KWAME NKRUMAH UNIVERSITY OF SCIENCE AND TECHNOLOGY-KUMASI

COLLEGE OF HEALTH SCIENCES, FACULTY OF PHARMACY AND PHARMACEUTICAL SCIENCES

INFLUENCE OF POLYSORBATE ON THE ACTIVITY OF SOME ANTIMICROBIAL PRESERVATIVES

THESIS SUBMITTED TO KNUST DEPARTMENT OF PHARMACEUTICS IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE AWARD OF MPHIL. IN PHARMACEUTICAL MICROBIOLOGY

BY

AYISI FELIX

2010

DECLARATION

I declare that the dissertation I am presenting is the result of my own research except for the references and quotations which have been given the due acknowledgement.

I hereby declare that, this work has never been submitted for any award elsewhere.

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AYISI, FELIX DATE

(STUDENT)

---------- ----------

PROF. G.H. KONNING DATE

(SUPERVISOR)

---------- ----------

DR. K. OFORI-KWAKYE DATE

(HEAD OF DEPARTMENT)

DEDICATION

This work is dedicated to my parents Mr. and Mrs. Ayisi for their continuous prayers and parental love shown me. This piece is also dedicated to Mrs. Betty Oppong Duku.

ACKNOWLEDGEMENT

My greatest appreciation goes to the God Almighty for being the source of my strength and inspiration. I wish to leave on record my immersed indebtedness to Prof. G.H. Konning for his time spent; correction, constructive criticism and patience in making this work a success.

To Mrs Rose Ayisi I wish to pronounce God's blessings for your love, money and care shown during the course of my studies.

I am also grateful to Mr. Mike Pobi, Mr. Andrews Gordon and Mr. Stephen Danson-Mensah who served as resource persons during part of the work. To all the technicians and assistants at the Microbiology Laboratory of the Pharmaceutics department I say God bless you.

Finally, I thank all my siblings Mr. Emmanuel Ayisi, Theophilus Ayisi, Ebenezer Ayisi and Ruth Ayisi for their morale support and to all friends who contributed to this work in diverse ways.

ABSTRACT

The antimicrobial activity of Chlorocresol and salicylic acid against *E. coli*, *Bacillus subtilis*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* were studied in the presence of Polysorbate. Polysorbate is a surfactant and it prevents coalescence by reducing interfacial tension of immiscible liquids. The activities of Chlorocresol and salicylic acid were considerably inhibited by the Polysorbate. The interactions of the antimicrobial with the Polysorbate and subsequent reduction in the availability of the preservatives to the bacterial cells were found to be the predominant mechanism in the reduction of the activities of the preservatives.

Physical shielding of the bacterial cells from the actions of the preservatives was the primary cause for the lower activities of the preservatives in the presence of the Polysorbate.

The physical influence of Polysorbate on the preservatives was also determined using the UV spectrophotometer. The reduction in the Spectrophotometric activity of the preservatives in the presence of Polysorbate was found to be caused by the physical binding of the Polysorbate to part of the active surfaces of the preservatives.

TABLE OF CONTENTS

Declaration i

Dedication ii

Acknowledgement iii

Abstract iv

Table of contents v-viii

Tables viii-ix

Figures x-xi

CHAPTER ONE

1.0 INTRODUCTION 1

1.1 General 1

1.2 Creams 1

1.3 Antimicrobial preservatives 2

1.4 Development pharmaceutics 3

1.5 Emulsions 3

1.6 Emulsifying agents 5

1.6.1 Synthetic emulsifying agents 6

1.6.2 Natural emulsifying agents 6

1.6.3 Hydrocolloid emulsifies 7

1.6.4 Finely divided solid particle emulsifiers 7

1.6.5 Auxiliary emulsifying agents 8

1.6.6 Polysorbate 80 8

1.7.0 Antimicrobial agents 9

1.7.1 Salicylic acid 9

1.7.2 Chlorocresol 10

1.8.0 Literature review 11

1.8.1 Influence of surfactants on Antimicrobials 11

1.8.2 Interactions of preservatives with macromolecules 11

1.8.3 Effect of nonionic surfactants on bactericidal activity of Cetylpyridinium chloride 12

1.8.4 Effect of surfactants on the antimicrobial activity of

preservatives 12

CHAPTER TWO

2.0 SCOPE OF WORK

14

CHAPTER THREE

3.0 MATERIALS AND METHODS 15

3.1 Instruments 15

3.2 Culture media 15

3.3Reagents 15

3.4 Source of microorganisms 15

3.5 Preparation of culture media 15

3.5.1 Nutrient broth 15

3.5.2 Nutrient agar 16

3.6.0 Cultivation of test microorganisms 16

3.7.0 Preparation of antimicrobial samples 16

3.7.1 Chlorocresol (0.3% w/v) 16

3.7.2 Salicylic acid (0.2% w/v) 17

CHAPTER FOUR

4.0 EXPERIMENTAL 19

4.1 Determination of zone of inhibition bactericides against test organisms 19

4.2 Determination of biological influence of Polysorbate on Bactericides 19

4.3 Determination of maximum wavelength 21

4.4 Spectrophotometric analysis of the bactericides 21

4.5 Determination of physical influence of polysorbate on Chlorocresol and Salicylic acid 21

4.6 Determination of rate of diffusion of bactericide. 22

CHAPTER FIVE
5.0 RESULTS 23
5.1 Activity of bactericide in model system 23
5.2 Effect of the presence of polysorbate on bactericides 27
5.3 Standardization of the bactericidal concentration as a measure Of UV absorbance 30
5.4 Effect of polysorbate on bactericide by Spectrophotometric Analysis 31
5.5 Estimation of bactericides available in the bactericide-polysorbate Admixture 35
5.6 The influence of physical interaction between polysorbate-Bactericide admixture on biological activity 39
5.7 Diffusion of bactericides. 39

CHAPTER SIX
6.0 DISCUSSION AND CONCLUSION 49
6.1 Discussion 49
6.2 Conclusion 51
6.2.1 Relativity 51
6.2.2 Mechanism 51
6.2.3 Diffusion rates 52
6.2.4 Solubilization. 52
6.2.5 Recommendations 53

CHAPTER SEVEN
7.0 REFERENCES 54
Appendix I Standardized graph of Spectrophotometric analysis Of salicylic acid 58
Appendix II Standardized graph of Spectrophotometric analysis Of chlorocresol 60

Appendix III Preparation of polysorbate-bactericide Admixture for Spectrophotometric analysis 62

Appendix IV Preparation of Admixture to determine the effect of Polysorbate on Chlorocresol diffusion 63

TABLES

Table	Title	Page
1	Preparation of varying Chlorocresol Concentration from a stock Solution of 0.30%w/v	17
2	Preparation of varying salicylic acid Concentration from a stock Solution of 0.20%w/v	18
3.	Preparation of Admixture to determine the effect of polysorbate On Chlorocresol biologically	20
4.	Preparation of Admixture to determine the effect of polysorbate On Salicylic acid biologically	20
5	Activity of Salicylic acid in model system against test Organisms	24
6	Activity of Chlorocresol in model system against test Organisms	24
7	Effect of 0.15% salicylic acid in the presence of polysorbate	28
8	Effect of 0.20% Chlorocresol in the presence of polysorbate	28
9	Effect of polysorbate on absorbance of Salicylic acid 0.12%w/v at 311.5nm	32
10	Effect of polysorbate on absorbance of Chlorocresol 0.20%w/v at 293nm	32
11	Determination of 0.12% w/v Salicylic acid free in the Presence of polysorbate	36

12	Determination of 0.15% w/v Chlorocresol free in the Presence of polysorbate	36
13	Effect of Chlorocresol concentration on diffusion	40
14	Effect of Salicylic acid concentration on diffusion	40
15	Relationship between Chlorocresol concentration and rate Of diffusion	43
16	Relationship between Salicylic acid concentration and rate Of diffusion	43
17	Determination of extent of Chlorocresol diffusion after Interacting with 0.5%v/v polysorbate	46
18	Determination of rate of diffusion by Chlorocresol + 0.5% Polysorbate	46
19	Relationship between Salicylic acid concentration and Absorbance	56
20	Relationship between Chlorocresol concentration and Absorbance	58
21	Preparation of Admixture to determine the effect of polysorbate on chlorocresol using the Spectrophotometer	62
22	Preparation of Admixture to determine the effect of polysorbate on Salicylic acid using the Spectrophotometer	62
23	Preparation of Admixture to determine the effect of Polysorbate on Chlorocresol diffusion	63

FIGURES

Figures	Title	Page
1	Activity of Salicylic acid in a model system against test Organisms	25
2	Activity of Chlorocresol in a model system against test Organisms	26
3	Activity of fixed Chlorocresol 0.2%w/v in the presence of Polysorbate	29
4	Effect of polysorbate on 0.12%w/v salicylic acid absorbance At 311.5nm	33
5	Effect of polysorbate on 0.15%w/v Chlorocresol absorbance At 293nm	34
6	Concentration of 0.12%w/v Salicylic acid free after Interactions with polysorbate	37
7	Concentration of 0.15%w/v Chlorocresol free after Interactions with polysorbate	38
8	Extent of Chlorocresol diffusion with time	41
9	Extent of Salicylic acid diffusion with time	42
10	Relationship between Chlorocresol concentration and rate Of diffusion	44
11	Relationship between Salicylic acid concentration and rate Of diffusion	45
12	Relationship between distance diffused by chlorocresol + 0.5%v/v polysorbate time taken	47
13	Relationship between rate of diffusion by chlorocresol + 0.5%v/v polysorbate.	48
14	Relationship between Salicylic acid concentration and Absorbance	59
15	Relationship between Chlorocresol concentration and Absorbance	61

CHAPTER ONE

1.0 INTRODUCTION

1.1 General

Microbial spoilage of pharmaceutical products has been known for many years. Spoilage may result in the deterioration of the products due to loss of potency or the initiation of an infection in the user. Sterile pharmaceutical products (single dose or multi dose forms) require the addition of an antimicrobial preservative when they have been manufactured under aseptic conditions from presterilised ingredients where the products are subject to a terminal sterilisation process. Only the multidose requires the addition of an antimicrobial agent as preservation. In the multidose the preservative is added to protect the product and the end user against the consequences of microbial entry during use. Chemical antimicrobial agents are thus added to all multidose sterile formulations and to aqueous and aqueous-based non-sterile pharmaceuticals. Gilbert and Allison (2006)

1.2 CREAMS

Formulated medicinal products include creams which could be pharmaceutical or cosmetic. Creams are for protection against sunlight, microbes and for moistening the skin. Most creams are easily contaminated, there by rendering them non-functional. Effective preservation during use is therefore an essential safeguard for users of both pharmaceutical and cosmetic products. Behravan *et al* (2000)

The tropical weather serves as a favourable breeding environment for microbes. Formulated products are mostly contaminated by microbes such as *Aspergillus niger*, *Candida albicans*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Escherichia coli* which are responsible for a number of infectious diseases, spoilage of food and pharmaceutical products. Contaminated products present a potential health hazard because they are unable to suppress the growth of organisms. In a situation where a nutritionally rich pharmaceutical product is

severely contaminated, rapid growth and multiplication would be expected. This could lead to biodegradation of the product and hence the risk of infection to consumers of the product. Product contamination may arise from raw materials or water used in the formulation or accidentally during use. Hugbo *et al* (2003)

1.3 ANTIMICROBIAL PRESERVATIVES

Antimicrobial preservatives are used to inhibit the growth of microorganisms which could present a risk of infection or degradation of the medicinal product. The microorganisms may proliferate during normal storage condition or during use by the patient. Preparations at greatest risk of contamination are those which contain water such as solutions, suspensions and emulsions to be taken orally, solutions for external use, creams and sterile preparations used repeatedly (e.g. injectable multidose preparations and eye-drops). The level of efficacy will vary according to the chemical structure of the preservative, its concentration, the physical and chemical characteristics of the medicinal product, especially the type and level of microbial contamination. The design of the pack and the temperature at which the product is stored will also affect the level of activity of any antimicrobial preservatives present. The antimicrobial efficacy of the preservative in the finished product should be assessed during product development using the European Pharmacopoeia test. If products do not contain a preservative and do not have adequate inherent preservative efficacy they must not be packaged in multidose presentations without a sound justification. The European Agency for the Evaluation of medicinal products (1997)

A preservative must be non-toxic, compatible and inexpensive and have an acceptable taste, odour and colour. It should be effective against a wide variety

of bacteria and fungi. International Journal of Pharmaceutical compounding (2005)

The preservative capacity of a product is the power or ability of the product to consistently maintain low and acceptable levels of microbial contaminants when challenged with fresh microbial loads. (Hugbo *et al* 2003).

The official specified microbial limits are not more than 1.0x103 g/ml for bacteria and 1.0x102 g/ml for moulds of the product. (B.P 1993)

1.4 Development pharmaceutics

During pharmaceutical development of a product, the necessity to add an antioxidant or a preservative to the finished product at the level chosen, the physical and chemical compatibility of the preservative with other constituents of the finished product must be demonstrated.

The concentration used must be notified in terms of efficacy and safety, such that the minimum concentration of the preservative used gives the required level of efficiency. This is used to determine whether the required level of activity is achieved. Parenteral infusions do not contain any added antimicrobial preservatives when the medicinal product is intended for administration by routes where for medical reasons an antimicrobial presevative is unacceptable. The European Agency for the Evaluation of medicinal products (1997)

1.5 EMULSION

An emulsion is a thermodynamically unstable two-phase system consisting of at least two immiscible liquids, one of which is dispersed in the form of small droplets throughout the other, and an emulsifying agent. Ted (2006)

The dispersed liquid is known as the internal or discontinuous phase, whereas the dispersion medium is known as the external or continuous phase. Where oils, petroleum hydrocarbons, and/or waxes are the dispersed phase, and water or an aqueous solution is the continuous phase, the system is called an

oil-in-water (o/w) emulsion. An o/w emulsion is generally formed if the aqueous phase constitutes > 45% of the total weight, and a hydrophilic emulsifier is used. Conversely, where water or aqueous solutions are dispersed in an oleaginous medium, the system is known as water-in-oil (w/o) emulsions. Emulsions are generally formed if the aqueous phase constitutes < 45% of the total weight and a lipophilic emulsifier is used. Emulsions tend to have a cloudy appearance, because the many phases (called the interface) scatter light that passes through the emulsion.

Emulsions are unstable and thus do not form spontaneously. Energy input through shaking, stirring, homogenizers, or spray processes are needed to form an emulsion.

