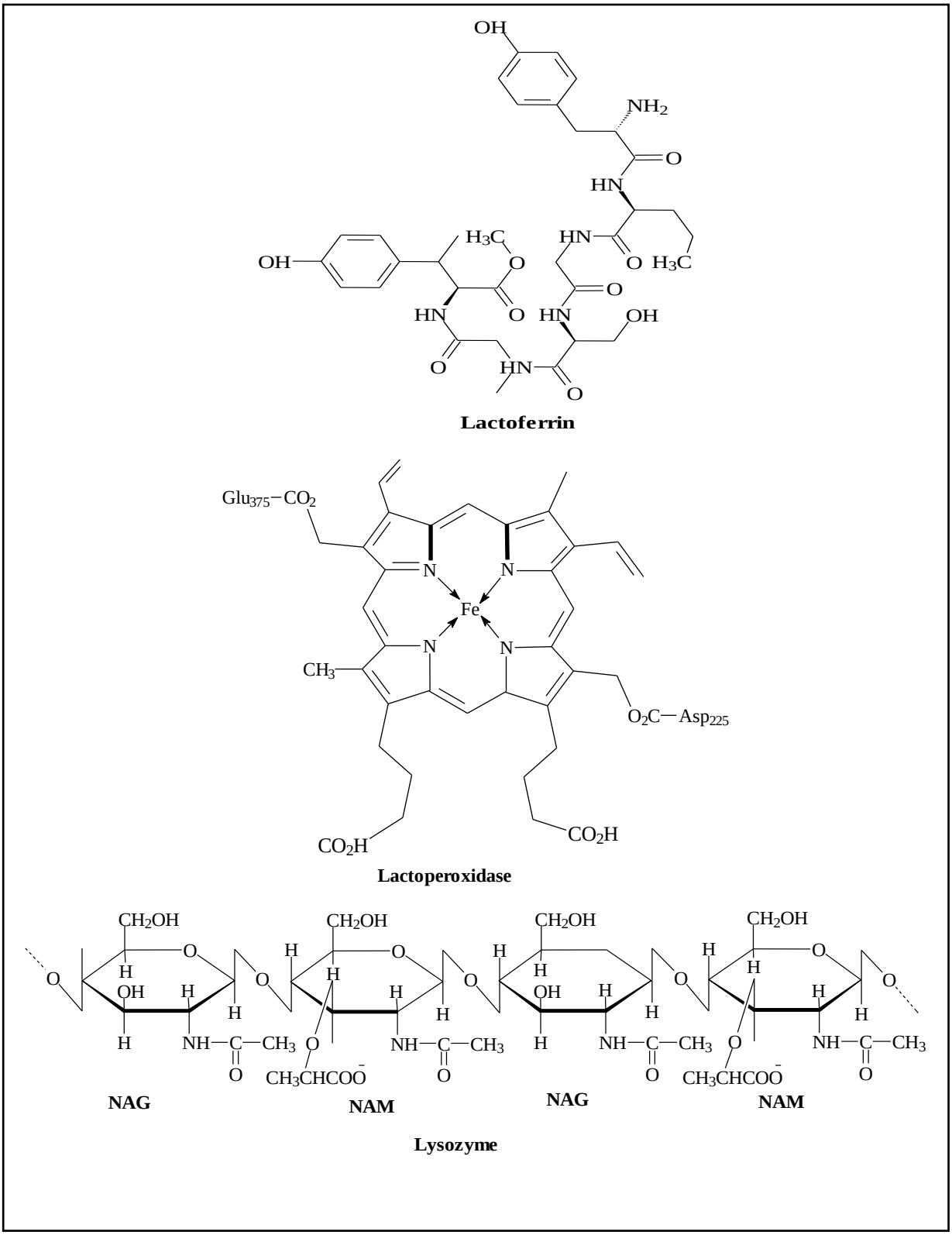
Sodium fluoride on the other hand is an inorganic ionic compound that dissolves in water to produce fluoride and sodium ions (Marinho *et al.*, 2016). The fluoride ions prevent dental carries by improving remineralization with fluoro-hydroxyapatite and fluorapatite, hence, improving the enamel resistance against the attack from acids. Sodium fluoride is prepared through neutralization of hydrofluoric or hexafluorosilicic acid (superphospate fertilizer by products) with neutralizing agents such as sodium carbonate and sodium hydroxide in presence of a precipitating agent (alcohols). The reaction produces sodium bifluoride precipitate, which is subsequently heated to release hydrogen fluoride and sodium fluoride (Aigueperse *et al.,* 2000). These mouthwashes and gargles are recommended for the patients

that are susceptible to dental caries and even those that experiences xerostomia after thermotherapy and irradiation (Marinho *et al.*, 2016).

1.2.6 Enzymes

Some of the enzymes that have been incorporated in mouthwashes include lysozyme, lactoferrin, and lactoperoxidase (Tenovuo, 2002). The lactoperoxidase system is composed of hydrogen peroxide and lactoperoxidase thiocyanate (Seifu, Buys and Donkin, 2005) Lactoferrin is found in seminal fluid, saliva and milk as well as in polymorphonuclear leukocytes. However, lactoferrin is largely found in bovine and human milk. The high affinity for iron is responsible for lactoferrin antibacterial activity. It binds to iron leading to iron deficiency in the microorganisms hence slows their growth (Cheng *et al.*, 2008)

Lysozyme is found in many biological tissues and fluids such as cervical secretions, saliva, respiratory secretions, milk, plant bacteria and avian egg. The enzyme lyses bacteria cell wall by splitting the link connecting *N*-acetymuramic acid and *N*-acetylglucosamine leading to bacterial death (Ibrahim, Hisham and Matsuzaki, Tetsuji and Aoki, 2001). Figure 1.4 shows some structures of enzymes found in mouthwashes.



NAM= N-acetylemuramic acid, NAG= N-acetylglucosamine

Figure 1.4: Chemical structure of lactoferrin, lactoperoxidase and lysozyme enzyme

1.2.7 Metal salts

Zinc (Zn^{2+}), stannous (Sn^{2+}) and copper (Cu^{2+}) are the most commonly used metal ions in dental preparations. The metal ions affect bacterial growth, bacterial enzymes, plaque formation, the glycolytic sequence in oral anaerobic bacteria, and prevent the conversion of urea to ammonia by plaque bacteria (Vranić *et al.*, 2004). Zinc is added to mouthwashes as zinc chloride ($ZnCl_2$), zinc fluoride (ZnF_2) and zinc citrate ($C_{12}H_{10}O_{14}Zn_3$) while stannous is added to mouthwashes as stannous fluoride (SnF_2) or stannous pyrophosphate ($O_7P_2Sn_2$) (Storehagen, Ose and Midha, 2003).

1.2.8 Natural products

Natural products have continued to provide mankind with medicinal compounds with about 80% of world relying on natural products for healthcare needs (Ekor, 2014). Some of the compounds from plants that are included in mouthwashes include: sanguinarine, apigenin, *tt*-farnesol (Awang, 2009).

Sanguinarine is an alkaloid present in the bloodroot sap which contains anti-inflammatory and anti-bacterial characteristics responsible for reduction of bleeding and gingival inflammation as well as preventing plaque formation (Foster and Duke, 2000). Research has shown that the alkaloid stays in the buccal cavity for an extended period upon brushing hence prolonging its effects (Maryand James, 2005).

Apigenin is present in vegetables and fruits. The common sources include chamomile, celeriac, celery, and parsley tea (Emily Caldwell, 2013). It has antibacterial impact on streptococcus mutans. Its use reduces dental caries and prevents the synthesis of water-soluble glucans (Koo et al, 2005).

tt-farnesol is found in propolis, a product normally found in beehives. It shows effect against streptococcal membranes through enhancement of proton penetrability as well as prevention of acid release by S. mutans inside biofilms. Koo *et al* demonstrated that its topical application decreased frequency of dental caries without affecting the viability of mouth's normal flora (Koo *et al.*, 2002). Figure 1.5 illustrates the chemical structure of some commonly used phytochemicals in oral hygiene products.

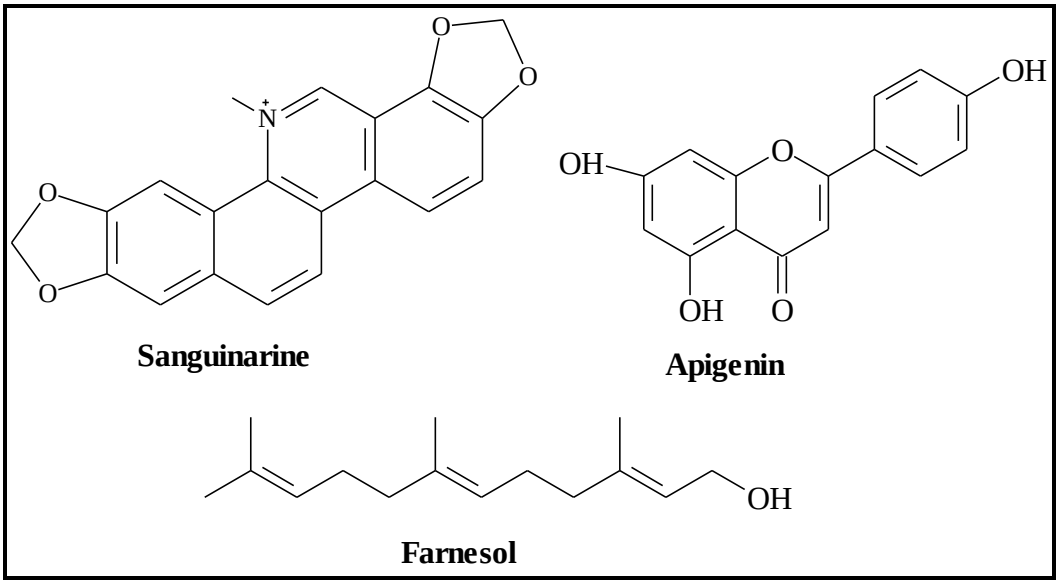


Figure 1.5: Chemical structures of herbal compounds

1.2.9 Alcohols

Monohydric and polyhydric alcohols are known to possess antimicrobial activity. However, when added in mouthwashes, alcohol is mainly used as a carrier for plaque penetration enhancers such as thymol, eucalyptol and menthol rather than an antimicrobial agent (Toedt, Koza and Van Cleef-Toedt, 2005). Specifically, a substantial quantity of alcohol at about 27% v/v may be used as a carrier for the flavor in the mouthwashes (Lachenmeier *et al.*, 2008). Alcohols are also known to have a preservative and antimicrobial property especially at high concentration (McCullough and Farah, 2008). The antimicrobial activities of alcohols on fungi, viruses and bacteria are a result of protein denaturation and dissolution of lipids (Parashar, 2015). Some of the alcohols that have been incorporated in mouth products include ethanol and isopropyl alcohol. Alcohols are however associated with adverse affects such as hypoglycemia, coma or tonic seizure activity especially in children (Lemos and Villoria, 2008).