Over time, emulsions tend to revert to the stable state of oil separated from water. Surface active substances (surfactants) can increase the kinetic stability of emulsions greatly so that, once formed, the emulsion does not change significantly over years of storage. Homemade oil and vinegar salad dressing is an example of an unstable emulsion that will quickly separate unless shaken continuously. This phenomenon is called coalescence, and happens when small droplets recombine to form bigger ones. Fluid emulsions can also suffer from creaming, the migration of one of the substances to the top of the emulsion under the influence of buoyancy or centripetal force when a centrifuge is used. The thermodynamic stability of formulated systems is a consequence of interfacial free energy that exists between the two phases. The free energy is the driving force for droplet coalescence and eventual phase separation. Surfactants improve the stability by decreasing interfacial free energy and providing a barrier to droplet coalescence. Collision of dispersed droplets with one another or with the walls of the container can lead to thinning and rupture of the surfactant interfacial film. Emulsions are part of a more general class of two-phase systems of matter called colloids. Although the terms colloid and emulsion are sometimes used interchangeably, emulsion

tends to imply that both the dispersed and the continuous phase are liquid. The Pharmaceutics and compounding Laboratory Report (1996)

There are three types of emulsion instability: flocculation, where the particles form clumps; creaming, where the particles concentrate towards the surface (or bottom, depending on the relative density of the two phases) of the mixture while staying separated; and breaking and coalescence where the particles coalesce and form a layer of liquid. Hannan 2007

W/O emulsions tend to be immiscible in water, not water washable, will not absorb water, are occlusive, and may be "greasy." This is primarily because oil is the external phase, and oil repels any of the actions of water. The occlusiveness is because the oil does not allow water to evaporate from the surface of the skin. Conversely, o/w emulsions are miscible with water, are water washable, will absorb water, are nonocclusive, and are nongreasy. Here water is the external phase and readily associates with any of the actions of water. Emulsions like creams are, by nature physically unstable; that is, they tend to separate into two distinct phases or layers over time. Several levels of instability are described in the literature. Creaming occurs when dispersed oil droplets merge and rise to the top of an o/w emulsion or settle to the bottom in w/o emulsions. In both cases, the emulsion can be easily redispersed by shaking. Coalescence (breaking or cracking) is the complete and irreversible separation and fusion of the dispersed phase. Finally, a phenomenon known as phase inversion or a change from w/o to o/w (or vice versa) may occur. This is considered a type of instability by some. The Pharmaceutics and compounding Laboratory Report (1996)

1.6 EMULSIFYING AGENTS (surfactants)

Surfactants have both a hydrophilic and a lipophilic part in their chemical structure. All emulsifying agents concentrate at and are adsorbed onto the oil:

water interface to provide a protective barrier around the dispersed droplets. In addition to this protective barrier, emulsifiers stabilize the emulsion by reducing the interfacial tension of the system. Some agents enhance stability by imparting a charge on the droplet surface thus reducing the physical contact between the droplets and decreasing the potential for coalescence. Troy *et al* (2006)

Surfactant is usually present in formulated systems in the form of monomers, micelles and liquid crystals. Some commonly used emulsifying agents include tragacanth, sodium lauryl sulfate, sodium dioctyl sulfosuccinate, and polymers known as the Spans and Tweens.

Emulsifying agents can be classified according to: 1) chemical structure; or 2) mechanism of action. Classification according to chemical structure are synthetic, natural, finely dispersed solids, and auxiliary agents. Classes according to mechanism of action are monomolecular, multimolecular, and solid particle films. Regardless of their classification, all emulsifying agents must be chemically stable in the system, inert and chemically non-reactive with other emulsion components, and nontoxic and nonirritant. They should also be reasonably odorless and not cost prohibitive. The Pharmaceutics and compounding Laboratory (1996)

1.6.1 Synthetic Emulsifying Agents

Synthetic agents fall into three main categories, namely

- Cationic, e.g., benzalkonium chloride, benzethonium chloride
- Anionic, e.g., alkali soaps (sodium or potassium oleate); amine soaps (triethanolamine stearate); detergents (sodium lauryl sulfate, sodium dioctyl sulfosuccinate, sodium docusate).
- Nonionic, e.g., sorbitan esters (Spans), polyoxyethylene derivatives of sorbitan esters (Tweens), or glyceryl esters

Cationic and anionic surfactants are generally limited to use in topical, o/w emulsions. Cationic agents (quarternary ammonium salts) are incompatible with organic anions and are infrequently used as emulsifiers. Soaps are subject to hydrolysis and may be less desirable than the more stable detergents. Troy *et al* (2006)

1.6.2 Natural Emulsifying Agents

A variety of emulsifiers are natural products derived from plant or animal tissue. Most of the emulsifiers form hydrated lyophilic colloids (called hydrocolloids) that form multimolecular layers around emulsion droplets. Hydrocolloid type emulsifiers have little or no effect on interfacial tension, but exert a protective colloid effect, reducing the potential for coalescence, by:

- providing a protective sheath around the droplets
- imparting a charge to the dispersed droplets (so that they repel each other)
- swelling to increase the viscosity of the system (so that droplets are less likely to merge) Troy *et al* (2006)

1.6.3 Hydrocolloid emulsifiers

These may be classified as:

- vegetable derivatives, e.g., acacia, tragacanth, agar, pectin, carrageenan, lecithin
- animal derivatives, e.g., gelatin, lanolin, cholesterol
- Semi-synthetic agents, e.g., methylcellulose, carboxymethylcellulose
- Synthetic agents, e.g., Carbopols

Naturally occurring plant hydrocolloids have the advantages of being inexpensive, easy to handle, and nontoxic. Their disadvantages are that they require relatively large quantities to be effective as emulsifiers, and they are subject to microbial growth and thus their formulations require a preservative. Vegetable derivatives are generally limited to use as o/w emulsifiers.

The animal derivatives in general form w/o emulsions. Lecithin and cholesterol form a monomolecular layer around the emulsion droplet instead of the typically multimolecular layers. Cholesterol is a major constituent of wool alcohols and it gives lanolin the capacity to absorb water and form a w/o emulsion. Lecithin (a phospholipid derived from egg yolk) produces o/w emulsions because of its strong hydrophilic character. Animal derivatives are more likely to cause allergic reactions and are subject to microbial growth and rancidity. Their advantage is in their ability to support formation of w/o emulsions. Semi-synthetic agents are stronger emulsifiers, are nontoxic, and are less subject to microbial growth. Synthetic hydrocolloids are the strongest emulsifiers, are nontoxic, and do not support microbial growth. However, their cost may be prohibitive. These synthetic agents are generally limited to use as o/w emulsifiers. Troy *et al* (2006))

1.6.4 Finely Divided (Dispersed) Solid Particle Emulsifiers

These agents form a particulate layer around dispersed particles. Most will swell in the dispersion medium to increase viscosity and reduce the interaction between dispersed droplets. Most commonly they support the formation of o/w emulsions, but some may support w/o emulsions. These agents include bentonite, veegum, hectorite, magnesium hydroxide, aluminum hydroxide and magnesium trisilicate. Troy *et al* (2006)

1.6.5 Auxiliary Emulsifying Agents

A variety of fatty acids (e.g., stearic acid), fatty alcohols (e.g., stearyl or cetyl alcohol), and fatty esters (e.g., glyceryl monostearate) serve to stabilize emulsions through their ability to thicken the emulsion. Because they have only weak emulsifying properties, they are always used in combination with other emulsifiers. Troy *et al* (2006)

1.6.6 POLYSORBATE 80

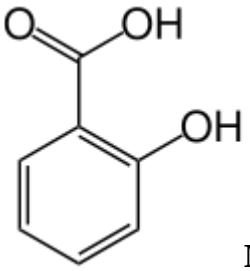
Polysorbate is also known as Polyoxyethylene (20) sorbitan monooleate, (x)-sorbitan mono-9-octadecenoate poly (oxy-1,2-ethanediyl), Tween 80, POE (20) sorbitan monooleate. Polysorbate 80 commercially also known as Tween 80, is a nonionic surfactant and emulsifier derived from polyoxylated sorbitan and oleic acid, and is often used in foods. It is a viscous, water-soluble yellow liquid. The hydrophilic groups in this compound are polyethers also known as polyoxyethylene groups which are polymers of ethylene oxide. Materials Safety data Sheet (2005)

In the nomenclature of polysorbates, the numeral designation following polysorbate refers to the lipophilic group, in this case the oleic acid.

It is often used in ice cream to prevent milk proteins from completely coating the fat droplets. This allows them to join together in chains and nets, to hold air in the mixture, and provide a firmer texture, holding its shape as the ice cream melts. Polysorbate 80 is also used in commercial pickle products. Dowin (2010)

1.7.0 Antimicrobial Agents

1.7.1 Salicylic acid



Molecular formula $C_7H_6O_3$

Salicylic acid also known as 2-hydroxybenzoic acid (from the Latin word for the willow tree, Salix, from whose bark it can be obtained) is a beta hydroxy acid (BHA) with the formula $C_6H_4(OH)CO_2H$, where the OH group is adjacent to the carboxyl group. This colorless crystalline organic acid is widely used in organic synthesis and functions as a plant hormone. It is derived from the metabolism of salicin. It is probably best known as a compound that is chemically similar but not identical to the active component of aspirin. Indeed salicylic acid is a metabolite of aspirin, the product of esterase hydrolysis in the liver. It has a melting point of 159^0C and is white odourless crystals.

Historically, the Greek physician Hippocrates wrote in the 5th century BC about a bitter powder extracted from willow bark that could ease aches and pains and reduce fevers. This remedy was also mentioned in texts from ancient Sumer, Lebanon, and Assyria. The Cherokee and other Native Americans used an infusion of the bark for fever and other medicinal purposes for centuries. Hemel *et al* (1975)

The medicinal part of the plant is the inner bark and was used as a pain reliever for a variety of ailments. The Reverend Edward (Edmund) Stone, a vicar from Chipping Norton, Oxfordshire, England, noted in 1763 that the bark of the willow was effective in reducing a fever. Stone 1963

The active extract of the bark, called *salicin*, after the Latin name for the white willow (*Salix alba*), was isolated in crystalline form in 1828 by Henri Leroux, a French pharmacist, and Raffaele Piria, an Italian chemist. Piria was able to convert the substance into a sugar and a second component, which on oxidation becomes salicylic acid. Diarmuid 2005

Salicylic acid reacts with acetic acid to produce acetylsalicylic acid the active ingredient in *aspirin*. It reacts with methanol to form methyl salicilate, more commonly known as oil of wintergreen.

It is common in many plants, such as broccoli, pepper, curry, cucumbers, and raisins, among many others. Chenhongxia (2009)

Medically, salicylic acid is used to treat acne, warts, dandruff, psoriasis, and similar conditions. Steele *et al* (1988)

In the treatment of acne, it slows the shedding of skin cells in hair follicles, so they do not clog the pores and cause pimples. Clearington (2009)

It also has a keratolytic effect, causing dead cells to slough off. The top layer of skin is removed, and pores unclogged. In treatments for warts, a stronger solution is used. This not only softens the wart so it can be rubbed off, but the irritation stimulates the immune system to attack the human papillomavirus that causes warts. Salicylic acid reduces inflammation in arteries to prevent hardening and narrowing. Because of its effect on skin cells, salicylic acid is used in several shampoos for treatment of dandruff. Roberts (2004)

1.7.2 Chlorocresol

Chlorocresol has a chemical formula of C_7H_7ClO and a melting point of 67^0C, it is also known as parachlorometacresol, 4-Chloro-3-methylphenol, 3-methyl-4-chlorophenol; 2-chloro-5-hydroxytoluene; p-chloro-m-cresol; p-Chlorocresol. Kburgess (2009)

Chlorocresol is found as white or slightly pink dimorphous crystals that have a phenolic odor. Glen (2004)

Chlorocresol is a man-made substance used as external germicide or bactericide and a preservative for cosmetics, glues, gums, paints, creams, lotions, inks, textiles and leather goods. It is also a potent disinfectant, antiseptic an antifungal agent used in eye drops. National Science Digital Library (2004)

It is used as a preservative in veterinary medicinal products and many pharmaceutical creams and lotions but especially steroid creams. It is used as an excipient in some veterinary preparations intended for both oral and parental use. It is also used as an active ingredient in some vapourizing fluids intended for the treatment of nasal congestion. It is considered less toxic to humans than phenols and serious adverse reactions are rare. Cases of hypersensitivity have occasionally been observed following the administration of injections containing Chlorocresol as a preservative. (The European Agency for the Evaluation of Medicinal Products, 1996)

Chlorocresol may interfere with perfumes in some cosmetic products. It is toxic to wildlife and water-dwelling organisms and irritant to the skin and eyes. It may be formed in waters which have undergone chlorination treatment. It may be released in the environment from evaporation, waste releases, use and production. National Science digital library (2004)

1.8.0 Literature review

1.8.1 Influence of Surfactant on Antimicrobials.

Anelich and Korsten (1996) reported the spoilage of creams and found, *Aspergillus niger*, *Candida albicans, Staphylococcus aureous* and *Pseudomonas aeruginosa* to be associated with the spoilage. In order to effect preservation the researchers incorporated into the composition a mixture of essential oils known to possess antimicrobial properties. To incorporate the oils, an emulsion was first formed using the solubilizer or dispersant agent polyoxyethlene sorbitan ester, which is available commercially under the "POLYSORBATE" name, i.e Polysorbate 20 and Polysorbate 80. The addition of the polysorbate clearly reduced antimicrobial activity of the polylysine and the essential oil. In a study addition of a surfactant particularly an ionic surfactant in large amount to polylysine caused significant inhibition in the antimicrobial activity

of polylysine. For achieving a higher effect at lower concentrations of polylysine, the content of the surfactant in the composition had to be reduced.

1.8.2 INTERACTIONS OF PRESERVATIVES WITH MACROMOLECULES

Kurup *et al* (1995) studied the interactions of preservatives with macromolecules. They found out that the antimicrobial activity of chlorocresol, methyl-p-hydroxybenzoate and Phenoxyethanol in the presence of different concentrations of methylcellulose, Sodium carboxymethylcellulose and hydroxypropylmethylcellulose reduced the activities of the preservatives against *Pseudomonas aeruginosa* to varying degrees. The activity of Chlorocresol was considerably inhibited by sodium carboxymethylcellulose and hydroxypropylmethylcellulose, while hydroxypropylmethylcellulose caused the maximum supression of the activity of methly-p-hydroxybenzoate. The activity of phenoxyethanol similarly was markedly reduced by each of the three cellulose derivatives.

1.8.3 Effect of nonionic surfactant on bactericidal activity of cetylpyridinium chloride

Bradshaw *et al* (1972) researched on the effect of non-ionic surfactants on bactericidal activity of cetylpyridinium chloride. The interaction of cetylpyridinium chloride with a nonionic surfactant was examined by equilibrium dialysis; and by using the simple aqueous phase saturation model, the effect of the surfactant upon the biological activity of the bactericide was predicted. Determination of the degree of reduction of bactericidal activity showed good agreement with the predicted result over a concentration range of 10-100 p.p.m. of bactericide. However, below a concentration of 10ppm they observed a discrepancy between the predicted and experimental results was possible.

1.8.4 Effect of surfactants on the antibacterial activity of preservatives.

Kurup *et al* (1991) published an article on the effect of surfactants on the antibacterial activity of preservatives. Antibacterial activities of methyl-p-hydroxybenzoate, phenoxyethanol and Chlorocresol against *Pseudomonas aeruginosa* were evaluated in the presence of varying concentrations of Tween 80. Below the critical micelle concentration (CMC) level, the bactericidal activities increased with decrease in the surface tension values of Tween 80 solutions and with interfacial tension values of Tween 80 solution/liquid paraffin systems. Linear relationships were found to exist between the concentrations of each preservative required to reduce the microbial population by a factor 10^3 within 48 hours and the values of surface tension and interfacial tension respectively. Reduction in surface tension and interfacial tension would have increased the adsorption and uptake of preservatives by bacterial cells thereby killing the cells at a faster rate. They further observed that concentrations of Tween 80 above CMC also enhanced the antibacterial activities of these preservatives. This was attributed to the increase in the permeability of bacterial membranes to preservatives.

CHAPTER TWO

2.0 SCOPE OF WORK

Most pharmaceutical and cosmetic products on the market today are contaminated with various microorganisms irrespective of the inclusion of desired preservatives in their preparation. The efficacy of these preservatives in the product could be affected by long shelf life, poor storage conditions, additives and concentration of the preservative and added surfactant in their preparations. Hugbo *et al* (2003)

To assess the impact of surfactants on these preservatives, some preservatives and surfactants will be selected and studied. This work seeks to investigate the effect of polysorbate on preservatives and to estimate the amount of the preservatives available in the system after interacting with the surfactants.

There will be an introduction to the source and uses of the antimicrobial preservatives; Salicylic acid and chlorocresol. A thorough study on types of emulsifying agents will be done, but particular attention will be given to polysorbate.

Previous work on the effect of non-ionic surfactants on the antimicrobial activity of different preservatives will be reviewed. Again, the interactions of preservatives with different macromolecules will be vividly reviewed to assess the effect macromolecules have on preservatives.

This work will assess the effect of two preservatives; salicylic acid and chlorocresol against four test microorganisms, *Escherichia coli*, *Bacillus subtillis*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*. The influence of a surfactant; polysorbate (Tween 80) on the bactericides against the test microorganisms will be investigated. The influence of polysorbate on the

bactericides will be investigated physically using the spectrophotometric analysis. The amount of bactericide available after interaction with the polysorbate will be estimated.

Finally, the influence of polysorbate on the rate of diffusion of the bactericides will also be estimated. This work will try to assign reasons to each of the activities to be performed.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Instruments

3.1.1 Stuart scientific flask shaker (SF1)

3.1.2 Cecil CE 2041 UV analyzer (2000 series)

3.3.5 Laminar flow cabinet. SKAN AG (post fach CH-4009 Basel)

3.2.0 Culture media

3.4.1 Nutrient Broth (Oxoid CM 1)

3.4.2 Nutrient Agar (Oxoid CM 3)

3.3.0 Reagents

Distilled water

Bromocresol purple Indicator

3.4.0 Source of Microorganisms

The microorganisms used were *Escherichia coli*, *Bacillus subtilis*, *Staphylococcus aureus* and *Pseudomonas aeruginosa*. All were obtained from the Microbiology Laboratory of the Faculty of Pharmacy and Pharmaceutical Sciences, KNUST.

3.5.0 Preparation of culture media.

3.5.1 Nutrient Broth

Nutrient broth (1.3g) granules were weighed into a beaker and about 60ml of sterile distilled water was added and stirred to dissolve. Enough of the water

was added to the 100ml mark then poured in 10ml quantities into already cleaned and dried text tube and plugged firmly with cotton wool. The tubes were then sterilized in an autoclave (Basilton) at 115^{0}C for 30minutes.

3.5.2 Nutrient agar

Nutrient agar granules (2.8g) were weighed with the electronic balance (WA 210 Adam equipment) into a beaker; about 60ml of sterile distilled water was added and stirred. The solution was heated in a hot water bath to a temperature of 100^{0}C while stirring for even distribution of heat. Enough distilled water was added to the 100ml mark. The mixture was taken off from the water bath after a distinctive colour change was observed. The solution was then poured in 20ml quantities into 5 cleaned test tubes and firmly stuffed with cotton wool. The tubes with the solutions were then sterilized in an autoclave at 121^{0}C for 15minutes. They were then stored and used when required.

3.6.0 Cultivation of test Microorganisms.

A loopful of the test microorganism was inoculated into 10ml nutrient broth. The inoculated broth was incubated at 37^{0}C for 24 hours. *Escherichia coli*, *Bacillus subtilis*, *Staphylococcus aureus* and *Pseudomonas aeruginosa* were all prepared using the above technique.

3.7.0 Preparation of Antimicrobial Samples (w/v)

3.7.1 Chlorocresol (0.3% w/v)

Pure Chlorocresol (0.3g) was weighed with an electronic balance 'Adams equipment WA 210' into a beaker. About 60ml of sterile distilled water was added and warmed using the Bunsen burner. Enough water was added to

make up 100ml after all crystals had dissolved to produce a 0.3% (w/v) solution.

This was serially diluted to produce 0.1, 0.2, 0.25 and 0.3%w/v.

To prepare concentrations of 0.10%, 0.20%, 0.25%, 0.30%w/v

Concentration of stock = C_1

Volume of stock= V_1

Concentration desired = C_2

Volume desired = V_2

The general formula used to calculate appropriate volumes of stock solution to use was

$$\therefore \text{Vol. of stock} = \frac{\text{Concentration desired X overall Vol. required}}{\text{Concentration of stock}}$$

$$C_1 = \frac{C_2 \times V_2}{C_1}$$

Various concentrations were prepared using the above equation

Table 1 Preparation of varying Chlorocresol conc. from stock solution of 0.3%

Concentration desired (%)	Volume of stock required (ml)	Total volume produced (ml)
0.10	33.33	100
0.15	50.00	100
0.20	66.67	100
0.25	83.33	100
0.30	100.00	100

3.7.2 Salicylic acid 0.2% w/v

Salicylic acid 0.2g was weighed with the Adams equipment WA 210 electronic balance into a beaker. 60ml sterile distilled water was added and heated over the Bunsen burner to dissolve the powder. Enough water was then added to produce 100ml mark. The solution then had a concentration of 0.2%. Various concentrations were prepared by serial dilution. The concentrations were calculated as follows.

$$C_1 = \frac{C_2 \; \mathbf{X} \; V_2}{C_1}$$

Table 2 Preparation of varying salicylic acid Conc. from stock solution of 0.2%

Concentration desired (%)	Volume of stock required (ml)	Total volume produced (ml)
0.05	25.0	100
0.10	50.0	100
0.12	60.0	100
0.15	75.0	100
0.18	90.0	100

CHAPTER FOUR

4.0 EXPERIMENTAL

4.1 Determination of antimicrobial activity of Chlorocresol and salicylic acid against test organisms

All four test organisms were cultured in nutrient broth for 24 hours .Four 20ml Nutrient agar in the tubes were melted and stabilized at 45^0C for 15minutes in a reciprocal water bath shaker (New Buns wick). The agar was aseptically inoculated with 1ml of a *Escherichia coli*, *Bacillus subtilis*, *Staphylococcus aureus and Pseudomonas aeruginosa* and mixed thoroughly and poured into a sterilized Petri dish. It was allowed to set for 30 minutes. A flamed sterilized cork borer No.6 (10mm diameter) was used to bore 4 wells in the agar in each plate and using a volume of 0.2ml of each concentration the drug was introduced into each well. The plates were incubated for 24 hours at 37^0C. The

Zones of inhibition were measured (mm) on the base of the plate. The measurement was between the edge of the bore and growth.

The above experiment was repeated using agar plates with the following concentrations of 0.05%, 0.10%, 0.15% and 0.18% salicylic acid.

4.2 Determination of the biological influence of Polysorbate on bactericide.

Chlorocresol 0.2% was prepared from the stock solution of 0.3%, varying concentrations of the Polysorbate were also prepared from the stock of 5%v/v. 10ml solution of 0.2% Chlorocresol or Salicylic acid 0.12% plus the various concentrations of Polysorbate were prepared

6.7ml of 0.2w/v% Chlorocresol was added to 1ml, 2ml, 2.4ml, 3ml, while 6ml of 0.2%w/v Salicylic acid was also added to 1ml, 2ml, 2.4ml, 3ml and 3.6ml of 5% Polysorbate and each diluted to a final volume of 10ml.

Table 3 Preparation of Admixture to determine the effect of Polysorbate on chlorocresol biologically

Vol. of stock of chlorocresol. (0.3%)	6.7ml	6.7ml	6.7ml	6.7ml
Vol. of stock of Polysorbate. (5%)	1ml	2ml	2.4ml	3.0ml
Vol. of distilled water	2.3ml	1.3ml	0.9ml	0.3ml
Final conc. Of chlorocresol Final conc. Of Polysorbate	0.2% 0.50%	0.2% 1.0%	0.2% 1.2%	0.2% 1.5%
Final vol. of solution	10.0ml	10.0ml	10.0ml	10.0ml

Table 4 Preparation of Admixture to determine the effect of Polysorbate on salicylic acid biologically

Vol. of stock of salicylic acid. (0.2%)	6.0ml	6.0ml	6.0ml	6.0ml	6.0ml
Vol. of stock of Polysorbate. (5%)	1ml	2ml	2.4ml	3.0ml	3.6ml
Vol. of distilled water	3.0ml	2.0ml	1.6ml	1.0ml	0.4ml
Final conc. Of salicylic acid Final conc. Of Polysorbate	0.12% 0.5%	0.12% 1.0%	0.12% 1.2%	0.12% 1.5%	0.12% 1.8%
Final vol. of solution	10.0ml	10.0ml	10.0ml	10.0ml	10.ml

The final solutions therefore contained a fixed 0.2% Chlorocresol or 0.12% Salicylic acid and 0.5%, 1.0%, 1.2%, 1.5% or 1.8% of the Polysorbate. 20ml Nutrient agar were melted and stabilized at 45^{0}C for 20minutes in a reciprocal water bath and labelled I, II, III, IV and V. They were inoculated with 1ml of 24hours culture of *Escherichia coli*, *Bacillus subtillis*, *Pseudomonas aeruginosa,* and *Staphylococcus aureus* respectively. Each was shaken to ensure uniform mixing and immediately poured into sterilized Petri dishes and allowed to set for 30minutes. Four holes created in each plate were filled with 0.2ml of 0.5%, 1.0%, 1.2%, 1.5% and 1.8%v/v Polysorbate containing 0.2% Chlorocresol or 0.12% Salicylic acid. They were incubated for 24 hours at 37^{0}C the zones of inhibition were measured and recorded.

4.3 Determination of maximum wavelength:

Different concentrations of Chlorocresol were prepared from the stock of 0.3%. One drop of Bromocresol purple indicator was added to each. Each of the

solutions was scanned using the Cecil CE 2041 UV analyzer. The maximum peaks were located and the corresponding wavelengths recorded. Chlorocresol recorded a wavelength of 293mm. This was repeated for salicylic acid. The maximum wavelength was found to be 311.5nm for salicylic acid.

4.4 Spectrophotometric analysis of the bactericides

From the 0.3% stock of freshly prepared Chlorocresol, sub-concentrations of 0.05%, 0.1%, 0.15%, 0.20% 0.25% were prepared. A drop of Bromocresol purple indicator was added to each of the five concentrations. Samples were drawn from each and run at 293nm using the UV analyzer. The various absorbance were recorded Table 19, Figure 14.

The experiment was repeated using 0.05%, 0.10%, 0.15% and 0.18% of salicylic acid at 311.5nm Table 20, Figure 15.

4.5 Determination of the physical influence of Polysorbate on Chlorocresol and salicylic acid:

2 drops of Bromocresol purple indicator was added to 50ml of 0.15% Chlorocresol prepared in five different bottles. The latex bags (condoms) were washed with boiling water and detergent to remove the oil and grease from them. 0.5%, 1.0%, 1.5%, 2.0% and 2.5% Polysorbate were prepared into the five respective latex bags. The latex bags were hung in the five bottles and tightly sealed. The bottles were shaken for 24 hours using the stuart scientific flasks shaker S F 1. Portions of the Chlorocresol were analyzed using the UV scan after 24 hours at 293nm Table 10, Figure 5.

The experiment was repeated using 50ml 0.12% salicylic acid at 311.5nm Table 9, Figure 4. The various absorbances were read and recorded. Graphs of Absorbance against concentration were plotted for both Chlorocresol and salicylic acid.

4.6 Determination of rate of diffusion of bactericide.

2 drops of Bromocresol purple indicator was added to 20ml nutrient agar and shaken to ensure a uniform mixture. The nutrient agar was poured into a Petri dish and allowed to set for. A number 6 cork borer was used to bore 4 different wells in the plate. The wells were filled with 0.10%, 0.12%, 0.15% and 0.20% salicylic acid respectively or 0.10%, 0.20%, 0.25% and 0.30% of Chlorocresol. The zone of diffusion indicated by a yellow region for salicylic acid and purple for Chlorocresol around the holes were measured (mm) hourly for four (4) hours. The rate of diffusion (Rd) of each concentration was determined by dividing Extent of diffusion(Ed) by the time taken (T).

$$R_d \text{ (mm/hr)} = \frac{E_d \text{ (mm)}}{T \text{ (hr)}}$$

CHAPTER FIVE

5.0 RESULTS

5.1 Activity of Bactericide in model system

The activity of salicylic acid in the model system against the four test organisms, *Escherichia coli*, *Staphylococcus aureus*, *Bacillus Subtilis* and *Pseudomonas aeruginosa* is shown in Table 5 Figure 1.

Below a concentration of 0.10%, salicylic acid had no activity as measured by the degree of inhibition. However when the concentration was increased above 0.10%w/v it showed activity against all four microorganisms. The highest activity of 4mm at 0.2%w/v was against Staph.

Chlorocresol was similarly effective against all the test organisms, but weakest on pseudomonas. Activity, again, increased with increase in concentration as shown in Table 6 Figures 2. However, Chlorocresol was found to be relatively more active than Salicylic acid. For instance while Salicylic acid 0.2%w/v produced inhibition zone of 2-4mm against the organisms, the same concentration of chlorocresol produced 6-11mm (Table 6).

Table 5 Activity of salicylic acid in model system against test organisms

	Zone of inhibition (mm)			
Salicylic acid conc.% w/v	E. *coli*	Staph *aureus*	B. *subt.*	Pseud *aer.*

0.05	-	-	-	-
0.10	1.0	-	-	-
0.12	2.0	2.0	1.0	1.0
0.15	2.0	3.0	1.0	1.0
0.20	3.0	4.0	2.0	2.0

Table 6 Activity of Chlorocresol in model system against test organisms

	Zone of inhibition (mm)			
Chlorocresol conc.% w/v	E. *coli*	Staph. *Aureus*	B. *subt.*	Pseud *aer.*
0.10	9	5	5	-
0.15	10	5	6	-
0.20	11	6	7	-
0.25	14	8	9	-
0.30	15	10	10	-