1.3 Povidone-iodine and chlorhexidine-based mouthwash products

1.3.1 Povidone-iodine

1.3.1.1 Description

Povidone-iodine possess antimicrobial properties against protozoa, mycobacteria, viruses, Gram-negative and Gram-positive bacteria and fungi. The free iodine that is produced from PV-I kills both prokaryotic and eukaryotic cells (Reddy, 2012). It is available in a reddish-brown powder that changes color to brown orange (Harry, 2002).

1.3.1.2 Chemistry

Povidone-iodine is a non-covalent complex comprising of iodine and polyvinylpyrrolidone. The percentage of iodine in the complex is at approximately 9 to 12 % (Reddy, 2012). The IUPAC name for PV-I is 1-ethenylpyrrolidin-2-one; molecular iodine. It has a molecular weight of 364.95 g/mole while its chemical formula is $C_6H_9I_2NO$. It is also known as isodine or PVP iodine (Reddy, 2012; National Center for Biotechnology Information, 2020). Figure 1.6 below illustrates the chemical structure of PV-I.

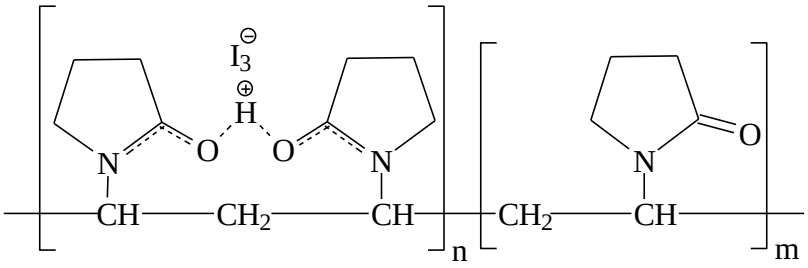


Figure 1.6: Chemical structure of PV-I

1.3.1.3 Synthesis

Povidone-iodine solution is formulated by directly mixing iodine powder into a mixture containing ethanol and polyvinylpyrrolidone. The iodine vapor route produces PV-I by passing the gaseous form of iodine (iodine vapor) into a fumed polyvinylpyrrolidone in a chamber. It can also be formulated by directly mixing the powders of the two components (iodine and polyvinylpyrrolidone) to produce the complex (Hong *et al.*, 2009).

1.3.1.3 Mode of action

Unbound ('free') iodine from the complex is responsible for the antimicrobial activity of PV-I. Polyvinylpyrrolidone has no antimicrobial activity but rather acts as the carrier for the iodine in the solution to sites of action (J. Kanagalingam, R. Feliciano, J.H. Hah, H. Labib, T.A. Le, 2015). Therefore, the mechanism of action of PV-I solely depends on the liberated iodine. Unbound iodine causes the oxidation of nucleic and amino acids in biological structures in reactions that are difficult to counteract leading to disruption of microbial metabolic pathways as well as structural weakening of pathogen's cell membranes which result in cell death. The consumed unbound iodine is then replenished by the iodine bound in the complex for continuity of microbicidal effect. Therefore, the antimicrobial strength of PV-I is directly associated with the concentration of the unbound iodine (Shirai *et al.*, 2000; Kumar *et al.*, 2006). Bactericidal activity of PV-I is due to structural damage on the

components of the nucleus and cytosol whereas in fungi, it damages the cell walls (Selvaggi *et al.*, 2003). For viruses, PV-I causes death by promoting degeneration of the nucleoproteins of the viral molecules as well as disruption of superficial proteins in the case of enveloped viruses. In addition, PV-I inhibits the release of pathogenic factors such as endotoxins and exotoxins in bacteria. It has also been found to have anti-inflammatory activity as iodine is a free oxygen radical scavenger (Beukelman *et al.*, 2008).

1.3.1.4 Clinical indications

There are various applications of PV-I in medicine. Some of the applications include disinfection of mucosal surfaces, hands, skin, and body. PV-I is also applied to treat wounds, eye applications, and rinsing of body joints and cavities (Reimer et al., 2002). It is used to reduce gingivitis and plaque as well as promotion of general oral hygiene. It is used in the reduction of duration, severity and occurrence of radiation mucositis (Farah, McIntosh and Lidija, 2009). Table 1.2 shows the various formulations containing PV-I in the market.

Table 1.2: Formulations of povidone-iodine in clinical use

Formulations	Clinical use	Active ingredients
PV-I solution	Preoperative site preparing, burn treatment, laceration treatment, wound antisepsis, catheter site disinfection, catheter care.	10% PV-I, surfactant (nanoxynol-9). The role of nonoxyl-9 in PV-I formulation is to decrease the surface tension of the product for easier infiltration and better contact of the product with the skin. It also gives foam which aid in cleansing process.
PV-I surgical scrub	Surgical hand scrub	7.5% PV-I, surfactant (ammonium nonoxyl-4 sulphate) and Lauramide
Skin cleanser	Laceration treatment	7.5% concentration PV-I
Ointment	Wound antisepsis	10% PV-I concentration
Swab stick	Preoperative skin preparation	10% PV-I concentration
Swab aid	Disinfection of catheter site	10% PV-I concentration
PV-I aerosol spray	Prepping of preoperative site	5% PV-I concentration
Mouthwash/gargle	Throat/mouth antisepsis	0.5% PV-I
PV-I douches	Antisepsis of vagina	0.3% PV-I, surfactant (nanoxynol-9)
PV-I perineal concentration	Anogenital area antisepsis	10% PV-I, surfactant (nanoxynol-9)

Adopted from (Daryl S. Paulson, 2002).

1.3.1.5 Adverse effects

Topical PV-I ointment (10%) together with 1% iodine in burn patients is associated with incidences of extreme metabolic acidosis. The use of topical PV-I in newborns has also been associated with hypothyroidism especially in iodine-deficient regions (Markou *et al.*, 2001). Exposure to PV-I at 10% concentration has been associated with primary irritant dermatitis and allergic dermatitis (Vandergriff *et al.*, 2006). Povidone-iodine based mouthwashes have also been linked to local mucosal irritation, rare type-I hypersensitivity allergic reaction and impaired renal function. Highly concentrated mouthwash solutions of PV-I may affect the thyroid gland whereby cases of thyroid adenoma, hypothyroidism/hyperthyroidism, goiter, and thyroid gland hyperplasia have been reported (Patil *et al.*, 2011).

1.3.2 Chlorhexidine

1.3.2.1 Description

Chlorhexidine is an antiseptic solution which has been in use since 1950 (Weinstein *et al.*, 2008). It has been shown to be efficacious and safe for vaginal antisepsis, pre-operative skin preparation, gingivitis treatment, body wash against normal sepsis, and in hand washing (Weinstein *et al.*, 2008). At 0.12 – 0.2 %, it is used as an anti-bacterial mouthwash as it prevents gingivitis and dental plaque formation (Yates et al., 2002).

1.3.2.2 Chemistry

Chemically, chlorhexidine contains two biguanide groups and two 4-chlorophenyl rings connected through a hexamethylene chain at the center. Chlorhexidine digluconate, a strong base, is the most stable salt of CHX with a high affinity for anionic elements due to its dicationic charge (Bascones *et al.*, 2005). The IUPAC name for chlorhexidine is 1, 6-di(4-chlorophenyldiguanido) hexane. It has a molecular weight of 505.45 g/mole and its empirical formula is $C_{22}H_{30}Cl_2N_{10}$. Figure 1.7 below illustrates structure of chlorhexidine.

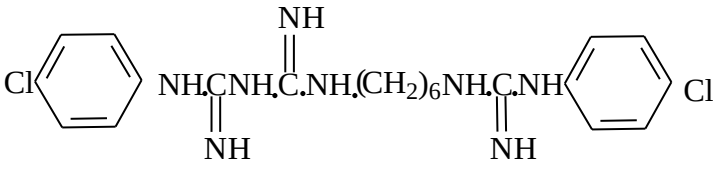


Figure 1.7: Chemical structure of chlorhexidine

1.3.2.3 Synthesis

Chlorhexidine is manufactured industrially in a two-stage process as shown in Figure 1.8. First, hexamethylenediamine (I) is reacted with hydrochloric acid to produce hexamethylenediamine dihydrochloride which is subsequently reacted with sodium dicyanamide (II) under reflux reaction and in an alcoholic condition at about 110°C to produce 1,6- hexamethylenebis(dicyanamide) intermediate (III). In the second step, 4-chloroaniline (IV) is reacted with 1, 6- hexamethylenebis (dicyanamide) intermediate (III) under reflux conditions in an alcoholic solvent such as ethanol, 2-ethoxyethanol or *iso*-propanol to produce chlorhexidine base. Addition of hot aqueous sodium hydroxide enables the separation of the chlorhexidine base from water soluble impurities (Werle, 2013). Chlorhexidine is then recrystallized using methanol (CH_3OH) to produce the purified chlorhexidine form.

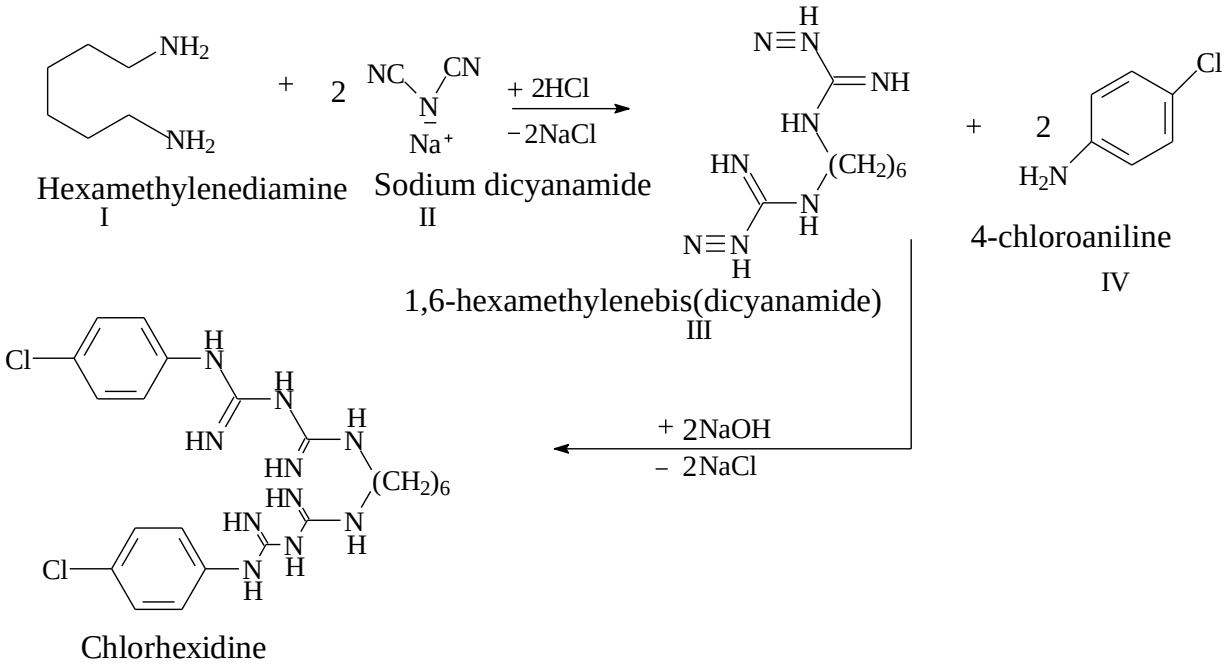


Figure 1.8: Synthesis of chlorhexidine

1.3.2.4 Stability

Chlorhexidine is chemically stable and exhibit maximum biological activity at a pH of 5 to 8 (Lunestad *et al.*, 2010). It is therefore pH-dependent whereby at pH greater than 8, the chlorhexidine base precipitates while in acidic pH less than 5, a reduction in its antibacterial effect is observed (Block, 2001). The diluted form of aqueous CHX solution (<1%) is heat-stable and can therefore be rendered sterile by autoclaving at 123 °C for up to 15 min. With

appropriate packaging, aqueous solutions of chlorhexidine are stable on storage at room temperature for more than a year (Lunestad, Hegstad and Scheie, 2010). However, prolonged exposure to extreme temperatures and light affects its stability (Block, 2001).