Figure1. Activity of Salicylic acid in model system against Organisms

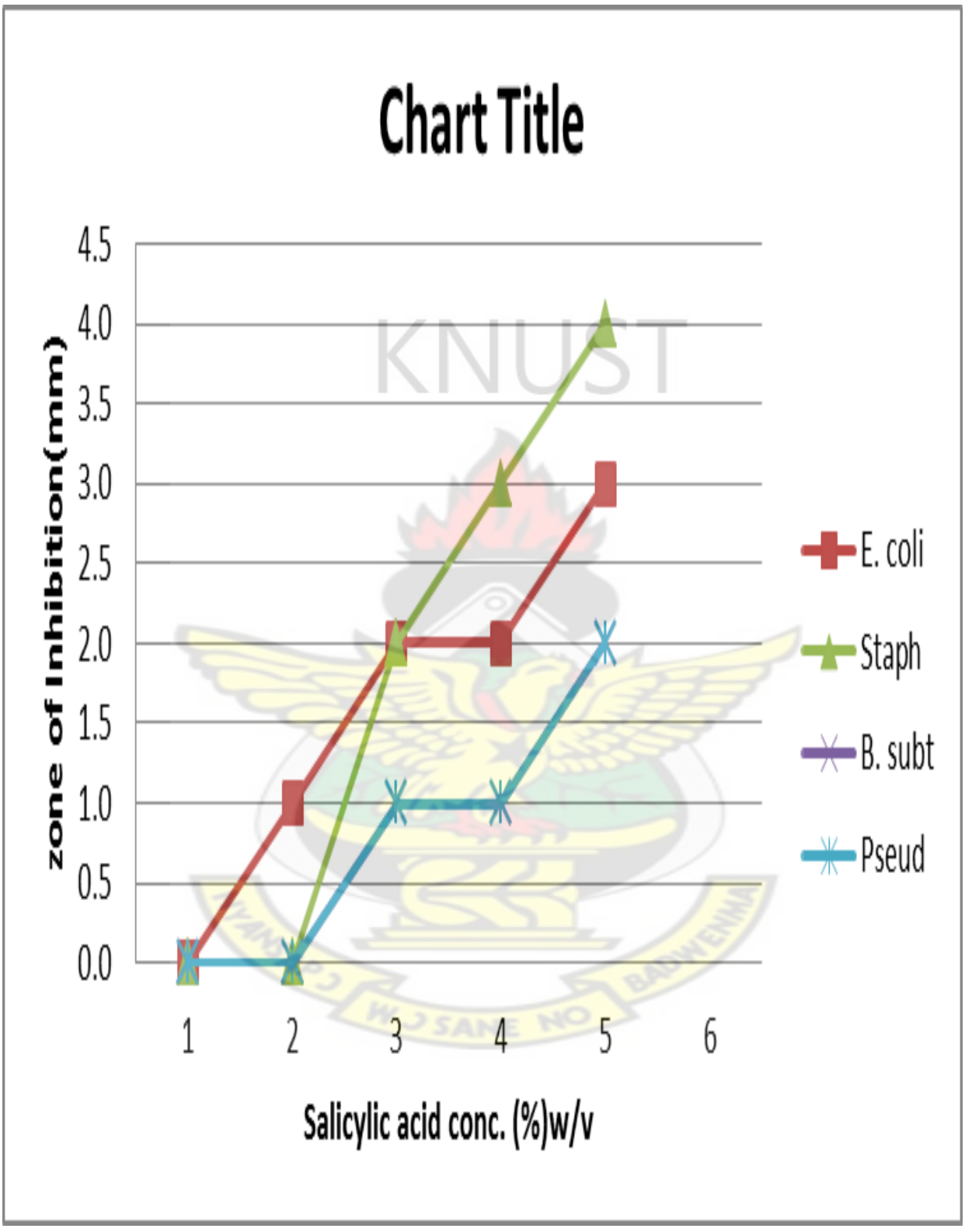
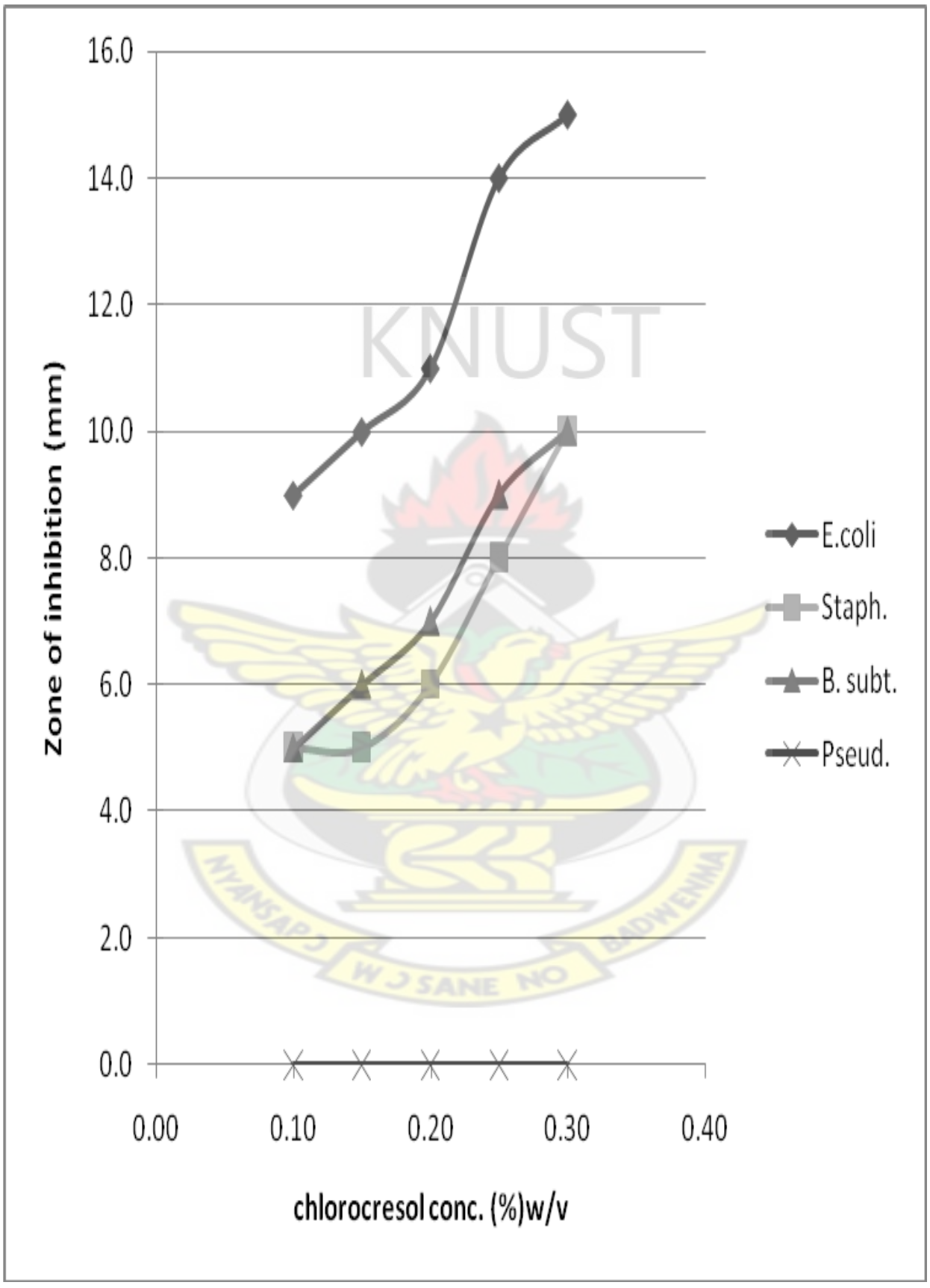


Chart Title
zone of Inhibition(mm)
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
1
2
3
4
5
6
Salicylic acid conc. (%)w/v
E. coli
Staph
B. subt
Pseud

Fig.2 Activity of Chlorocresol in model system against test organisms


16.0
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
Zone of inhibition (mm)
0.00
0.10
0.20
0.30
0.40
chlorocresol conc. (%)w/v
E.coli
Staph.
B. subt.
Pseud.

5.2 Effect of the presence of polysorbate on Bactericide.

The effect of a fixed concentration of salicylic acid 0.12%w/v was initially established. The addition of polysorbate was determined and it was found to have a dramatic effect. The results showed that concentrations of polysorbate added ranging from 0.5 to 1.8%v/v abolished the initial antimicrobial activity of 2.0mm and 1.0mm and the bactericide showed no inhibitions against *E. coli*, *Ps aeruginosa*, *B. Subtilis* and Staph. *aureus* (Table 7), all organisms were therefore found to grow in the surfactant-bactericide mixture. Table 8 and graphically represented in Figure 3, again, with respect to Chlorocresol initial activities of 11mm, 7mm, 6mm and 0mm for E. *Coli.*, B. *Subt.*, Staphylococcus and Pseudomonas respectively the addition of polysorbate ranging from 0.5 to 1.5%v/v drastically diminished activity for E. *coli* from 11mm to 3mm, for Staph from 6mm to 0mm and B. *subtillis* from 7mm to 0mm. The marked decrease in the activity due to the presence polysorbate in the model system, even though the amount of bactericide was maintained at a fixed level, suggested that polysorbate in some way interfered with the bactericide adversely. This observation derived from the biological effect led to an exercise to evaluate systematically physico-chemical effect of polysorbate on the bactericide, if any.

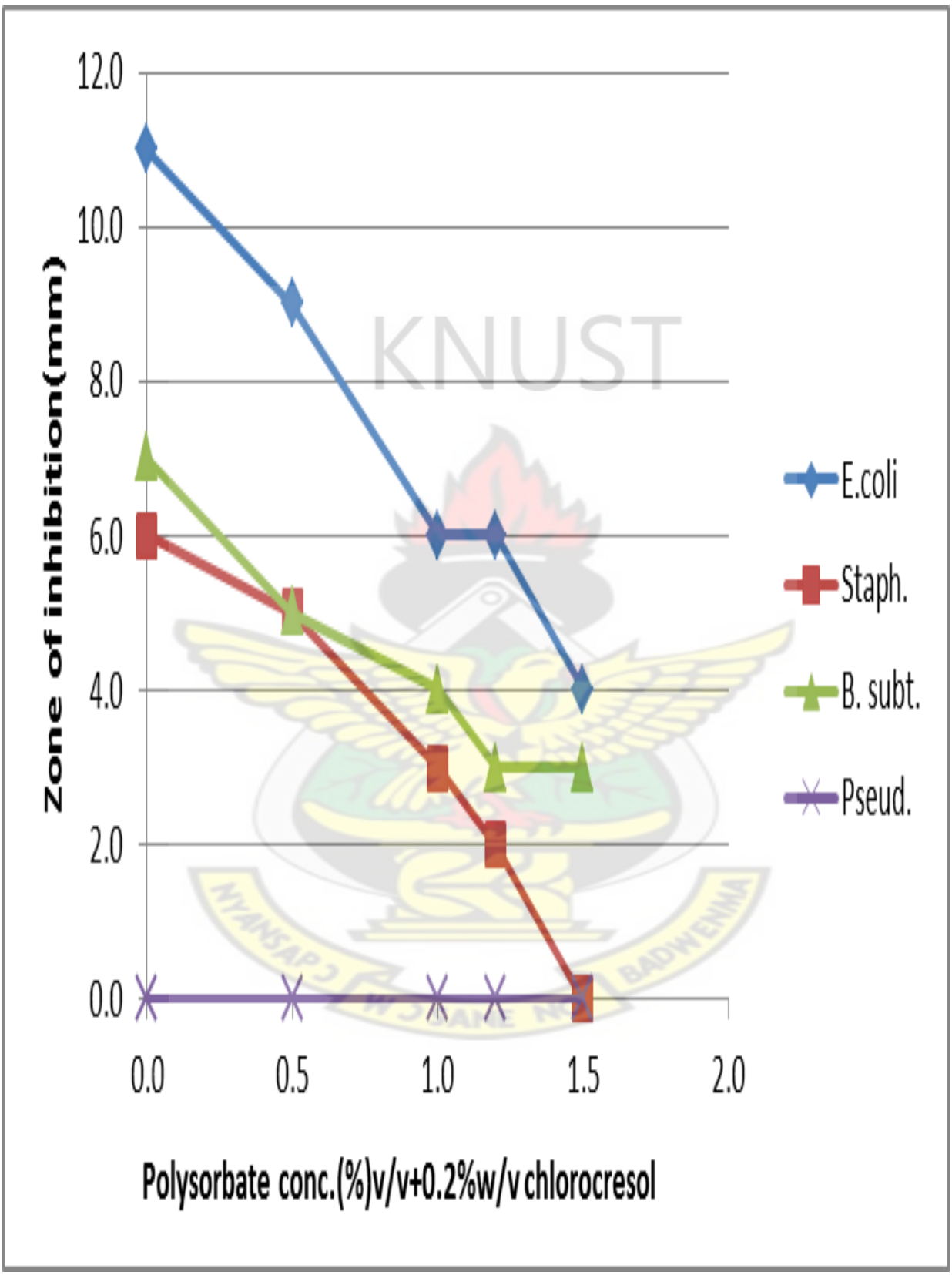
Tables 7 Effect of fixed 0.12% salicylic acid in the presence of polysorbate

	Zone of inhibition (mm)			
Polysorbate conc. % w/v	E. *coli*	Staph. *aureus*	B. *subt.*	Pseud. *aer*
0.0	2.0	2.0	1.0	1.0
0.5	0.0	0.0	0.0	0.0
1.0	0.0	0.0	0.0	0.0
1.2	0.0	0.0	0.0	0.0
1.5	0.0	0.0	0.0	0.0
1.8	0.0	0.0	0.0	0.0

Table 8 Effect of fixed 0.2% chlorocresol in the presence of polysorbate

	Zone of inhibition (mm)			
Polysorbate conc. % w/v	E. *coli*	Staph. *Aureus*	B. *subt.*	Pseud. *aer*
0.0	11.0	6.0	7.0	0.0
0.5	9.0	5.0	5.0	0.0
1.0	6.0	3.0	4.0	0.0
1.2	6.0	2.0	3.0	0.0
1.5	4.0	0.0	3.0	0.0

Figure 3 Activity of fixed 0.2%w/v Chlorocresol in the presence of Polysorbate.


12.0
10.0
8.0
6.0
4.0
2.0
0.0
Zone of inhibition(mm)
0.0
0.5
1.0
1.5
2.0
Polysorbate conc.(%)v/v+0.2%w/v chlorocresol
E.coli
Staph.
B. subt.
Pseud.

5.3 Standardization of bactericidal concentration by measurement of UV absorbance.

Spectrophotometric analysis was adopted for use in the study of effect of polysorbate on the two bactericides.

The UV absorbance of salicylic acid was determined at a previously determined λ_{max} of 311.5nm. Increases of salicylic acid concentrations from 0.0% to 0.14% Table 19 Appendix 1 were accompanied by persistent increases of absorbances. Similarly, the absorbance of Chlorocresol at λ_{max} 293nm followed the same trend as the salicylic acid. As the chlorocresol concentration increased the absorbance also increased Table 20 Appendix 2.

A plot of absorbance against concentration showed a straight line for salicylic acid Appendix 1 Fig. 14 and chlorocresol Appendix 2 Fig.15 both passed through the origin. The trend observed obeyed the Beer Lambert's law which states that "The intensity of absorption is proportional to the concentration of the dilute solution of an absorbing compound",

$$A = \varepsilon cd$$

Where A= absorbance, c= concentration, d= width of the sample cell and ε= molar absorptivity (L/mole-cm). Absorbance therefore is directly proportional to the total quantity of the absorbing compound in the path of the light through the cuvette. At high concentrations the law is not obeyed.

Figures 14 and 15 were therefore used as reference graphs for determining unknown concentrations of the bactericides when the absorbance was known spectrometrically.

5.4 Effect of polysorbate on bactericide by Spectrophotometric analysis.

The results of the physical influence of Polysorbate on a fixed concentration of bactericide salicylic 0.12%w/v acid and Chlorocresol 0.15%w/v are presented, Table 9 and Table 10 respectively. The graphical representations Figures 4 and 5 showed that, for instance, 0.0% to 2.0% v/v polysorbate drastically reduced the absorbance of 0.12% salicylic acid from 1.1800A to 0.2176A. For chlorocresol 0.0% to 2.5%v/v of polysorbate reduced the absorbance from 1.2200A to 0.1260A. The results determined indicated that the addition of polysorbate had very significant influence on the absorbances of both salicylic acid and chlorocresol. Since absorbance is directly related to concentration, the true concentrations could be determined.

Table 9 Effect of Polysorbate on absorbance of salicylic acid 0.12%w/v at 311.5nm

Polysorbate conc (%) v/v	Absorbance (A)
0.00	1.1800
0.50	0.9590
1.00	0.6230
1.50	0.3956
2.00	0.2176

Table 10 Effect of Polysorbate on absorbance of Chlorocresol 0.15%w/v at 293nm

Polysorbate conc. (%) v/v	Absorbance (A)
0.00	1.2200
0.50	0.5459
1.00	0.4451
1.50	0.3192
2.00	0.2268
2.50	0.1260

Figure 4 Effect of polysorbate on 0.12%w/v salicylic acid absorbance at 311.5nm

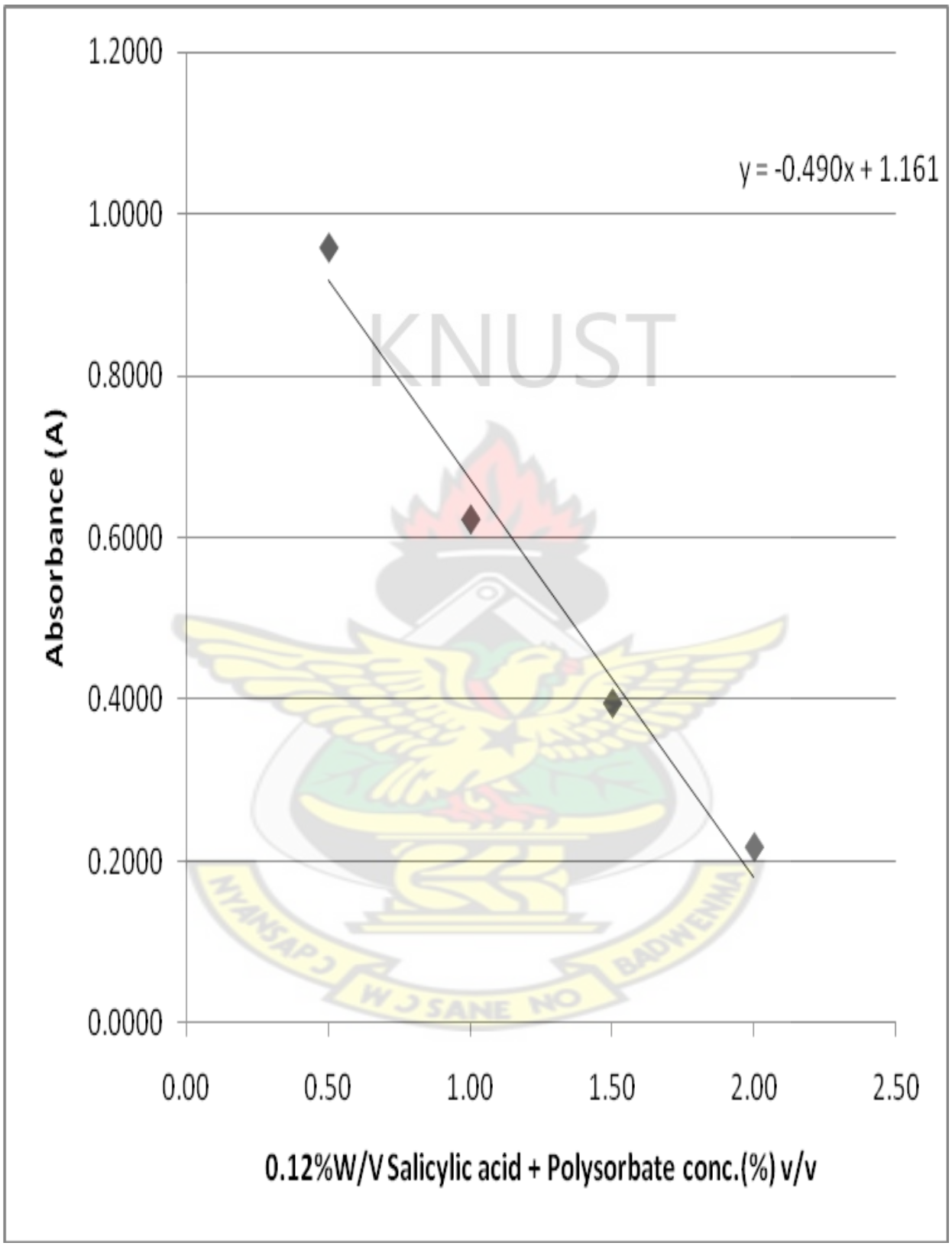
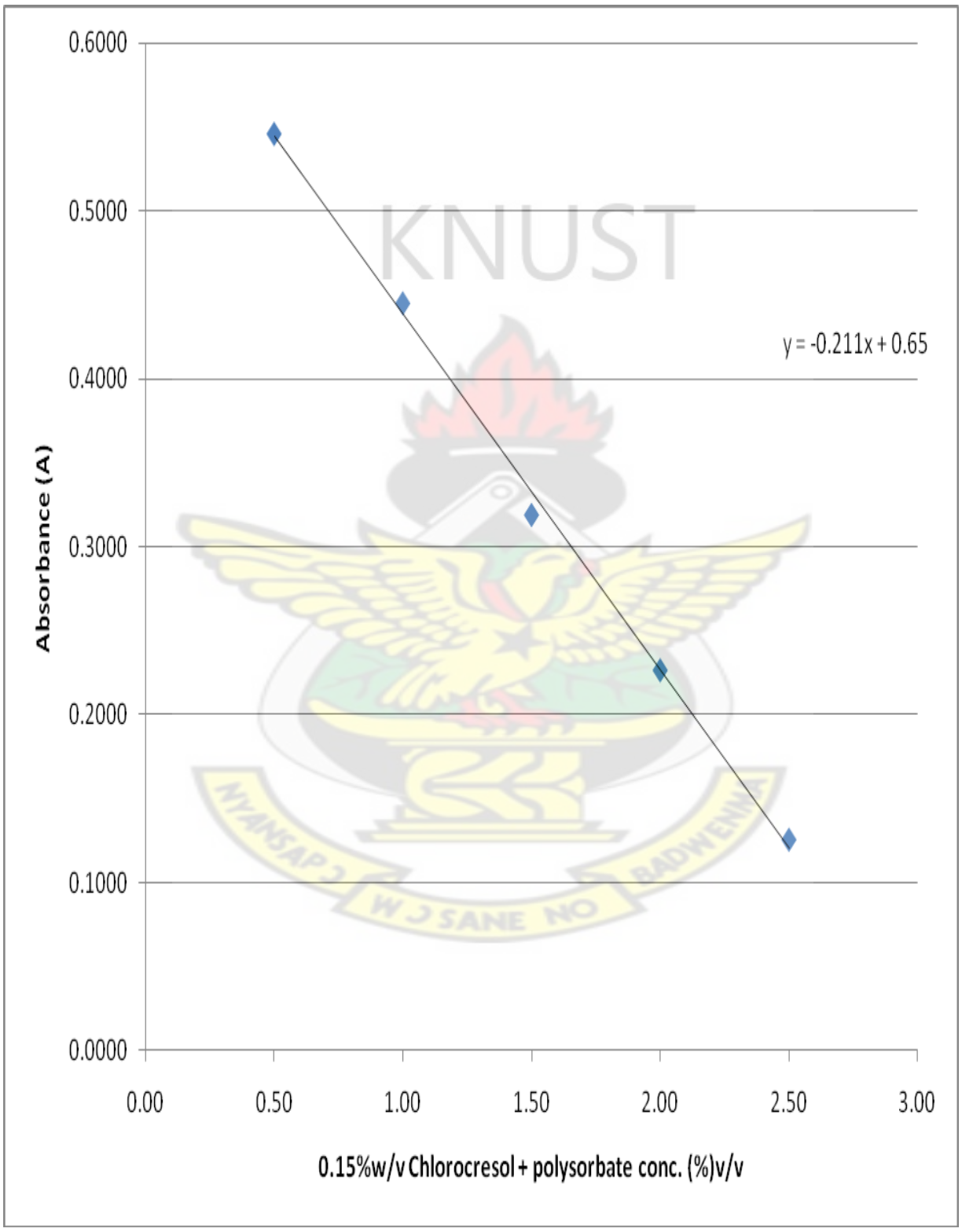

y = -0.490x + 1.161
Absorbance (A)
1.2000
1.0000
0.8000
0.6000
0.4000
0.2000
0.0000
0.00
0.50
1.00
1.50
2.00
2.50
0.12%W/V Salicylic acid + Polysorbate conc.(%) v/v

Figure 5 Effect of polysorbate on absorbance of 0.15%w/v chlorocresol at 293nm



5.5 Estimation of bactericides available in the bactericide-polysorbate admixture

From the results of absorbances obtained and recorded in Tables 9 and 10 it was possible to determine the actual concentration of bactericide available in any particular polysorbate-bactericide admixture using as reference the Beer Lambert plots already constructed (appendix 1 and 2). The results for salicylic acid Tables 11 and chlorocresol Table 12 indicated that the chlorocresol determinable in the system declined successively with an increase in the amount of polysorbate. For instance, by increasing polysorbate concentration from 0.0% to 2.0% the determinable residual salicylic acid concentration dropped sharply from 0.12%w/v to 0.022%w/v representing 100% down to 18.3% v/v. For chlorocresol the corresponding figures were down from 100% to 10% for 0.0% to 2.5%v/v of polysorbate concentration. The amount of determinable bactericide was 18.3% for salicylic acid and 10% for chlorocresol. The results clearly indicated that as much as 81.7% salicylic acid and 90% chlorocresol were lost to the polysorbate in the bi-phase system and was unavailable. The amount determinable in the system reflects only the amount of bactericide free and available in the system.

Table 11 Determination of 0.12%w/v Salicylic acid free in the presence of polysorbate

Polysorbate conc. (%)v/v	absorbance (A)	residual salicylic acid (%w/v)	% of residual salicylic acid conc.	% of salicylic acid bound
0.0	1.1800	0.120	100.0	00.0
0.5	0.9590	0.097	80.3	19.7
1.0	0.6230	0.063	52.5	47.5
1.5	0.3956	0.040	33.3	66.7
2.0	0.2176	0.022	18.3	81.7

Table 12 Determination of 0.15%w/v chlorocresol free in the Presence of polysorbate

Polysorbate conc.(%) v/v	absorbance (A)	Residual chlorocresol conc. (%) w/v	% of residual chlorocresol conc.	% of chlorocresol bound
0.0	1.2200	0.150	100.0	0.00
0.5	0.5459	0.065	43.3	56.7
1.0	0.4451	0.053	35.3	64.7
1.5	0.3192	0.038	25.3	74.7

2.0	0.2268	0.027	18.0	82.0
2.5	0.1260	0.015	10.0	90.0

FIGURE 6 Concentration of 0.12%w/v Salicylic Acid free after interaction with Polysorbate

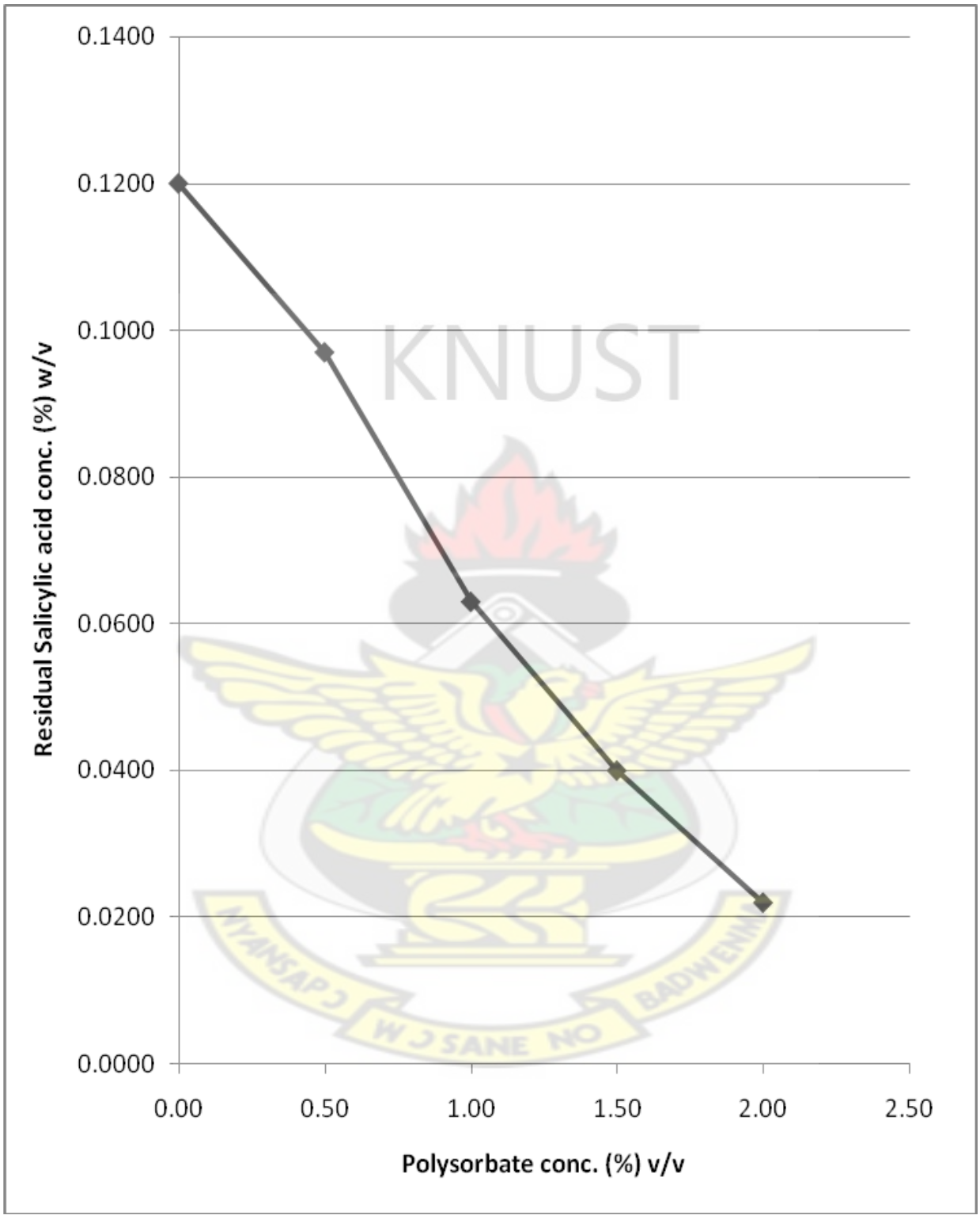
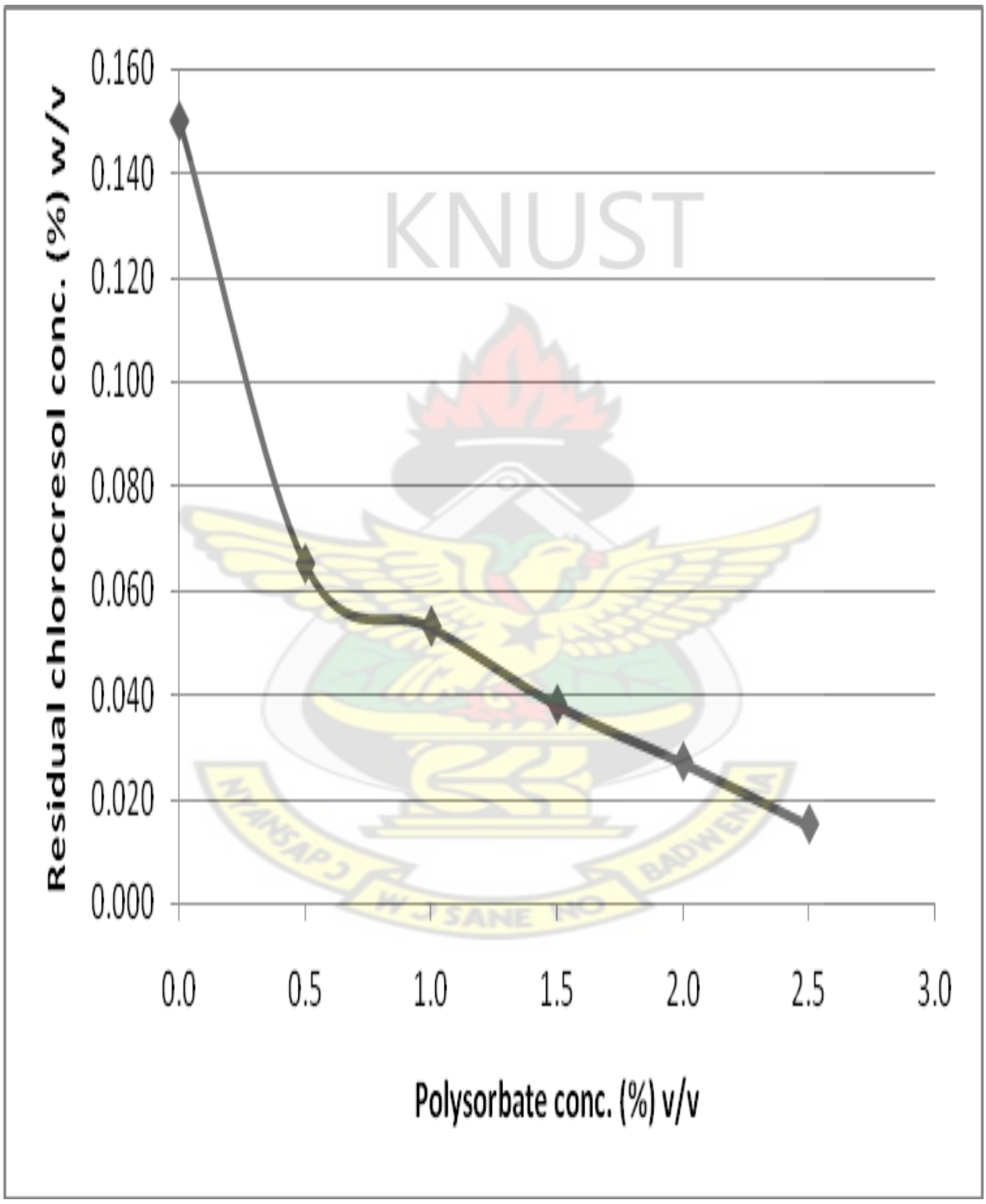

0.1400
0.1200
0.1000
0.0800
0.0600
0.0400
0.0200
0.0000
Residual Salicylic acid conc. (%) w/v
0.00
0.50
1.00
1.50
2.00
2.50
Polysorbate conc. (%) v/v

FIGURE 7 Concentrations of 0.15% w/v Chlorocresol free after interaction with Polysorbate.