1.3.2.5 Mode of action

Chlorhexidine works by increasing the permeability across micro-organism's cell membranes causing a change in the concentration of intercellular potassium (Bascones *et al.,* 2005). It also provides effect by inhibiting glycosidic and proteolytic enzymes in microorganisms (Addy, M. and Moran, 2008). Concentrated CHX is a bactericidal agent through the induction of cytoplasmic precipitation that resulting in cell death. It remains active for over 8 to 12 hours in body tissues (across both hard and soft tissues) due to its chemical structure. This property is called substantivity (Bascones *et al.*, 2005).

Chlorhexidine is active against bacteria including aerobes, anaerobes as well as other Gram-positive and Gram-negative bacteria and fungi. Chlorhexidine has been shown to retain its activity against Gram-negative anaerobes for up to 2 years (Addy, M. and Moran, 2008). It is available in the market at 0.12% and 0.2% (Rath and Singh, 2013). Studies have shown that the two concentrations produce maximum clinical results. However, adverse effects are concentration/dose dependent. Therefore, chlorhexidine at 0.12% has been recommended as it provides for an increase in patient compliance (Rath and Singh, 2013). Alcohols can be added to chlorhexidine as a solvent as well as an antiseptic (Borrajo., 2002).

1.3.2.6 Clinical indications

Chlorhexidine reduces skin flora by 86-92% when applied for hand washing (Weinstein *et al.*, 2008). The residual effect on the skin prevents proliferation of skin's normal flora and therefore prolongs antisepsis. Chlorhexidine mouthwashes has been used to decrease the duration and discomfort of minor aphthous ulceration which in turn leads to the increase of ulcer free days (Sajjan *et al.*, 2016). When used as a mouthwash especially for preoperative and postoperative teeth extraction procedures, it lowers the oral microflora leading to low incidences of post-extraction bacteremia (Šečić, Sadeta; Prohić, Samir; Komšić, Sanja; Vuković, 2015). In addition, it improves preoperative gingival healing (Hibbard, Mulberry and Brady, 2002).

Chlorhexidine has also been used in urinary tract catheterization where the urinary bladder is irrigated with 0.005% solution to lower the likelihood of urinary tract infection in prolonged urinary catheterization (Hessen, Kaye and Zuckerman, 2000). It is also used in newborn skin cleaning, maternal vaginal lavage and umbilical cord cleaning to prevent neonatal infections (Lumbiganon *et al.*, 2014). Chlorhexidine has also been used as a hand sanitizer and as a surgical area preparation product during surgical procedures to prevent wound infection as it lowers the number of normal skin flora prior to skin cut (Mulberrry *et al.*, 2001).

1.3.2.7 Adverse effects

After prolonged use as a mouthwash, it is associated with transient and tongue taste impairment and browning of teeth (Pippi, 2017). It also increases the likelihood of calculus formation (Rath and Singh, 2013).

1.4 Study Justification

Breakthrough in disease management is dependent on various factors. The quality of the medicines is one of the most important of these factors. Therefore, in the management of conditions such as sores, gum swelling, and halitosis using gargles and mouthwash products containing PV-I and CHX, the quality of these products should be determined and ascertained for better outcomes.

Most gargles and mouthwashes are available locally as over-the-counter medications due to less safety concerns. Chlorhexidine-based mouthwash products are available in the market at two concentrations (0.1% and 0.2%). At the lowest concentration (0.1%), chlorhexidine is ineffective in plaque removal while at the highest concentration (0.2%), it is effective but is however associated to causing mucosal erosion and teeth discoloration (Strydonck et al., 2012).

Chlorhexidine is unstable when exposed to conditions of extreme temperature and light (Block, 2001). It therefore necessary to ascertain the quality of chlorhexidine-based mouthwashes as substandard preparations may lead to economic losses especially to the patients. Quality products are effective and also provide assurance that they are free from contamination by pathogens which may lead to poisoning or further ailments (Newton, Green and Fernández, 2010; Roger, 2012).

Povidone-iodine based mouthwashes and gargles are effective in preventing the formation of plaque as well as reducing the severity of radiation mucositis and gingivitis. The concentration of PV-I in mouthwashes differ from product to product. Products with a high concentration of PV-I have been associated with thyroid gland issues such as hypothyroidism/hyperthyroidism, thyroid gland hyperplasia, goiter and thyroid adenoma (Patil *et al.*, 2011). However, products with a low concentration of PV-I have been shown to be ineffective in reducing plaque formation (Kanagalingam et al., 2017). In Kenya, there is no literature on studies or published data that have been conducted to establish the quality of chlorhexidine and PV-I based mouthwash and gargle products in the market. The study therefore sought to determine the quality of mouthwash and gargle products containing chlorhexidine and PV-I in the Kenyan market.

1.5 Objectives of the study

1.5.1 General objective

The study was conducted to assess the quality of povidone-iodine and chlorhexidine-based mouth rinse/gargle products in Nairobi County.

1.5.2 Specific objectives

Specific objectives of this study were:

i. To determine the amount of chlorhexidine in samples of chlorhexidine mouthwashes and gargles.
ii. To determine the amount of iodine in samples of povidone iodine mouthwashes and gargles.

CHAPTER 2

LITERATURE REVIEW

2.1 Survey of analytical methods for povidone-iodine

A number of methods have been documented for the analysis of povidone-iodine. From the literature, PV-I has been analyzed via High Performance Liquid Chromatography (HPLC), X-ray photoelectron spectroscopy and potentiometric titration.

2.1.1 High-Performance Liquid Chromatography

Ohshiro, Hokama and Hobara utilized a HPLC method using a C-18 reversed phase octadecylsilane (ODS) column with a UV-Vis detector set at 355 nm to determine the content of iodine and iodide in povidone-iodine containing products such as gargles, solutions and ointments. In this method, the flow rate of the mobile phase was set at 1 mL per min. Povidone-iodine had retention time of 3.9 min. For gargles and solutions, povidone-iodine content was above 95 % while in the ointments, the content correlated (R=0.9999) with the iodine content obtained via titration method (Garg, Jambu and Vermani, 2007; Ohshiro, Hokama and Hobara, 2011).

2.1.2 X-ray photoelectron spectroscopy

Yai and Yai developed X-ray photoelectron spectroscopy method to analyze PV-I topical spray. The spectra of the sample were recorded using PHI 5000 versa Probe II with Al K α ($h\nu$ = 1486.3 eV) detector. The results of the analysis showed that the spray had iodine content with the acceptable range of 85% to 120%. This method of analysis confirmed that the ionized form of iodine was higher than the unionized (Yai, Yai and Yai, 2017).

2.1.3 Potentiometry

In the potentiometric method, a methyl violet tri-iodide sensor is used to determine the content of iodine. Potentiometric analytical methods are preferred because of simplicity, selectivity and sensitivity (David et al., 2014). Sequential flow injection-based potentiometric method has been used for the assay of iodide and iodine in povidone-iodine containing products. In this method, iron (II) trisbathophenanthroline iodide ion pair complex is used as the exchange site while the sandwich cell is in a PVC membrane. The sensor has a linear range response of 10^{-1} to 10^{-6}. This method is known to be highly sensitive and precise over a wide range of concentration in the analysis of iodine and iodide in the analysis of povidone-iodine containing products (David et al., 2014). However, according to the 2017 British

Pharmacopoeia, povidone-iodine is analyzed using titrimetry (redox titration) with sodium thiosulfate as a titrant. The titrant serves as a reducing agent in acidic media as shown in the half reaction (Equation 1). Before titration, the titrant is standardized using potassium bromate and potassium iodide in presence of hydrochloric acid as shown in Equation 2 and 3.

$2S_2O_3^2 \quad 2e- \rightarrow \quad S_4O_6^{2-}$ ..... Equation 1

(Thiosulfate ion) (Tetrathionate ion)

$I_{2(g)} + 2Na_2S_2O_{3(aq)} \rightarrow 2NaI_{(aq)} + Na_2S_4O_{6(aq)}$ .....

Equation 2

$KBrO_{3(s)} + 6KI_{(s)} + 6HCl_{(aq)} \rightarrow KBr_{(aq)} + 6KCl_{(aq)} + 3H_2O_{(l)} + 3I_{2(g)}.$

Equation 3

The standardization reaction releases iodine gas which is titrated with sodium thiosulfate as shown in Equation 3.

2.2 Analytical methods for chlorhexidine

Various methods to analyze CHX such as chromatography, spectrometry, capillary electrophoresis and solid phase extraction have been developed and described in literature. However, HPLC (a BP method) is the most preferred method of analysis.

2.2.1 Chromatography

High Performance Liquid Chromatography is the official method (as described in the BP and European Pharmacopoeia) of analysis for chlorhexidine and its salts in irrigation solutions and mouthwashes. The USP29-NF24 describes the HPLC method for the analysis of chlorhexidine gluconate mouthwashes (USP, 2014). The BP 2019 describes gas chromatography as the method of analysis of *p*-chloroaniline (related substance) while USP29-NF24 describe HPLC as the compendia method (USP, 2014; BP, 2017b). In literature, HPLC has been cited as a method of analysis for chlorhexidine associate impurities and related substances as a non-compendia method (Masquio Fiorentino, Correa and Nunes Salgado, 2010).

Havlikova and Nov developed a novel isocratic reverse-phase HPLC for the simultaneous determination of chlorhexidine gluconate and its related substance (p-chloroaniline). This method utilized SB Zobrax Phenyl column (75mm x 4.4mm, 3.5µm) for the separation. The mobile phase consisted of a buffer solution of 0.08M sodium phosphate monobasic (with 5 mL triethylamine) and acetonitrile. The mobile phase was adjusted to a pH of 3 with 85 % phosphoric acid. The flow rate of the mobile phase was set at 0.6 mL/min. Detection was via a UV method at 239 nm for about ten min. This method was proven to be fast (separation occurred below 6 min) and suitable for use in routine quality control laboratory (Havlikova and Nov, 2007).

Cardoso *et al* came up with and validated, modest, fast, sensitive and isocratic reverse phase high performance liquid chromatography method to estimate chlorhexidine and *p*-chloroaniline in several pharmaceutical preparations including mouthwashes. A compound partition was accomplished under ten minutes using XBridge C18 column at a temperature of 40 °C. The mobile phase consisted of 32:68 (v/v) of acetonitrile and phosphate buffer, pH 3.0. The samples were analyzed at a flow rate of 2 mL/min and at 239 nm wavelength. Selectivity, accuracy, linearity, precision and robustness were achieved during the analysis. However, it was noted that the method was very sensitive to pH of the mobile phase buffer. The method was successively validated as per International Conference on Harmonization guidelines and therefore suitable for determination of chlorhexidine in the respective formulations (Cardoso *et al.*, 2011).

Dubal *et al* developed ultra-performance liquid chromatography (UPLC) with a photodiode array detector to analyze chlorhexidine gluconate in a throat spray. They used Waters Acquity BEH C18, (2.4 × 50 mm, 1.7µm) at flow rate of 0.3 mL/min and detection at 215 nm. The binary mobile phase used composed of acetonitrile and 20 mM sodium phosphate buffer at pH 3. This method was applied in the analysis of lidocaine hydrochloride and chlorhexidine gluconate in a throat spray sample. The method was sensitive, fast plus had a good resolution, therefore reducing the analysis time of chlorhexidine gluconate and lidocaine hydrochloride to between 1.3 min and 4.5 min respectively (Dubal *et al.*, 2016).

2.2.2 Derivative spectrophotometry

Derivative spectrophotometry has a higher selectivity than common spectrophotometry and provides a stronger method for resolution of band overlaying in quantitative assays of multi-

component blends. The spectra can be produced by processing the output from the spectrophotometer. With derivative spectra, detection sensitivity is improved while errors that may arise from overlapping spectra bands from other components in the samples are reduced. The outright value of the derivative of the sum curve can be determined at a wavelength equivalent to a zero-crossing of the other constituents in the sample mix. Zero-crossing derivative spectroscopy method improves the resolution of the spectra of an analyte because it is recorded at a wavelength which does not pick the signals of other components in the sample (Karpińska, 2004).

Gan and Aziz determined the content of chlorhexidine digluconate in microbicidal preparations using first derivative spectrophotometry. This method was observed to be simple, rapid and direct for analysis of chlorhexidine products. In the method, the spectra were read at a wavelength $\lambda = 276.1$ nm in which the Beers' Law was adhered to in the range of 0 to 50 µg/mL (linearity). Its application was shown to eliminate interferences from other ingredients used in complex matrices (Gan and Aziz, 2011).

2.2.3 Conductometric titration

Conductometric titrations are based on the principle of ions replacement where one ion in a solution is replaced by another with a different conductivity. The overall effect is seen as variation of the conductivity of the solution during the titration process. The endpoint is determined graphically from a graph containing the values of the change in conductance as a function of the titrant volume (Leaflets, 2019).

Conductometric titrations are mostly applied for colored and very dilute solutions as well as systems that involve comparative incomplete reactions. Calatayud, Falcó, and Martí conducted a Conductometric determination of chlorhexidine and proguanil in water/ethanol using copper acetate as the titrant (1986). The procedure was based on the copper-biguanide reaction which gives a pink solid at endpoint. Conductometric titrations are short, selective with similar accuracy and precision as that of standard titrations (Calatayud, Falcó, and Martí, 1986)**.**

2.2.4 Capillary electrophoresis

Capillary electrophoresis method of analysis is suitable for small sample volumes with complex ionic matrix. Fluorescence and UV- absorption are the most commonly used optical

detectors in this method of analysis although they are not suitable for most analyte. Therefore, detection using conductivity method especially axial capacitive joined contactless conductivity detector has been adopted for routine use. Abad-villar *et al* utilized capillary electrophoresis with contactless conductivity detection method for the assay of chlorhexidine digluconate and polyhexamethylene biguanide in eye drops. The study showed that the method had a satisfactory precision of about 3 - 6 % in peak area. Detection limit was comparable with that of the compendia HPLC method of assay for chlorhexidine gluconate. Moreover, capillary electrophoresis is simple, sensitive, compatible with low sample volumes and has a high tolerance to the associated high salt background with the samples (Abad-villar *et al.*, 2006).

2.2.5 Voltammetry

Wang and Tsai used voltammetry analysis to determine the behavior of chlorhexidine in cosmetic and oral hygiene products using film mercury electrodes (glass carbon and electrode pasted mercury electrodes) in an aqueous medium. The basic principle for voltammetry assay is based on adsorptive accumulation of chlorhexidine onto hanging mercury drop electrode. During the analysis, various factors such as time, deposition material, concentration of mercury and other interfering factors that may have interfered with the precision of the method were explored. Chlorhexidine had a peak height at -1.88 V after the analysis. The results of the study had a 98 % similarity index when compared with those obtained using HPLC method (compendia method) and they showed a 98 % similarity (Wang and Tsai, 2001). Table 2.1 gives a summary of analytical methods used in the assay of chlorhexidine-based mouthwashes and gargles.

Table 2.1: Published methods of analysis of chlorhexidine and its salts

Method	Wavelength (nm)	Mobile phase/solvent	Column	Linearity	Sample
Liquid chromatography	239	**Solution A**: sodium phosphate buffer pH 3.0, acetonitrile triethylamine, water. **Solution B**: acetonitrile	L1 (octadecyl silane chemically bonded to porous silica/ceramic micro-particles, 3 to 10 m in diameter)		Gluconate salt of chlorhexidine
Titrimetry		Perchloric acid			Chlorhexidine salts
Gas chromatography		^{63}Ni detector	Supelco®	50 to 200 ppm	Chlorhexidine acetate
Gradient HPLC	230	**Phase A**: ammonium acetate pH 5.0 **Phase B**: acetonitrile	Nucleosil C_{18}	10 µg/mL to 10 mg/mL	Chlorhexidine gluconate

Adopted from (Masquio Fiorentino, Correa and Nunes Salgado, 2010).

CHAPTER 3
EXPERIMENTAL

3.1 Study design

The study was carried out in Nairobi County which is one of the 47 counties in Kenya and the most populous county according to the recently conducted census with about 4.4 million people (Census, 2019). Nairobi is one of the largest and fastest growing cities in Africa as an administrative, economic and cultural hub. Nairobi is also the Kenyan capital city which harbors most of the pharmaceutical industries, distributors and wholesalers of pharmaceuticals and therefore is an ideal sampling location for the study.

The sampled products were analyzed in the Drug Analysis and Research Unit (DARU) within Kenyatta National Hospital complex at the Department of pharmaceutical chemistry, as well as at the Centre for Traditional Medicine and Drug Development Program at Kenya Medical Research Institute (KEMRI) along Mbagathi way, Nairobi.

3.2 Sample collection

A total of 34 samples drawn from 15 brands of PV-I containing mouthwashes and gargles were collected from retail pharmacies within Nairobi County through convenient sampling. Likewise, a total of 15 samples drawn from 9 brands of CHX containing mouthwashes and gargles were collected using the same sampling approach. Where possible, three (3) batches of each product were sampled. All the collected samples were coded based on the brand and batch number on the products to avoid biasness during the analysis. Sample collection took a period of 4 months starting from February 2019 to May 2019. Table 3.1 and 3.2 shows the sample information for PV-I and CHX mouthwashes and gargles respectively.

Table 3.1: Sample information for povidone-iodine mouthwashes and gargles

CODE	BATCH NO	LABEL CLAIM
PI 1a	P406	PV-I USP 1% w/v (equivalent to 0.1% iodine).
PI 1b	P25	
PI 1c	P447	
PI 2a	64408	PV-I containing 0.1% available iodine.
PI 2b	53009	
PI 2c	60108	
PI 3a	181167	Each 100ml contains 1g PV-I which is equivalent to 0.1% available iodine.
PI 3b	1806192	
PI 3c	1806193	
PI 4a	5804805	PV-I BP 1% w/v.
PI 4b	5804244	
PI 4c	5801826	
PI 5a	518104	PV-I BP 1% w/v.
PI 5b	1217013	
PI 5c	219004	
PI 6a	28R0001	Contains 2% w/v PV-I USP equivalent to 0.2% available iodine.
PI 7a	18D227K41	One gram PV-I with a content of 10% available iodine.
PI 7b	18D232N41	
PI 7c	18D229K41	
PI 8a	NR1832	PV-I USP 2% w/v (available 0.2% w/v).
PI 9a	1801238	Each 100 ml contains 1g PV-I which is equivalent to 0.1% available iodine.
PI 9b	1801264	
PI 9c	1801255	
PI 10a	11653	PV-I USP 1% w/v.
PI 10b	11593	
PI 10c	11609	
PI 11a	17-XDGS-022	Iodinated povidone BP 2% w/v (0.2% w/v available iodine).
PI 11b	17-XDGS-021	
PI 12a	EAM1-001	PV-I IP 1% w/v (equivalent to available iodine 0.1% w/v).
PI 12b	EAM1-002	
PI 13a	NC 002	PV-I USP 10% w/v (equal to available iodine 1%w/v).
PI 14a	119002	Each 100 mL contains 1g PV-I which is equivalent to 0.1% available iodine.
PI 14b	118015	
PI 15a	139	PV-I USP 10 mg.

Table 3.2: Sample information for chlorhexidine mouthwash and gargles

CODE	BATCH NO	LABEL CLAIM
C1a C1b C1c	RAJ3583 RAJ1J73 RAJ2F83	Chlorhexidine gluconate solution BP diluted to 0.2 % w/v.
C2a C2b C2c	109853 109779 109779	Each 15 ml contains chlorhexidine gluconate 18 mg.
C3a	5145543	Chlorhexidine digluconate 0.2 % w/v.
C4a C4b	BC2557 BM2560	Chlorhexidine gluconate solution BP diluted to 0.2 % w/v.
C5a	372	Dilution of chlorhexidine gluconate solution BP to chlorhexidine gluconate 0.2 % w/v
C6a	5145248	Chlorhexidine digluconate 0.2 % w/v.
C7a	8522038A	Chlorhexidine digluconate at 0.12 %.
C8a	E868	Chlorhexidine gluconate solution BP diluted to 0.2 % w/v.
C9a C9b	E862 E867	Chlorhexidine gluconate solution BP diluted to 0.2 % w/v.

3.3 Labeling

To ensure that there was conformity to labeling standards the samples were assessed to check for the presence of batch/lot numbers, presence of package inserts for the patients, information on storage conditions, manufacturing and expiry dates, manufactures official address, any precaution on use, market authorization number, list of excipients as well as (KEBS) standardization marks (S-mark) and permit numbers.

3.4 Materials

3.4.1 Chemicals, reagents and solvents

Analytical grade sodium thiosulfate and potassium bromate were from Sigma-Aldrich (Steinheim, Germany) while hydrochloric acid and HPLC grade methanol were from Loba Chemie Pvt. Ltd (Mumbai, India). Other reagents used include sodium octanesulfonate and potassium iodide from Oxford Lab Chem (Thane, India) and glacial acetic acid from VWR international SAS (Fontenay sous Bois, France). Distilled water was prepared using a Thermo scientific water distillation system (Smart2Pure 3UV/UF, Niederelbert, Sweden) at KEMRI, Nairobi.

3.4.2 Chlorhexidine working reference standard

Chlorhexidine gluconate, a working reference standard (96.7% w/w) was a kind donation by the National Quality Control Laboratory.