5.6 The influence of physical interaction between polysorbate-bactericide on biological activity

The work suggests a direct correlation between the reduction in bactericidal concentration for salicylic acid (Table 11) and chlorocresol (Table 12) in the presence of polysorbate and the drastic decline in activity observed earlier for salicylic acid (Table 7) and chlorocresol (Table 8) in the presence of the surfactant, even though the bactericides concentrations were maintained at a fixed level.

5.7 Diffusion of bactericides

Tables 13 and 14 represent the relationship between time and the distance diffused by the bactericides. It is seen from figures 8 and 9 that the distance diffused is directly proportional to the time taken. The diffusion continued with time until equilibrium was reached because a concentration gradient was set between the systems and the agar. The reduction in the rate of diffusion of chlorocresol is probably due to micellar solubility. That is in agreement with the claim that the surfactant binds to the chlorocresol and salicylic acid reducing the concentration of the free drug that will act on organisms.

Table 13 Effect of Chlorocresol concentration on diffusion

	Distance moved by chlorocresol concentration (mm)			
Time (hr)	0.10%	0.20%	0.25%	0.30%
0	0.00	0.00	0.00	0.00
1	2.00	3.00	3.00	4.00
2	3.00	3.50	4.00	4.50
3	3.00	4.00	5.00	5.00
4	3.00	4.00	5.00	6.00

Table 14 Effect of Salicylic Acid concentration on diffusion

	Distance (mm) moved by salicylic Acid concentration			
TIME (hr)	0.10%	0.12%	0.15%	0.20%
0	0.00	0.00	0.00	0.00
1	1.00	2.00	2.00	3.00
2	2.00	2.50	3.00	4.00
3	2.00	3.00	4.00	5.00
4	3.00	3.00	4.00	5.00

FIGURE 8 Extent of chlorocresol diffusion with time taken.

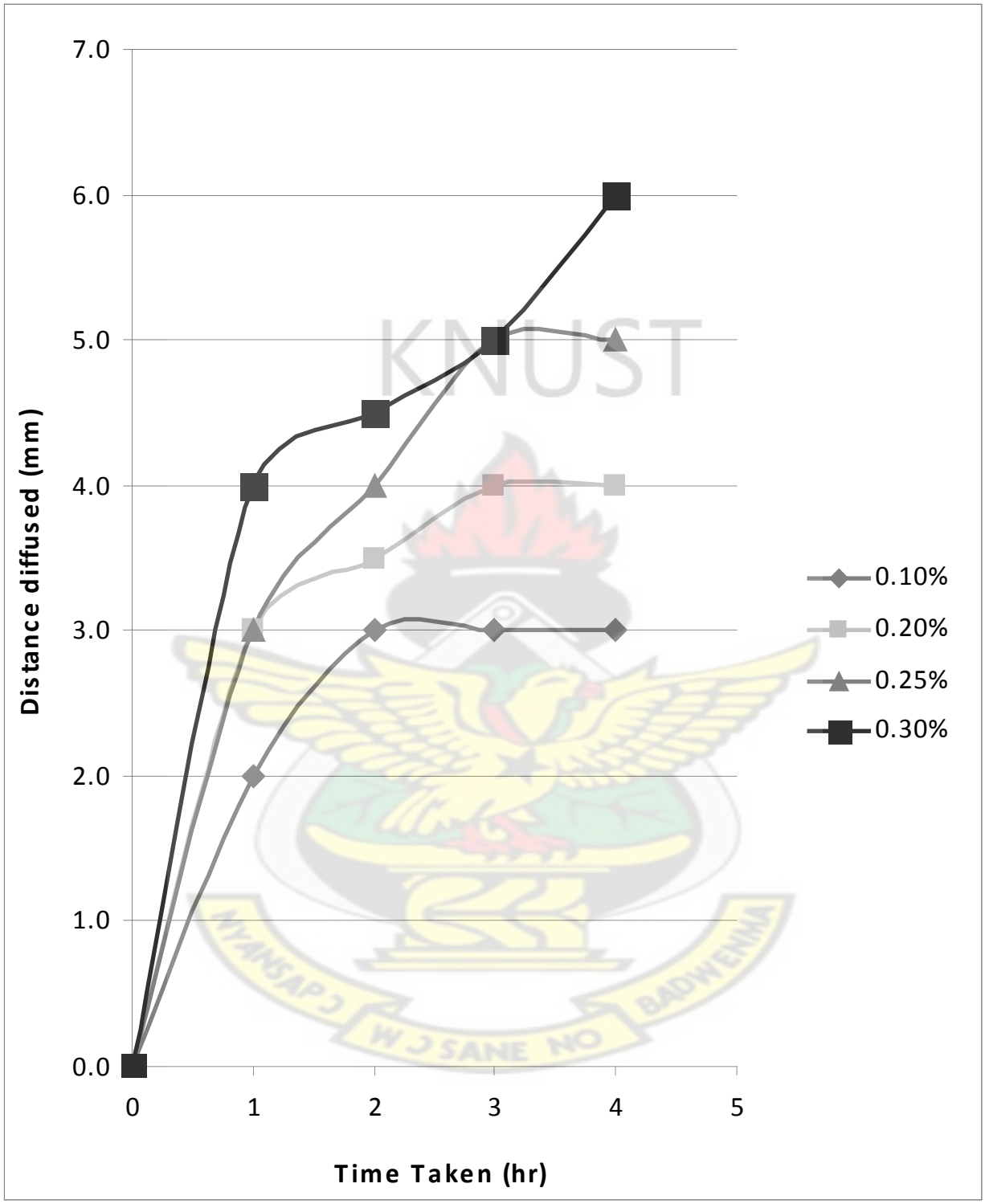

7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
Distance diffused (mm)
0
1
2
3
4
5
Time Taken (hr)
0.10%
0.20%
0.25%
0.30%

Figure 9 Extent of Salicylic Acid diffusion with time

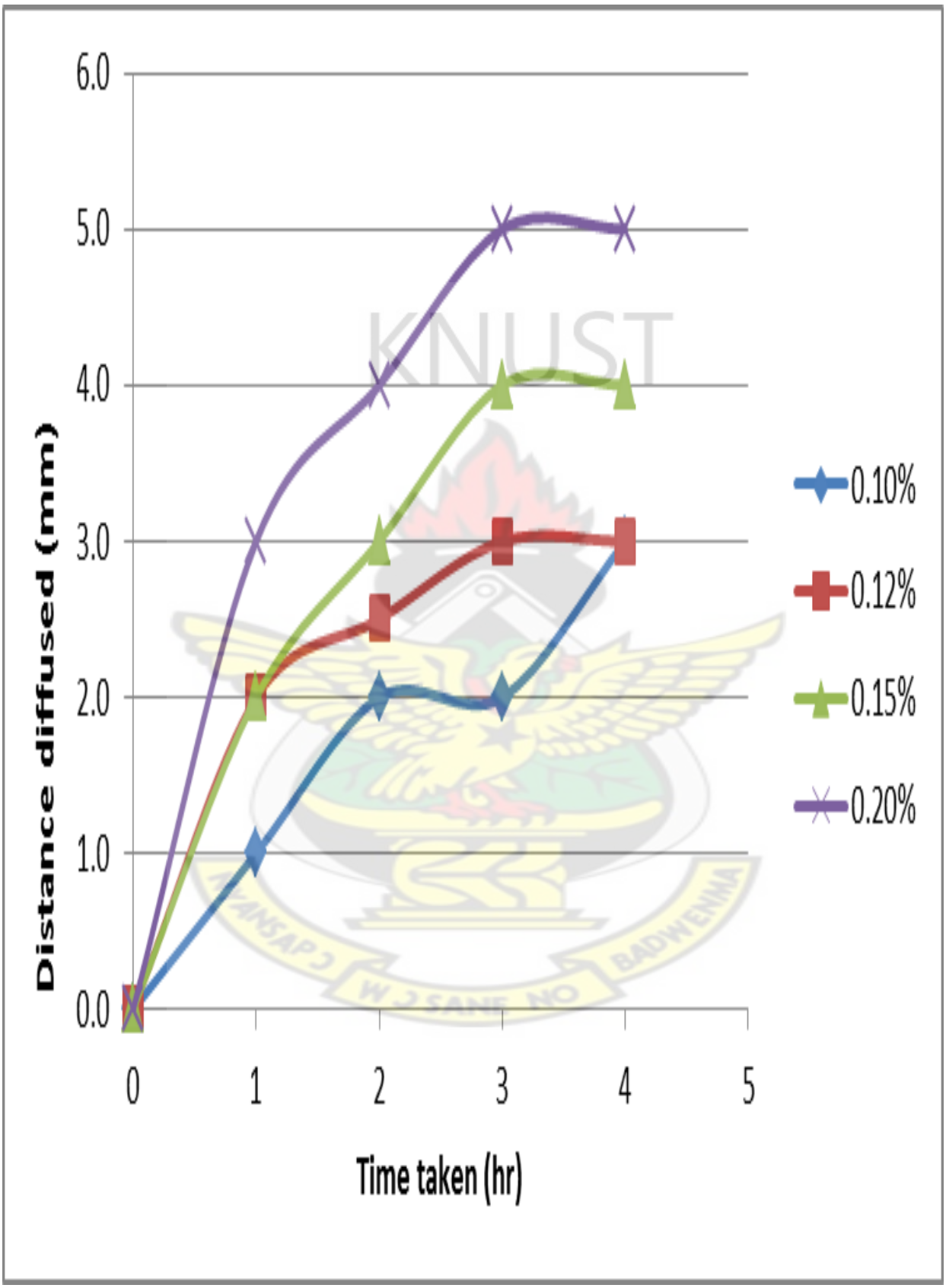

6.0
5.0
4.0
3.0
2.0
1.0
0.0
Distance diffused (mm)
0
1
2
3
4
5
Time taken (hr)
0.10%
0.12%
0.15%
0.20%

Table 15 Relationship between chlorocresol concentration and rate of diffusion

	Rate of chlorocresol diffusion (mm/hr)			
CONCENTRATION (%)W/V	1hr	2hrs	3hrs	4hrs
0.00%	0.00	0.00	0.00	0.00
0.10%	2.00	1.50	1.00	0.75
0.20%	3.00	1.75	1.33	1.00
0.25%	3.00	2.00	1.67	1.25
0.30%	4.00	2.25	1.67	1.50

Table 16 Relationships between rate of diffusion and salicylic Acid concentration

	Rate of Salicylic Acid diffusion (mm/hr)			
CONCENTRATION (%)W/V	1hr	2hrs	3hrs	4hrs
0.00%	0.00	0.00	0.00	0.00
0.10%	1.00	0.50	0.67	0.50
0.12%	2.00	1.25	1.00	0.75
0.15%	2.00	1.50	1.33	1.00
0.20%	3.00	2.00	1.67	1.25

FIGURE 10 Relationship between chlorocresol concentration and rate of diffusion

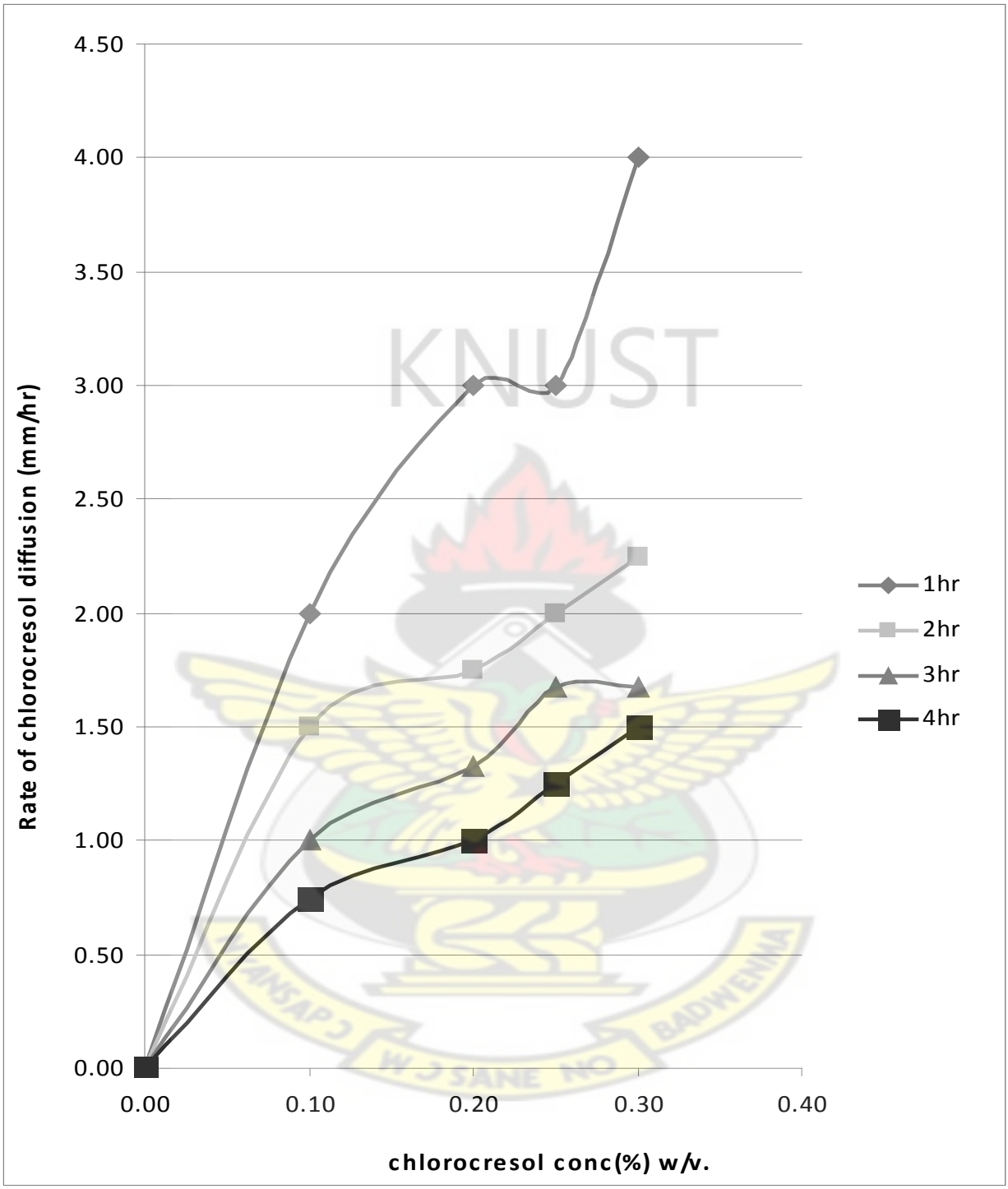

4.50
4.00
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
Rate of chlorocresol diffusion (mm/hr)
0.00
0.10
0.20
0.30
0.40
chlorocresol conc(%) w/v.
1hr
2hr
3hr
4hr