3.5 Equipment

3.5.1 Titro Processor

The titration of iodine-based samples was performed on an automated titrator TitroLine® 6000 (Si Analytics GmbH, Mainz, Germany), with 3.5 inch -1/4 VGA TFT display equipped with stirrer.

3.5.2 Liquid chromatograph

Analysis of chlorhexidine was conducted using an Agilent 1260 Infinity liquid chromatography system (Agilent technologies, California, USA) which was equipped with a 1260 quaternary pump G1311C (S/N: DEAB818928), 1260 standard auto sampler G1329B (S/N: DEAAC39869), 1260 thermostated column compartment G1316A (S/N: DEACN4229), multiple wavelength detector G1315C (S/N: DEAAX08605) and a 1260 diode array. Separation was on LICHrospher® 100 RP-18 end capped Merck KGaA, Darmstadf, Germany), column. All mobile phase were degassed with the help of MRC DC 200H ultrasonic bath and then filtered using 0.45 μm PTFE filter (BP, 2017a).

3.6 Analytical procedures

Chlorhexidine containing products were analyzed using HPLC as per BP (2017) specifications for CHX content limit at 95% to 105% (BP, 2017a). The assay of iodine in PV-I containing products was performed using redox (potentiometric) titration as prescribed in BP (2017) at a range of 85% to 120%.

3.6.1 Analysis of chlorhexidine

Chlorhexidine samples were analyzed using HPLC as prescribed in BP (2017). The procedure involved preparation of the mobile phase, sample, working reference standard and analysis of the samples in the HPLC system. The analysis was performed in triplicate against the working reference standard (BP, 2017a).

3.6.1.1 Preparation of mobile phase for the analysis of chlorhexidine

The mobile phase for chlorhexidine gargles and mouthwashes was prepared using 270 mL purified water, 120 mL glacial acetic acid (HPLC grade),730 mL methanol (HPLC grade) and 2g sodium octanesulfonate. The mobile phase was subsequently passed through mobile phase filtration unit prior to sonification for about 20 min.

3.6.1.2 Sample preparation

Five mL of chlorhexidine sample was put into a 100 mL volumetric flask and subsequently diluted using adequate mobile phase and made to volume. The solution was filtered through 0.45 μm nylon filters and sonicated for about 20 min before transferring into HPLC vials.

3.6.1.3 Preparation of chlorhexidine working reference standard

A working reference standard solution was prepared by diluting 20 mg of chlorhexidine gluconate chemical reference standard with the mobile phase in a 25 mL volumetric flask. Ten milliliters of the resultant solution were then pipetted into a 100 mL volumetric flask and made to volume using the mobile phase to achieve 0.08 mg/mL concentration.

3.6.1.4 Assay

Chlorhexidine samples were analyzed using HPLC with a flow rate set at 1.50 mL/min and with an injection volume of 20 μL using LICHrospher® 100 RP-18 end capped column (Merck KGaA, Darmstadt, Germany). The detection wavelength for chlorhexidine was set at 254 nm while the temperature for the column was set at 30 °C.

3.6.2 Analysis of povidone-iodine containing products

Povidone-iodine containing samples were analyzed using potentiometric titration as per BP (2017) specifications to determine iodine content at a limit of 85% to 120%. The procedure involved standardization of sodium thiosulfate (titrant), sample preparation and analysis of samples to a potentiometric end point.

3.6.2.1 Standardization of 0.1 M Sodium thiosulphate

Standardization procedure for 0.1M NaS_2O_3 was as per BP (2017) specifications whereby, about 40 mL of distilled water was transferred into a 250 mL beaker together with 10 mL potassium iodide, 5 mL 7M hydrochloric acid, and 20 mL 0.0167M potassium bromate.

The resulting solution was titrated using 0.1M sodium thiosulfate to a potentiometric endpoint (BP, 2017). The factor of 0.1M NaS_2O_3 was calculated as shown in Equation 4.

$$Factor = \frac{Amount\ of\ potassium\ bromate\ in\ 20\ mL}{Volume\ of\ sodium\ thiosulfate \times Equivalence} \quad \text{..... Equation 4}$$

3.6.2.2 Sample preparation

One hundred milliliters of samples containing povidone-iodine were transferred into a 250 ml beaker. To the sample, 40 mL of distilled water and 10 mL of 0.1M hydrochloric acid were added.

3.6.2.3 Assay

Povidone-iodine samples were assayed potentiometrically using an automatic titrator TitroLine® 6000 (Si Analytics GmbH, Mainz, Germany) to determine iodine content. The titrant containing the standardized 0.1M sodium thiosulfate was delivered from a 20 mL syringe burette into a beaker containing the sample solution while stirring using a magnetic stirrer. Sodium thiosulphate platinum electrode with a 3M potassium chloride bridging solution was used throughout the analysis as the potentiometric probe.

Figure 3.1: Picture of TitroLine® 6000 automated titrator.

CHAPTER 4
RESULTS AND DISCUSSION

4.1 Introduction

There is lack of a comprehensive database at Kenya Bureau of Standards (KEBS) and PPB containing a list and properties of gargles and mouthwashes. Lack of regulatory information concerning the products shows that there may be insufficient regulation which could lead circulation of substandard/counterfeit products. The study intended to survey the market to establish the types and quality of gargles and mouthwashes available in Nairobi County by determining the content of their API through analysis and labeling requirements.

4.2 Labeling

Table 4.1 and 4.2 shows the labeling information of the sampled povidone-iodine and chlorhexidine containing products respectively. Figure 4.1 shows the percent conformity to labeling standards on the sampled PV-I products. All PV-I samples had labels on the packages that contained information of their batch numbers as well as the manufacturing and expiry dates. However, 71 % of the samples lacked permit number, 6.7 %, of the samples lacked indication details of the active pharmaceutical ingredient (API) on the package although the information was present in the packaging insert, 20 % lacked information on storage conditions and 20 % lacked details of the manufactures' address. Interestingly, there were no details of the formulation excipients in either the primary or secondary package labels as well as KEBS standardization mark (S-mark) in all the sampled PV-I products. All chlorhexidine containing samples had on their packages, the details of the batch numbers, manufacturing and expiry dates, address to the manufacturer, excipients information and the indication for the API as shown in Figure 4.2. However, 60 % of the samples lacked PPB permit number, 77% lacked KEBS S-mark while details about storage conditions were absent in 44.4 % of the sampled products.

Poor labeling has been associated with medication errors as it is difficult for healthcare providers, patients and/or guardians to easily locate and comprehend critical safety information (Aronson, 2009). Specifically, lack of information on storage conditions is likely to affect the stability of products especially those that require storage at special environmentally friendly conditions such as temperature and humidity. Failure to indicate the address to the manufacturer makes it difficult to locate the manufacturer in cases of products recall. Lack of the indication details of the API on the label can lead to inappropriate

prescribing. It is important to provide the details of the excipients in formulations because it is likely for the API to be either in competition with, chemically bound to or rendered inactive by other ingredients (Yang and Chong, 2000).

According to current Good Manufacturing Practices (cGMP) and especially PPB, all pharmaceutical products should be clearly labeled to indicate the name of the product (brand and generic), the quantity/percentage of the API, the name and address of the manufacturer, indication, batch number, storage conditions, and any relevant caution that is related with the use of the drug including the directions of use. Concerning packaging, all products should have the relevant patient information on an insert inside the secondary container. In Kenya especially, the PPB requires that the information be written in English or Kiswahili, the official languages. Where the products are not originally meant for the Kenyan market, the original label as well as a translated copy should be availed (PPB, 2012). According to a study conducted by Nyamweya and Abuga in Nairobi County to check for the compliance of hand sanitizers to packaging, labeling and regulatory standards, products lacking the KEBS S-mark and PPB permit number were regarded as either counterfeit or substandard (Nyamweya and Abuga, 2020).

Table 4.1 Details of labeling for povidone-iodine samples

Code	Label claim	Indication	Storage conditions	Batch number	Manufacturing and expiry date	Manufacturer's address	Excipients	KEBSS-mark	Permit number
PI 1a PI 1b PI 1c	PV-I USP 1% w/v (equivalent iodine 0.1%).	✔	×	✔	✔	×	×	×	×
PI 2a PI 2b PI 2c	PV-I containing 0.1% available iodine.	✔	×	✔	✔	×	×	×	×
PI 3a PI 3b PI 3c	Each 100ml contains 1g PV-I which is equivalent to 0.1% available iodine.	✔	×	✔	✔	✔	×	×	✔
PI 4a PI 4b PI 4c	PV-I BP 1% w/v.	✔	✔	✔	✔	✔	×	×	×
PI 5a PI 5b PI 5c	PV-I BP 1% w/v.	✔	✔	✔	✔	✔	×	×	✔
PI 6a	Contains 2% w/v PV-I USP equivalent to 0.2% available iodine.	✔	✔	✔	✔	✔	×	×	×
PI 7a PI 7b PI 7c	One gram PV-I with a	✔	✔	✔	✔	✔	×	×	×

	content of 10% available iodine.								
PI 8a	PV-I USP 2% w/v (available 0.2% w/v).	✔	✔	✔	✔	✔	×	×	✔
PI 9a PI 9b PI 9c	Each 100 ml contains 1g PV-I which is equivalent to 0.1% available iodine.	✔	✔	✔	✔	✔	×	×	✔
PI 10a PI 10b PI 10c	PV-I USP 1% w/v.	✔	✔	✔	✔	✔	×	×	×
PI 11a PI 11b	Iodinated povidone BP 2% w/v (0.2% w/v available iodine).	✔	✔	✔	✔	✔	×	×	×
PI 12a PI 12b	PV-I IP 1% w/v (equivalent to available iodine 0.1% w/v).	✔	✔	✔	✔	✔	×	×	×
PI 13a	PV-I USP 10% w/v (equal to available iodine 1%w/v).	×	✔	✔	✔	✔	×	×	×
PI 14a PI 14b	Each 100 mL contains	✔	✔	✔	✔	×	×	×	×

	1g PV-I which is equivalent to 0.1% available iodine.								
PI 15a	PV-I USP 10mg.	✔	✔	✔	✔	✔	×	×	×

KEY

✔ - Details present - Details absent

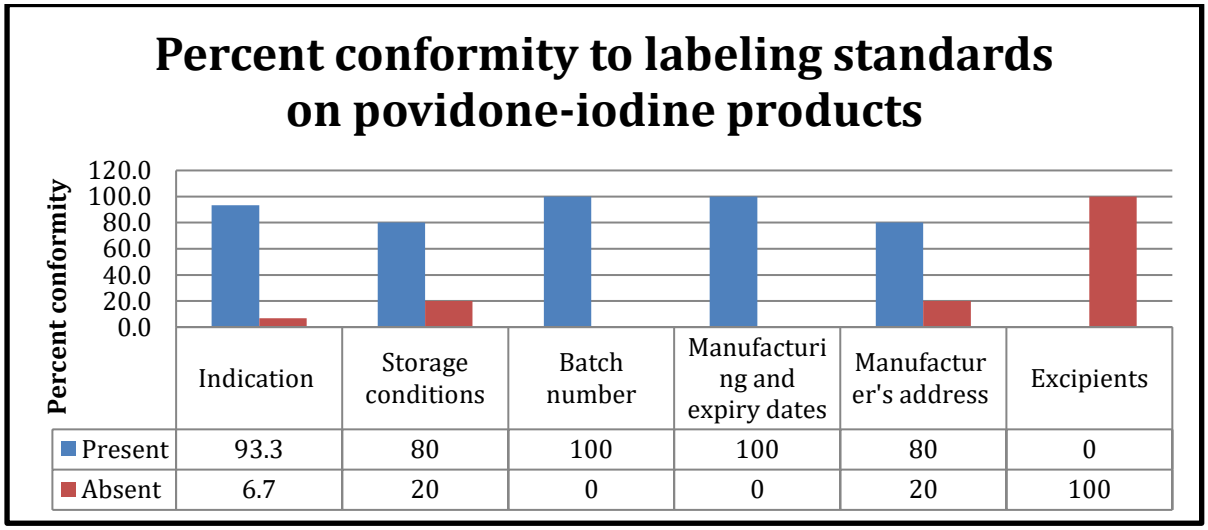


Figure 4.1: Overall percentage conformity of the sampled povidone-iodine products to selected labeling standards parameters.