FIGURE 11 Relationship between salicylic acid concentration and rate of Diffusion

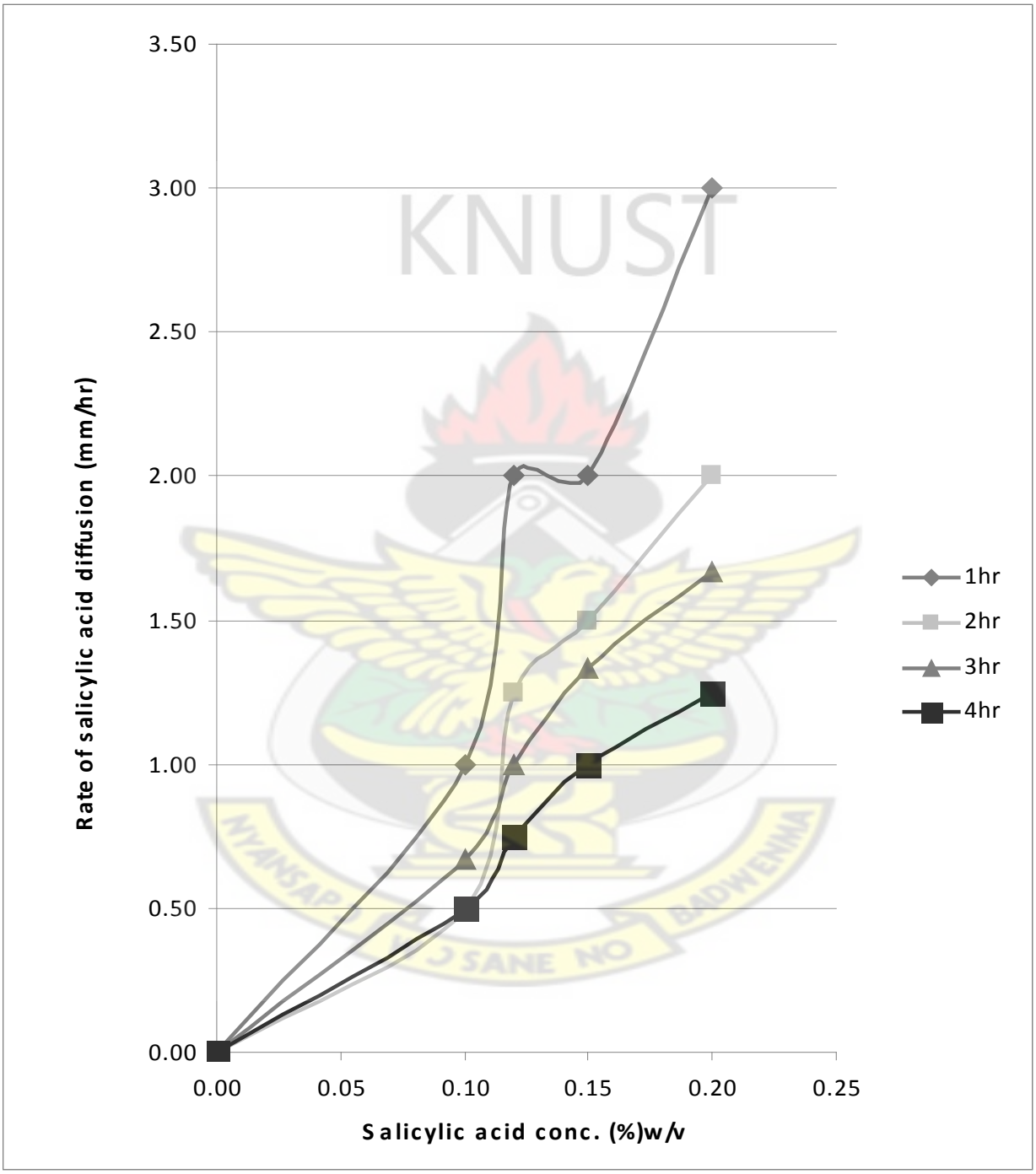


TABLE 17 Extent of Chlorocresol diffusion after interacting with 0.2% Polysorbate

	distance diffused by chlorocresol conc. (%)w/v + 0.2% polysorbate			
Time (hrs)	0.10%	0.20%	0.25%	0.30%
0	0.00	0.00	0.00	0.00
1	1.00	1.00	2.00	2.00
2	1.00	2.00	3.00	3.00
3	1.00	2.00	3.00	4.00
4	1.00	2.00	3.00	4.00

TABLE 18 Determination of rate of diffusion by chlorocresol + 0.2% polysorbate.

	rate of chlorocresol diffusion			
Conc. Of Chlorocresol + 0.2% Polysorbate.	1hr	2hr	3hr	4hr
0.00	0.00	0.00	0.00	0.00
0.10	1.00	0.50	0.33	0.25
0.20	1.00	1.00	0.67	0.50
0.25	2.00	1.50	1.00	0.75
0.30	2.00	1.50	1.33	1.00

FIGURE 12 Relationship between distances diffused by Chlorocresol + 0.2% polysorbate and time taken (hrs)

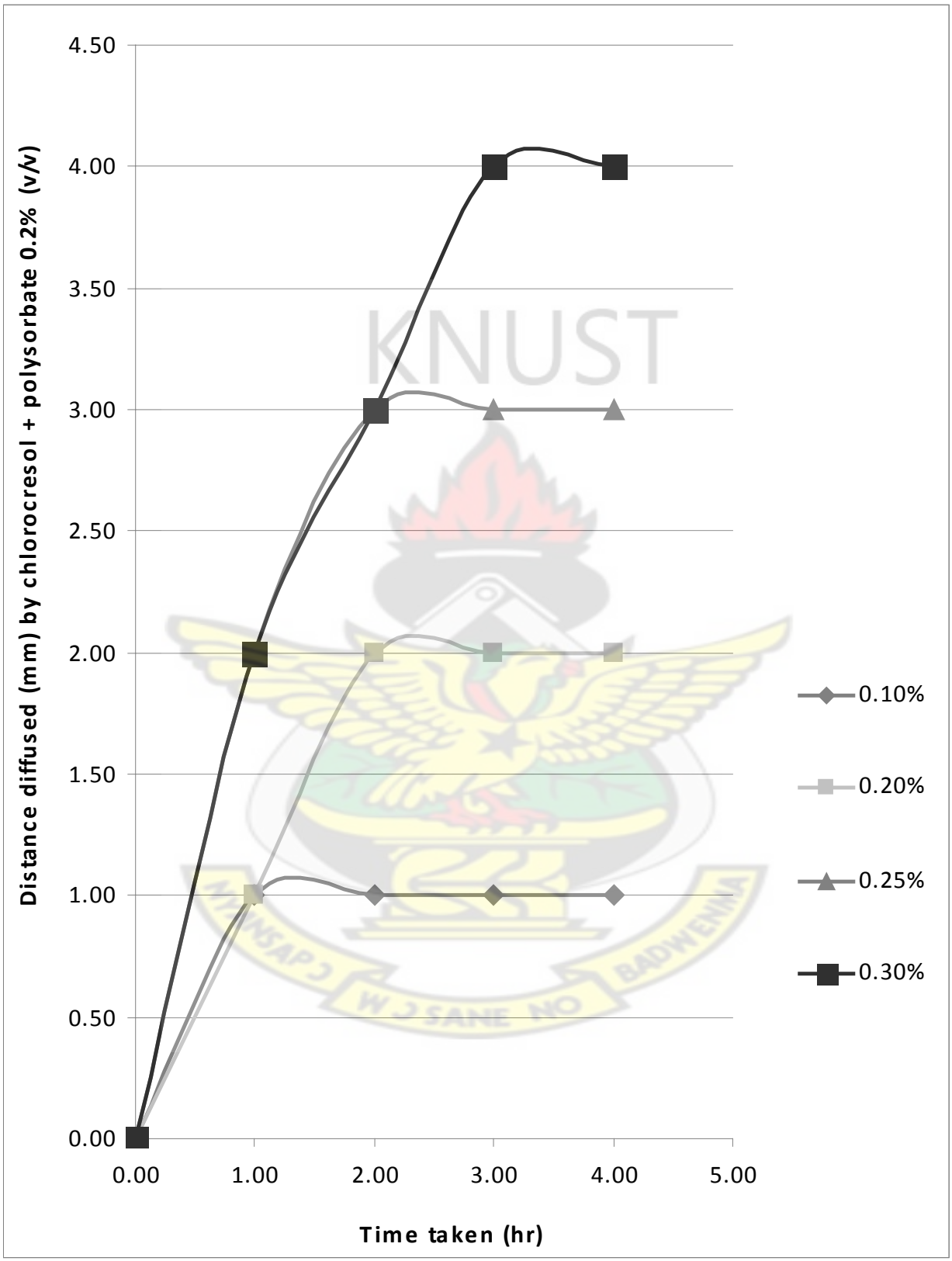
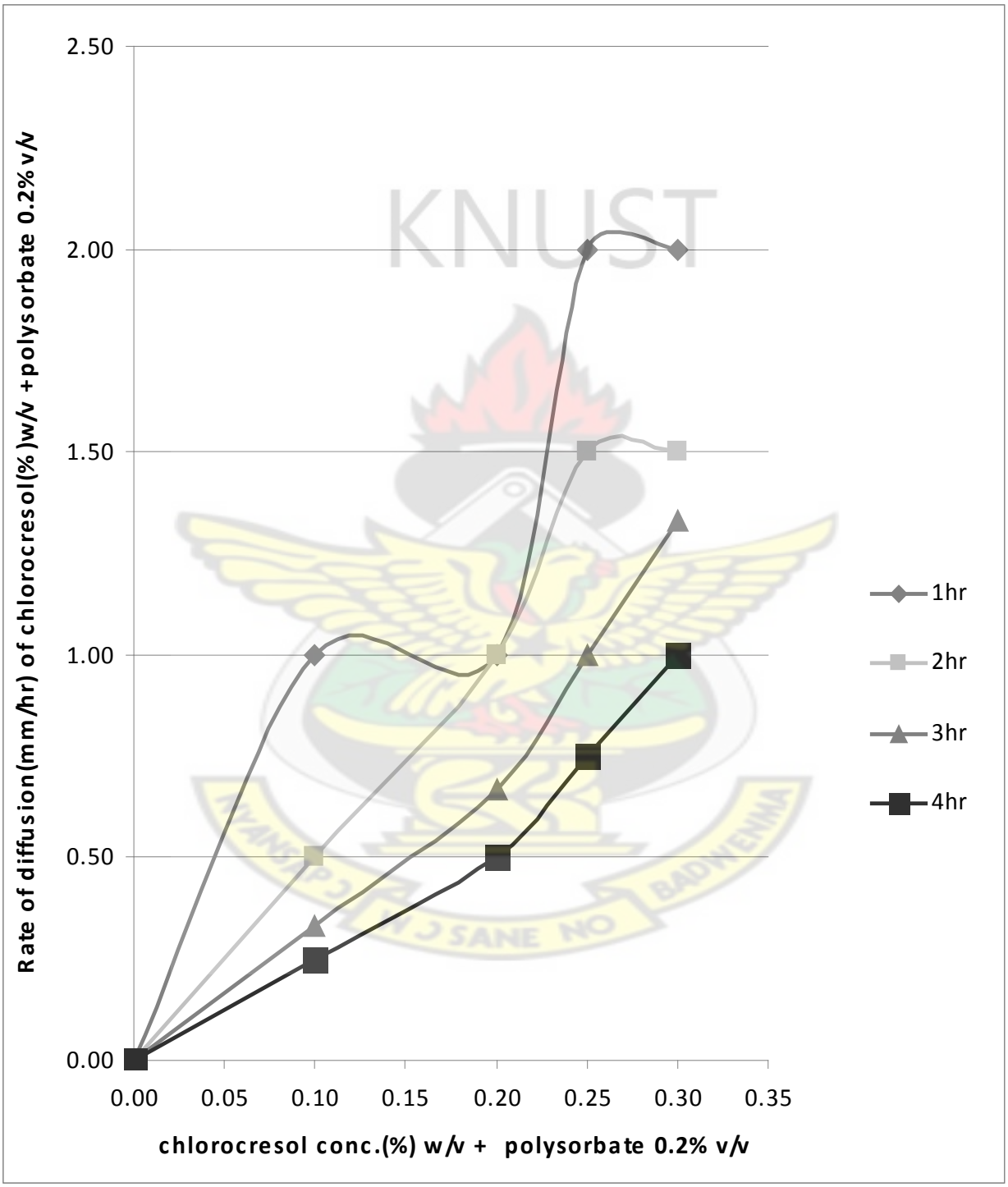

4.50
4.00
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
Distance diffused (mm) by chlorocresol + polysorbate 0.2% (v/v)
0.00
1.00
2.00
3.00
4.00
5.00
Time taken (hr)
0.10%
0.20%
0.25%
0.30%

FIGURE 13 Relationship between rates of diffusion of Chlorocresol (%) w/v + Polysorbate 0.2%v/v concentration



CHAPTER SIX

6.0 Discussion and conclusion

6.1 Discussion

Salicylic acid and chlorocresol have been used as antimicrobial agents in many pharmaceutical dosage forms. Both were found in this work to be effective against the indicator organisms. In low concentrations the activities were low but increased with increasing concentrations.

Relatively chlorocresol was found to be a more active bactericide than salicylic acid resulting in a relatively wider zone of inhibition being recorded for chlorocresol.

A concentration of 0.10% w/v salicylic acid showed inhibition against only *Escherichia coli* as shown in Table 5 and Figure 1 but the same concentration of chlorocresol exhibited inhibition against *E. coli*, *staph aureus* and *Ps aeruginosa* as shown in Table 6 and Fig 2. Thus, for a given concentration, chlorocresol portrayed a relatively wider spectrum of activity than salicylic acid.

Studies using nutrient agar medium as the challenging barriers showed that chlorocresol diffused faster through the agar than the salicylic acid (Table 13 Figure 8 and Table 14 Figure 9). For instance, the rate of diffusion (Table 15 Fig. 10) for chlorocresol was faster as against salicylic acid (Table 16.Fig. 11). The indication is that chlorocresol would reach and diffuse across the microbial cell membrane relatively faster than the salicylic acid.

The addition of polysorbate to the chlorocresol and salicylic acid systems was found to drastically reduce their activities as compared with their activities without the polysorbate. It is seen from Table 7 that addition of concentration range from 0.5% to 1.8%v/v polysorbate to 0.12% w/v salicylic acid abolished activity completely indicating there was not enough salicylic acid in the model system to act on the microorganisms leading to the increase in microbial growth.

Similarly, the same range of polysorbate concentration reduced activity of chlorocresol 0.2% w/v markedly as shown in Table 8 and Figure 3. There is therefore, a clear indication that the polysorbate micelles interacted with the antimicrobials affecting its activity. The polysorbate could be metabolized by the microorganisms or acted like a nutrient carrier between the aqueous phase and the cellular membrane of the microorganisms promoting its growth or extending microbial survival.

This behaviour can also be attributed to the polysorbate binding the surface of the preservative; therefore, an important part of the preservatives in the aqueous phase was entrapped in the polysorbate micelles and was not available to act on the organisms.

In relation to the decrease in the activity of preservatives due to the presence of a surfactant, Bean *et al* 1969 also reported that an addition of 0.4%v/v polysorbate to oil-water mixture containing 25% liquid paraffin and 0.1%w/v total chlorocresol reduced preservative activity one hundred-fold.