Table 4.2 Details of labeling on chlorhexidine samples

Code	Label claim	Indication	Storage conditions	Batch number	Manufacturing and expiry date	Manufacturer's address	Excipients	KEBS S-mark	Permit number
C1a C1b C1c	Chlorhexidine gluconate solution BP diluted to 0.2 % w/v.	✔	✔	✔	✔	✔	✔	×	✔
C2a C2b C2c	Each 15 ml contains chlorhexidine gluconate 18 mg.	✔	✔	✔	✔	✔	✔	×	✔
C3a	Chlorhexidine digluconate 0.2 % w/v.	✔	✔	✔	✔	✔	✔	✔	×
C4a C4b	Chlorhexidine gluconate solution BP diluted to 0.2 % w/v.	✔	×	✔	✔	✔	✔	✔	×
C5a	Chlorhexidine gluconate solution BP diluted to 0.2 % w/v.	✔	✔	✔	✔	✔	✔	×	×
C6a	Chlorhexidine digluconate 0.2 % w/v.	✔	×	✔	✔	✔	✔	×	×
C7a	Chlorhexidine digluconate at 0.12 % w/v.	✔	✔	✔	✔	✔	✔	✔	✔
C8a	Chlorhexidine gluconate solution BP diluted to 0.2 % w/v.	✔	×	✔	✔	✔	✔	✔	✔
C9a C9b	Chlorhexidine gluconate solution BP diluted to 0.2 % w/v.	✔	×	✔	✔	✔	✔	✔	✔

KEY

✔ - Details present

× - Details absent

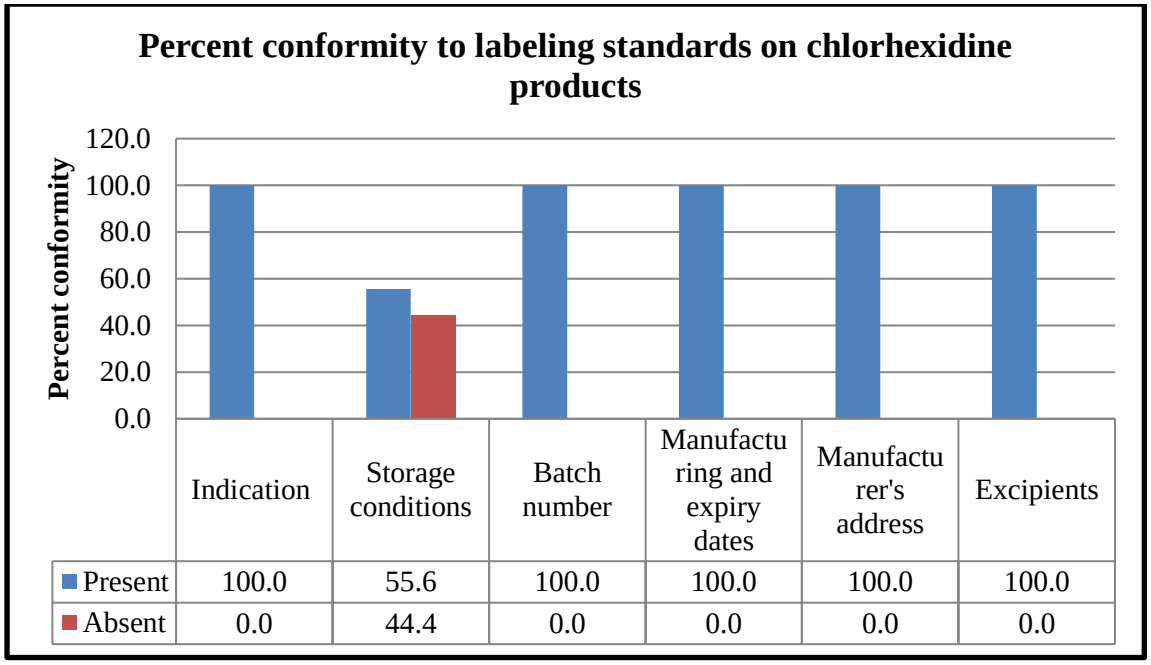


Figure 4.2: Overall percentage conformity of all sampled chlorhexidine products to selected labeling standards parameters.

4.3 Assay

According to the BP (2017) specifications, povidone-iodine gargles and mouth washes should have iodine content at the range of 85 % to 120 % while chlorhexidine content in chlorhexidine containing gargles and mouthwashes should be within a limit of 95 % – 105 % of the labeled claim when stored at recommended conditions and time.

4.3.1 Povidone-iodine

Table 4.3 displays the results of the assay for povidone-iodine gargles and mouthwashes. Iodine content in povidone-iodine containing products ranged from 24.4 % to 145.4 %.

Table 4.3: Assay results of povidone-iodine gargle and mouthwash products

SAMPLE CODE	BATCH NUMBER	ASSAY (%)	COMPLIANCE (C)	LIMIT (%)
PI 1a	**P406**	**83.9 (0.96)**	**NC**	85 -120
PI 1b	**P25**	**63.2 (1.18)**	**NC**	
PI 1c	**P447**	**36.2 (1.26)**	**NC**	
PI 2a	**64408**	**58.3 (1.83)**	**NC**	85 -120
PI 2b	**53009**	**44.8 (1.52)**	**NC**	
PI 2c	**60108**	**49.4 (1.54)**	**NC**	
PI 3a	1811167	115.1 (1.09)	C	85 -120
PI 3b	1806192	98.5 (1.18)	C	
PI 3c	1806193	96.2 (1.33)	C	
PI 4a	5804805	112.5 (1.46)	C	85 -120
PI 4b	5804244	114.8 (0.32)	C	
PI 4c	5801826	109.2 (1.33)	C	
PI 5a	**518104**	**59.8 (1.44)**	**NC**	85 -120
PI 5b	**1217013**	**24.4 (1.18)**	**NC**	
PI 5c	**219004**	**71.2 (1.54)**	**NC**	
PI 6a	28R0001	111.6(0.52)	C	85 -120
PI 7a	18D227K41	94.0 (0.53)	C	85 -120
PI 7b	18D232N41	94.7 (0.38)	C	
PI 7c	18D229K41	93.3 (1.35)	C	
PI 8a	NR1832	99.5 (1.15)	C	85 -120
PI 9a	**1801238**	**70.9 (0.78)**	**NC**	85 -120
PI 9b	**1801264**	**77.7 (1.33)**	**NC**	
PI 9c	**1801255**	**80.5 (0.88)**	**NC**	
PI 10a	**11653**	**57.6 (0.58)**	**NC**	85 -120
PI 10b	**11593**	**24.5 (1.66)**	**NC**	
PI 10c	**11609**	**33.6 (1.74)**	**NC**	
PI 11a	17-XDGS-022	114.8 (0.07)	C	85 -120
PI 11b	17-XDGS-021	114.5 (0.41)	C	
PI 12a	**EAM1-001**	**63.7 (1.84)**	**NC**	85 -120
PI 12b	**EA.M1-002**	**60.3(1.94)**	**NC**	
PI 13a	**NC 002**	**145.4 (0.10)**	**NC**	85 -120
PI 14a	119002	101.4(1.94)	C	85 -120
PI 14b	118015	104.7 (1.80)	C	
PI 15a	139	93.8 (0.25)	C	85 -120

Values in parenthesis represent the relative standard deviation (RSD); C represents assay results that complied while NC represents assay results that did not comply with pharmacopoeia specifications. The highlighted areas show non-compliant samples.

Figure 4.3 shows that 47.1 % (sixteen samples from 8 brands) of the samples had iodine content within the acceptable range as per BP (2017) specifications while 52.9 % (Eighteen samples from 7 brands) of samples were outside the range. Percentage compliance to the assay test for PV-I samples. From the analysis, sixteen samples (about 47.1 %) from 8 brands complied with the assay test for povidone-iodine-based gargles and mouthwashes as per BP pharmacopoeia specifications while 18 samples (52.9 %) from 7 brands of povidone-iodine failed to comply with the assay test as shown in Figure 4.1.

Siddege and Saadalla demonstrated the effect of temperature and light on the stability of 10 % PV-I solution at Sudan University of Science and Technology. From the study, it was evident that iodine chemically degraded leading to a reduction in the amount of available iodine when exposed to different temperature conditions (30 ° and 40 °C). The highest rate of chemical degradation was at 40 °C than in 30 °C. It was also evident that the chemical degradation was time-dependent. In the case of the effect of light, chemical degradation was observed to be higher in samples packed in colorless containers than in brown containers. Interestingly, the color, smell and the pH of the solutions were not affected by the conditions under test (Siddege and Saadalla, 2014). Therefore, it is likely that the samples that had iodine content below the set range might have been exposed for an extended period to high temperature (above 40 °C) and probably direct sunlight on storage. Sample PI 13a which had higher iodine content than the set limit could have resulted from additional of high active ingredient than recommended during preparation.

According to Patil *et* al, products containing high concentration of iodine have been associated with thyroid gland issues such as hypothyroidism/hyperthyroidism, thyroid gland hyperplasia, goiter and thyroid adenoma (Patil *et al.*, 2011). Products with iodine content below the set limit have been shown to be ineffective in reducing plaque formation (Kanagalingam et al., 2017).

In general, 52.9 % of PV-I samples had iodine content outside pharmacopoeial set limit of 85 % to 120 % (Table 4.3). In a similar study conducted to evaluate the quality of PV-I solutions

in public health services in Tucuman, Argentina, only 50 % of the collected samples had iodine content within the set limit (Lorenzo et al., 2016). However, a similar study to assess the quality of sampled marketed PV-I antiseptic solutions products in Iraq showed that all the samples apart from riodine antiseptic solution complied with the assay of iodine (Bayoumi and Al-haideri, 2019). Samples with iodine content below the set limit cannot guarantee maximum antimicrobial activity. According to Shirai *et al* and Kumar *et al*, the antimicrobial strength of PV-I is directly linked to the concentration of the unbound iodine (Shirai *et al.*, 2000; Kumar *et al.*, 2006). On the other hand, when the iodine content in a product is above the set limit as in the case of PI 13a (Table 4.3), the high iodine content may worsen the associated side effects such as primary irritant dermatitis and allergic dermatitis (Vandergriff *et al.*, 2006). In addition, since iodine is absorbed through the oral trans-mucosal route, it has been observed to interfere with the levels of serum thyroid stimulating hormone which may affect the thyroid gland. Therefore, cases of thyroid gland diseases such as hypothyroidism, hyperthyroidism, thyroid adenomas and goiter may arise with products which have higher than recommended iodine (Murugesan and Venkat, 2019).

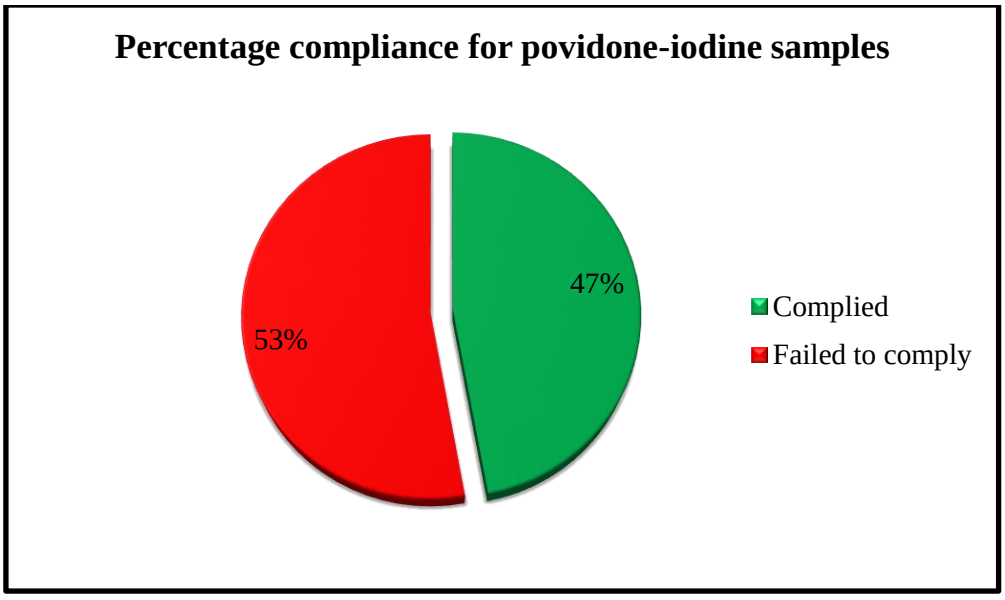


Figure 4.3: Percentage compliance for povidone-iodine products.

4.3.2 Chlorhexidine

Table 4.4 shows the results of the assay for chlorhexidine-based gargles and mouthwashes. The assay (label claim) results for the chlorhexidine-based gargle and mouthwash products ranged from 25.5 % to 102.8 %.

Table 4.4: Assay results of chlorhexidine-based mouthwash and gargles products

Code	Batch no	Assay (%)	Compliance	Limit (%)
C1a	RAJ3583	96.0 (0.00)	C	
C1b	RAJ1J73	101.6 (0.14)	C	95 – 105
C1c	RAJ2F83	96.9 (0.01)	C	
C2a	109853	100.4 (0.07)	C	
C2b	109779	96.0 (0.01)	C	95 – 105
C2c	**109779**	**92.7 (0.03)**	**NC**	
C3a	5145543	95.7 (0.03)	C	95 – 105
C4a	**BC2557**	**65.8 (0.01)**	**NC**	95 – 105
C4b	**BM2560**	**25.5 (0.01)**	**NC**	
C5a	**372**	**90.0 (0.01)**	**NC**	95 – 105
C6a	5145248	101.8 (0.01)	C	95 – 105
C7a	8522038A	96.0 (0.03)	C	95 – 105
C8a	E868	102.8 (0.02)	C	95 – 105
C9a	**E862**	**92.7 (0.03)**	**NC**	95 – 105
C9b	E867	101.7 (0.03)	C	

Values in parenthesis represent the relative standard deviation (RSD); C represents assay results that complied while NC represents assay results that did not comply with pharmacopoeia specifications. The highlighted areas show non-compliant samples.

Figure 4.4 shows the percentage compliance of the sampled products to the assay of chlorhexidine mouthwashes and gargles. Five batches (33.3 %) gave chlorhexidine results below the set limit while 10 batches (66.7 %) had chlorhexidine content within the limit. Chlorhexidine is known to be unstable under conditions of extreme temperature and direct sunlight. Therefore, it is likely that the samples which failed to comply with the assay test might have been exposed to extreme temperature and direct light especially at prolonged period of storage. Samples with CHX content below the set limit may not provide the anticipated antimicrobial effect.

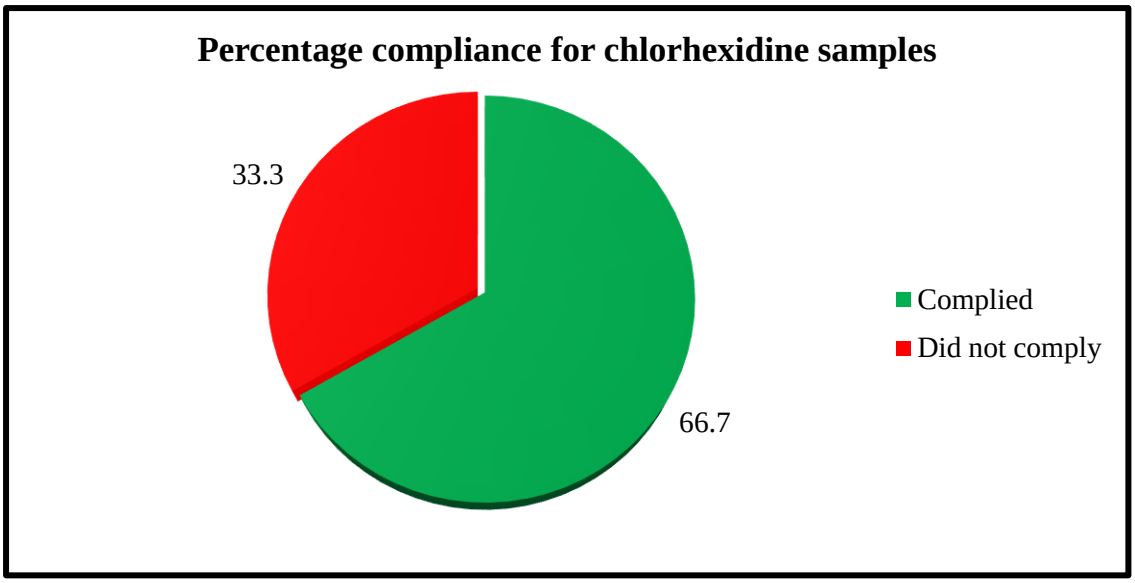


Figure 4.4: Percentage of compliance for chlorhexidine-based products to assay test.

Figure 4.5 and 4.6 shows the typical chromatogram of the standard and sample C1c respectively. Other relevant chromatograms are shown in the appendices.

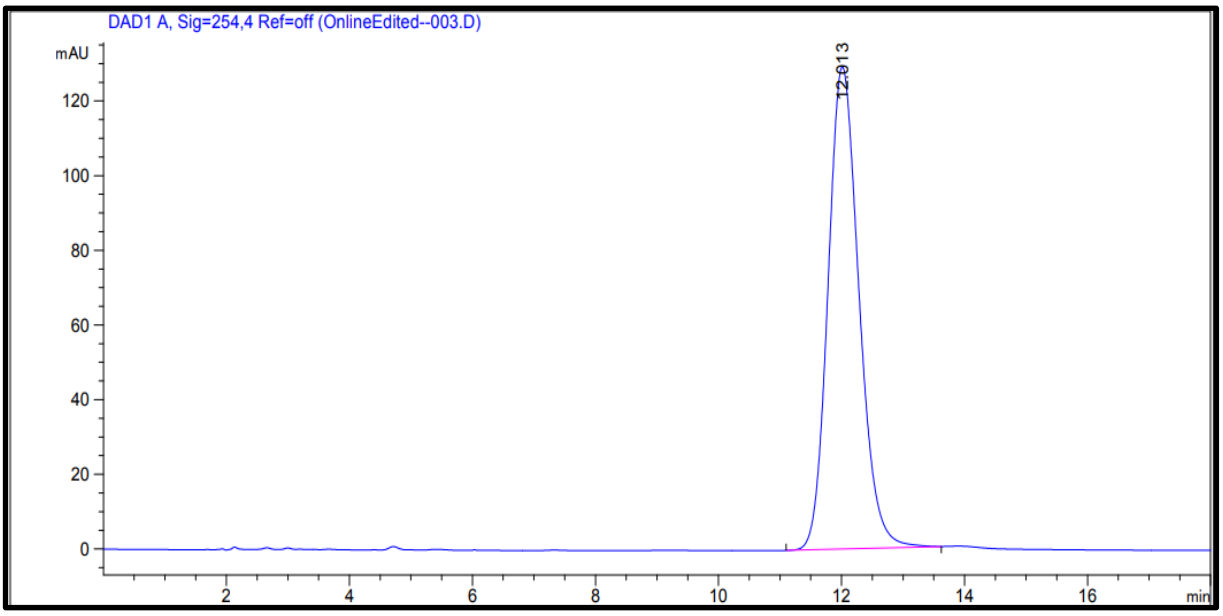


Figure 4.5: typical chromatogram of the chlorhexidine gluconate standard.

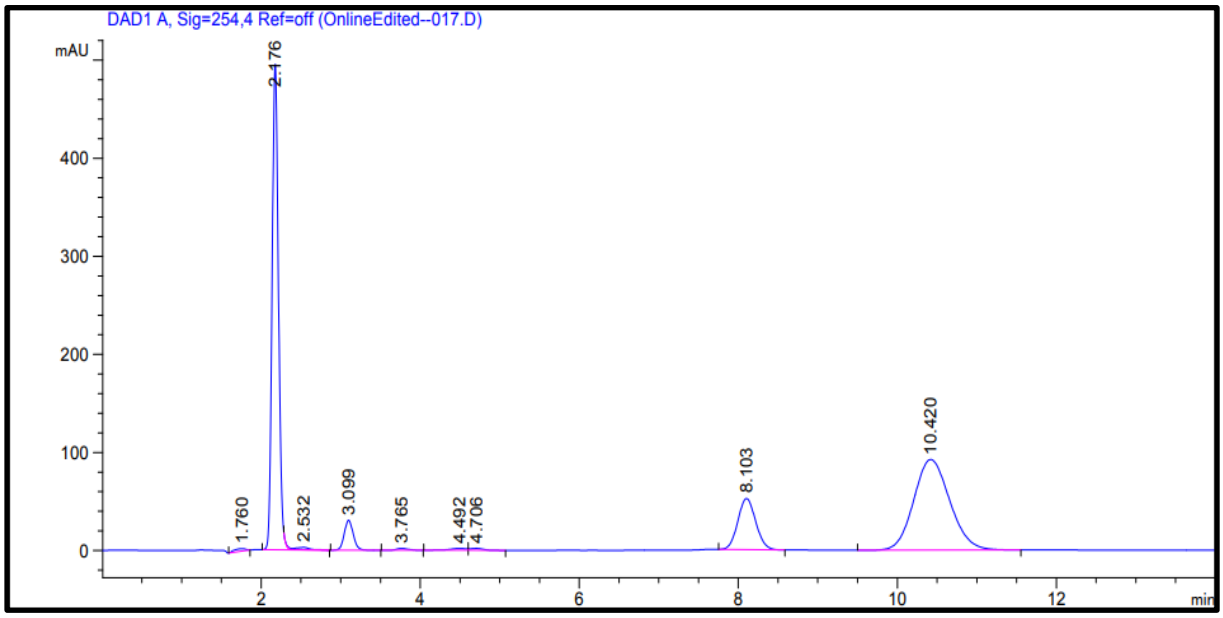


Figure 4.6: typical chromatogram for sample C1c

CHAPTER 5
CONCLUSION AND RECOMMENDATIONS

5.1 Introduction

Conformity to assay tests and labeling standards are some of finished product's critical quality attributes that influence performance within desired efficacy, quality and safety (Mesut, Özsoy and Aksu, 2015). Assessment of critical quality attributes can therefore be used to assess for quality as well as batch-to-batch variation within products.