Appendix 1 figure 14 and Appendix 2 figure 15 are the standard graphs used as reference to the spectrophotometric analysis. Addition of varying polysorbate concentrations to 0.12% w/v salicylic acid saw the absorbance reducing from 1.1800A when there was no polysorbate to 0.9590A to 0.2176A when 0.50% to 2.0% v/v polysorbate respectively was added (Table 12).

These figures give an indication of a reduction in the total amount of the preservatives left in the model system after the addition of the polysorbate. The decrease in the Spectrophotometric absorbance induced by the presence of the polysorbate could be attributed to the partition of the preservatives between the polysorbate micelles and the water. This behaviour can be attributed to the polysorbate binding part of the chlorocresol and the salicylic acid leaving a small fraction free in the system to be read by the spectrophotometer.

The fraction of 0.12% w/v salicylic acid bonded to the polysorbate and fraction free is represented in Table 11 Figure 6, while the fractions of 0.15% v/v chlorocresol that is free and bond to the polysorbate are in Table 12 Figure 7. When the polysorbate level was 0.5% w/v 19.7% of the 0.12% salicylic acid was

bonded while 80.3% was free but 81.7% was bonded leaving 18.3% free when the polysorbate level was increased to 2.0%.
90.0% of the 0.15%w/v chlorocresol was bonded leaving 10% free when polysorbate level was increased to 2.5%.
This trend had been reported by Nedzicha *et al (*1991) for Tween 80 – sunflower oil-water system containing sorbic acid. In this case the amount of sorbic acid bonded to the micelles was 36% when the level of Tween 80 was 2% and increased to 51% when the level of Tween 80 was 4%.

6.2 Conclusion

Salicylic acid and chlorocresol have been used in many pharmaceutical dosage forms for topical and parenteral products as preservative or bactericides respectively (B.P, 2007). The current work confirmed both as effective and active antimicrobial agents against the test organisms. Low concentrations were characterised by low activities; with higher concentrations activity increased accordingly.

6.2.1 Relativity

For any specified concentration, chlorocresol quantitatively was found to be relatively more active than salicylic acid. Quantitatively, while salicylic acid (0.1%w/v) was active against only E. coli chlorocresol even below this was effective against all three organisms, namely, *Escherichia coli*, *Staphylococcus aureus* and to a lesser extent, *Pseudomonas aeruginosa* Table 5 Figure 1 for salicylic acid and Table 6 Figure 2 for chlorocresol. The work seemed to suggest that chlorocresol might have a relatively wider spectrum of antimicrobial activity than salicylic acid.

6.2.2 Mechanism

For any chemical to exert antimicrobial effect and cause damage it must penetrate into the cell structure-firstly, sensitive receptors on the lipoprotein

cell membrane as the first line of action, and then ultimately targets within the cytosol. The physicochemical reaction would be adsorption on membrane followed by diffusion through it to the cytosol.

6.2.3 Diffusion Rates

Simulated studies carried out on diffusion of the bactericides through nutrient broth/cell interface showed there was a much higher diffusion rate for chlorocresol Table as shown 15 Figure 10 than for salicylic acid Table 16 Figure 11. This suggests therefore that chlorocresol is likely to reach out from solution, adsorb and penetrate a cell causing cell injury and death more readily than salicylic acid.

6.2.4 Solubilisation

Both salicylic acid and chlorocresol are phenolic compounds but with very poor solubilities in water of 0.20% salicylic acid and 0.3% for chlorocresol (B.P 2007).

Pharmaceutical formulations often employ additives or solubilising agents to improve solubility of the biologically active ingredients, one of such being polysorbate 80 (Tween 80). Muse (2005)

Incorporation of the polysorbate in the bactericidal systems had a dramatic adverse effect. A range of 0.5-1.5%w/v polysorbate added to fixed amount of chlorocresol (0.2%) drastically diminished its activity (Table 8 Figure 3) but for salicylic acid (0.12%) it abolished activity completely (Table 7).

A plausible cause of the decline might be that polysorbate forms a layer of protective coat surrounding the cells and thus limits normal bactericide-bacteria contact and adsorption as an earlier work by Bean *et al* 1969 of studies on oil-water-bacteria emulsified system with polysorbate as the emulsifier also recorded a decline in activity. The work further showed that the added bactericide portioned itself between the three phases of the system thus causing a reduced amount of it in the water phase for antimicrobial activity. Subsequent investigations in this current work demonstrated that in tandem

with increases in polysorbate, chlorocresol and salicylic acid free and detectable using UV Spectrophotometric analysis (Table 11 Figure 6 for salicylic acid and Table 12 Figure 7 for chlorocresol) persistently diminished an observation that accords with the observed in biological effect as recorded earlier (Table 8 Figure 3 for chlorocresol and Table 7 for salicylic acid) and available for hitting appropriate targets on the cells.

Account should be taken into the antimicrobial that would compensate for losses to other phases other than the aqueous. The surfactant therefore is dependent upon the antimicrobial and amount of additives, this is because the additives may protect cells from contact with bactericide or bind/absorb a proportion of the antimicrobial.

6.2.5 Recommendations

- To assess the bactericide required for any Pharmaceutical formulation, some preliminary evaluations need be undertaken.
- Manufacturers should take into account bactericide that would compensate for losses to other phases other than the aqueous.

CHAPTER SEVEN

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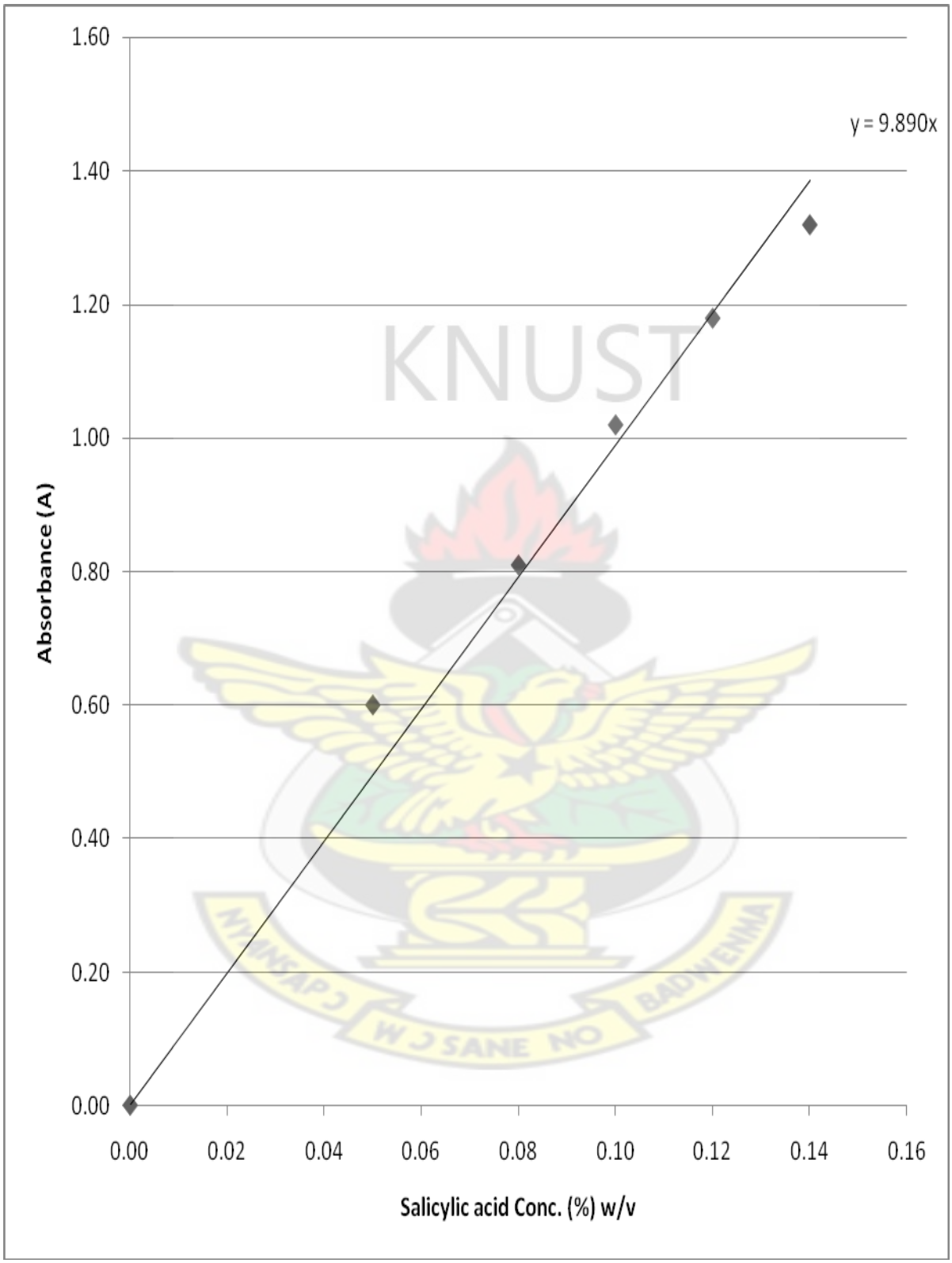
APPENDIX 1

TABLE 19 Relationship between salicylic acid concentration and absorbance

Salicylic Acid Conc. (%) w/v	Aborbance (A)
0.00	0.000
0.05	0.600
0.08	0.820
0.10	1.040
0.12	1.180
0.14	1.300

FIGURE 14 Standard graph of salicylic acid by UV Spectrophotometric analysis at 311.5nm

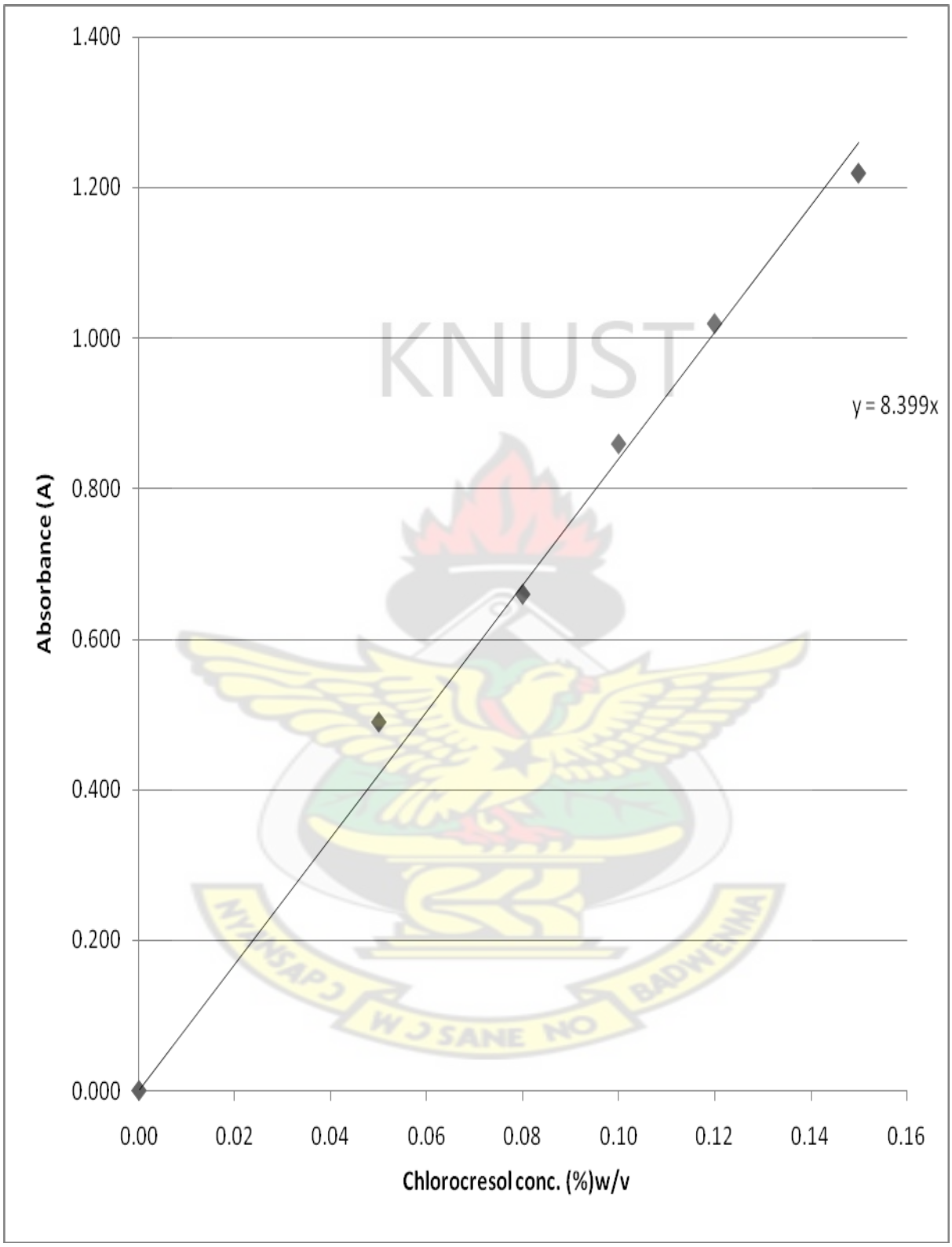

y = 9.890x
Absorbance (A)
Salicylic acid Conc. (%) w/v
1.60
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
0.00
0.02
0.04
0.06
0.08
0.10
0.12
0.14
0.16

APPENDIX 2

Table 20 Relationships between chlorocresol concentration and absorbance

Chlorocresol concentration (%)w/v	Absorbance (A)
0.00	0.000
0.05	0.490
0.08	0.660
0.10	0.860
0.12	1.020
0.15	1.220

FIGURE 15 Standard graph of salicylic acid by UV Spectrophotometric analysis at 293nm.


1.400
1.200
1.000
0.800
0.600
0.400
0.200
0.000
Absorbance (A)
y = 8.399x
0.00
0.02
0.04
0.06
0.08
0.10
0.12
0.14
0.16
Chlorocresol conc. (%)w/v

APPENDIX 3

Table 21 Preparation of Admixture to determine the effect of Polysorbate on salicylic acid

Vol. of stock of salicylic acid. (0.2%)	6mml	6mml	6mml	6ml
Vol. of stock of Polysorbate. (5%)	1ml	2ml	3ml	4ml
Vol. of distilled water	3ml	2ml	1ml	0ml
Final conc. Of salicylic acid	0.12%	0.12%	0.12%	0.12%
Final conc. Of Polysorbate	0.50%	1.00%	1.50%	2.00%
Final vol. of solution	10ml	10ml	10ml	10ml

Table 22 Preparation of Admixture to determine the effect of Polysorbate on chlorocresol

Vol. of stock of chlorocresol. (0.3%)	5ml	5ml	5ml	5ml	5ml
Vol. of stock of Polysorbate. (5%)	1ml	2ml	3ml	4ml	5ml
Vol. of distilled water	4ml	3ml	2ml	1ml	0ml
Final conc. Of chlorocresol	0.15%	0.15%	0.15%	0.15%	0.15%
Final conc. Of Polysorbate	0.50%	1.0%	1.5%	2.0%	2.5%
Final vol. of solution	10ml	10ml	10ml	10ml	10ml

APPENDIX 4

Table 23 Preparation of Admixture to determine the effect of Polysorbate on Chlorocresol diffusion

Vol. of chlorocresol stock. (0.3%)	3.3mml	6.7mml	8.3mml
Vol. of Polysorbate stock. (5%)	1ml	1ml	1ml
Vol. of distilled water	5.7ml	2.3ml	0.7ml
Final conc. Of chlorocresol	0.10%	0.20%	0.25%
Final conc. Of Polysorbate	0.50%	0.50%	0.50%
Final vol. of solution	10ml	10ml	10ml