5.2 Conclusion

The aim of the study was to carry out the evaluation of the quality of chlorhexidine and povidone-iodine based mouthwash and gargle products in Nairobi County. The quality of both products was successfully established using the BP (2017) assay specifications and assessment of conformity to the labeling requirements. From the non-compliance rates that were recorded, a wide range of problems of the antiseptics were noted. The poor-quality products would affect the objective of controlling the spread of infections for household and healthcare systems. Similarly, poor quality products may lead to wastage of financial benefit to the consumers which may cause lack of trust in the regulatory work from the relevant bodies.

5.3 Recommendations

5.3.1 Policy

From the study, it is evident that there is confusion on which regulator between KEBS and PPB is supposed to regulate the marketing of mouthwashes and gargles as they are said to be borderline products. The confusion in the registration may have led to insufficient registration of the products in the market which has eventual detrimental effect to quality. There is need to specify which regulatory body should carry out the registration of the products. The regulator should also ensure that during registration, the relevant certificates of analysis from recognized institutions such as the National Quality Control Laboratory, Drug Analysis and Research Unit and other relevant institutions are attached. The regulator as well as the companies which hold market authorization for gargles and mouthwashes product especially chlorhexidine and PV-I should carry out continuous market surveillance to ensure adherence to quality standards.

5.3.2 Further research

Since the study was conducted in Nairobi County, there is need to carry out the study in other parts of the country to ensure that there is adherence to quality standards for improved efficacy, and safety with the use of PV-I and chlorhexidine-based gargles and mouthwashes. Further research is required to check the effect of temperature, light and extended storage period of the quality of PV-I in gargles and mouthwashes which may have contributed to high amount of non-compliance to the assay test in the selected samples in the study.

5.4 Study limitation

This study had limitations in a number of ways such as failure to obtain a list of all the mouthwashes and gargles containing PV-I and chlorhexidine that have been registered with the regulator (PPB) in Kenya as they were reluctant. The convenience sampling method was used in the study to obtain the samples. Nevertheless, it is associated with biasness and the samples that are obtained are not representative of the entire population. A comprehensive batch to batch comparison was not possible because not all products had multiple batches.

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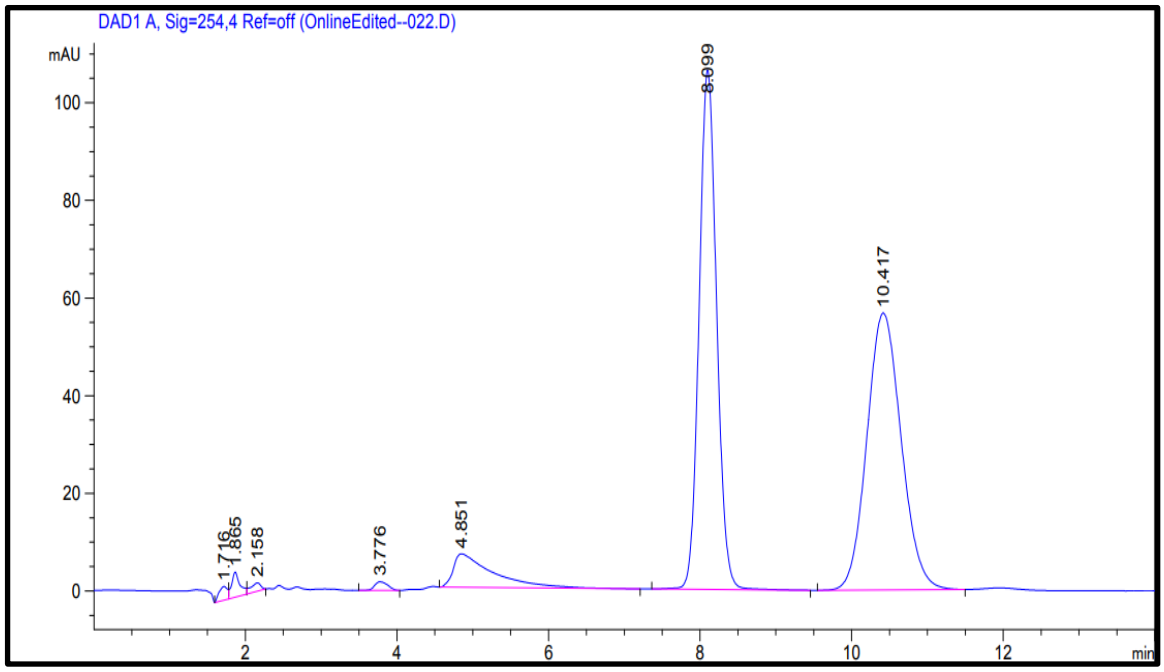
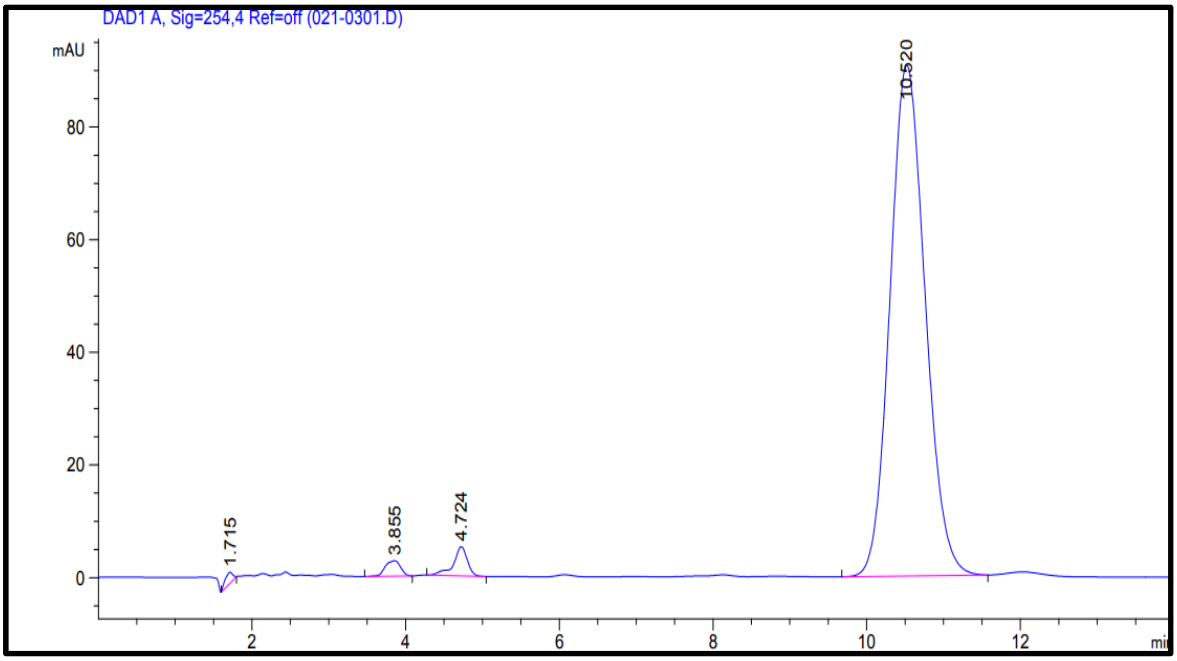
APPENDICES

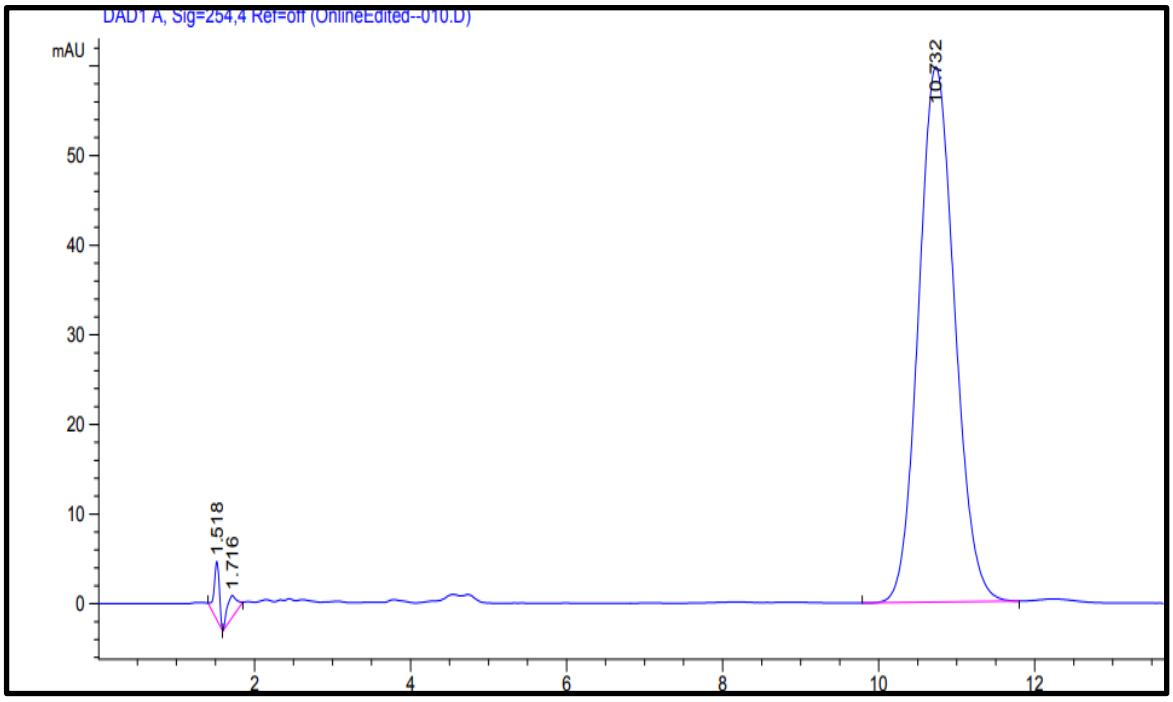
Appendix 1. Chromatogram for sample C2A at 254 nm



Appendix 2. Chromatogram for sample C3A at 254 nm



Appendix 3. Chromatogram for sample C4A at 254 nm



Appendix 4. Chromatogram for sample C5A at 254 nm