The Development of *Phyllanthus emblica* Extract-Loaded BSA Nanoparticles Production to Improve Bioavailability

By
Ms. Nwe Nwe Win
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A special project summited to the Faculty of Biotechnology, Assumption University in part of fulfillment of the Requirements for the Degree of Bachelor of Science in Biotechnology

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Title	:	The Development of *Phyllantus emblica* Extract-Loaded BSA Nanoparticles Production to Improve Bioavailability
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Ms. Nwe Nwe Win

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Abstract

Phyllanthus emblica or Indian gooseberry is a traditional and medicinal plant that can be commonly found in Asia and Southeast Asia. It has been widely used to cure various disease especially in India as an Ayurvedic medicine. It is reported to possess free radical scavenging, antioxidant, anti-inflammatory, antimicrobial, and anti-mutagenic. The preliminary aim of this research is to develop *Phyllanthus emblica* extract-loaded BSA nanoparticles (PBNP) production to improve bioavailability. PBNP was prepared by using desolvation method with three different ratio of *Phyllanthus emblica* 95% ethanol crude extract: BSA (1:2, 1:3, 1:4). The releasing behavior of crude extract of *P.emblica* using 95% ethanol and PBNP will be invested by performing in-vitro simulated gastrointestinal digestive system. The antioxidant activity of in-vitrosimulated gastrointestinal digestive systemwill be determined by DPPH radical scavenging activity.The result showed that the releasing behavior of PBNP tended to be higher at low pH condition in artificial gastrointestinal digestive system. However, *Phyllanthus emblica* 95% ethanol crude extract was more stable than PBNP. Antimicrobial activity is determined by using well agar diffusion method to evaluate the effectiveness of inhibition of PBNP with three different ratio between *P.emblica* 95% crude extract:BSA (1:2, 1:3, 1:4) and *P.emblica* 95% ethanol crude extractagainst seven food borne pathogens *Salmonella enterica* Typhimurium U302 (DT104b), *Salmonella enteric* Enteritidis (human), *Salmonella enterica* 4,5,12:i:-(human) US clone, *Bacillus cereus*, *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichai coli* ATCC25822. The results prove that PBNP using three different ratio (1:2,1:3,1:4) and three concentration (100,200,300µg/ml) had no significantly different inhibition against the growth of seven food borne pathogens ($p>0.05$). bacteria.

Keywords: Phyllanthus emblica, Nanoparticles, bioavailability, antioxidant, antimicrobial.

Contents

Acknowledgement i

Abstract ii

List of Figures v

Introduction 1

Objectives 2

Literature Review 3

Antioxidant Activity 4

Phytochemical Compound of *Phyllantus emblica* (Tannins) 5

Antimicrobial activity 5

Design of nanotechnology-based drug delivery system 6

Materials and methods 7

Sample preparation 7

Preparation of *P. emblica* Crude 95% Ethanol Extract 7

Preparation of *P. emblica* Crude Extract-Loaded BSA Nanoparticles 7

In-vitro Simulated Gastrointestinal Digestion (SGS) Release of *P. emblica* Crude Extract-Loaded BSA Nanoparticles (PBNP) and *P.emblica* 95% Crude Extract 8

Antioxidant activity of In-vitro SGS Release by DPPH radical scavenging activity 8

Antimicrobial activity 9

Statistical analysis and Experimental Design 9

Result and Discussion 10

Antioxidant activity of In-Vitro Simulated Gastrointestinal Digestive System or SGS 10

Antimicrobial activity 12

Conclusion 14

Reference 15

Appendix 19

Appendix A 19

Appendix B 22

Appendix C 26

List of Tables

Table 1: The Percentage of DPPH reduction of PBNP and P.emblica 95% ethanol crude extract in SGS. 11
Table 2: The outcome of DPPH as absorbance 517nm at pH6.9 in in-vitro SGS release 23
Table 3: The outcome of DPPH as absorbance 517nm at pH5.5 in-vitro SGS release 23
Table 4: The outcome of DPPH as absorbance 517nm at pH4.6 in-vitro SGS release 23
Table 5: The outcome of DPPH as absorbance 517nm at pH2.8 in-vitro SGS release 24
Table 6: The outcome of DPPH as absorbance 517nm at pH3.8 in-vitro SGS release 24
Table 7: The outcome of DPPH as absorbance 517nm at pH2.3 in-vitro SGS release 24
Table 8: The outcome of DPPH as absorbance 517nm at pH2.0 in-vitro SGS release 25
Table 9: The outcome of DPPH as absorbance 517nm at pH5.0 in-vitro SGS release 25
Table 10: The outcome of DPPH as absorbance 517nm at pH6.5 in-vitro SGS release 25

List of Figures

Figure 1: The fruit of Phyllantus emblica. 3
Figure 2: Active compounds of Phyllantus emblica. 5
Figure 3: The release of PBNP in in-vitro simulated gastrointestinal digestive system at 37°C over the period of 3.6 hour. 10

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Introduction

Indian gooseberry (*Phyllanthus emblica*) is a natural herb that is commonly found in India, the Middle East and some Southeast Asian countries. It belongs to the family Euphorbiacea[10].It has been used for thousands of years in India as an Ayurvedic medicine which is an ancient medical system. In the present days, it is used for treating eye problems, bloody diarrhea, high cholesterol, joint pain, cancer, stomach protective and obesity[1]. BSA is a natural protein able to form complexes in various shapes. This protein is biocompatible, biodegradable, nontoxic, and nonimmunogenic2. It can be obtained from a variety of sources including egg white (ovalbumin), bovine serum albumin (BSA)[3]. Albumin can be soluble in water and diluted in salt solution really well[5].

P. emblica pulp is particularly well known for its bioactive compound and its rich vitamin C content. The present work is to understand the antioxidant activity in in-vitro simulated gastrointestinal digestion system of PBNP and *P.emblica* 95% ethanol crude extract.To the best of current available knowledge, there have been no reports of local delivery of *P.emblica*-loaded albumin particles into the digestive system. Some study shows that the fruit of *P. emblica* has a potential against gram positive bacteria especially *Staphylococcus aureus*[16].Gallic acid and tannic acid are the major phytochemical compounds of *P.emblica*, that responsible for antimicrobial activity.

The development of *P. emblica* extract-loaded BSA nanoparticles (PBNP) would be a guideline to show that it could increase the bioavailability of chemical complexity of *P.emblica*.

Objectives

- To study the development of *P.emblica* extract-loaded BSA nanoparticles production to improve bioavailabilty.
- To determine the antioxidant activity of in-vitro simulated gastrointestinal digestion release of *P. emblica* crude extract-loaded BSA nanoparticles (PBNP).
- To study the antimicrobial activity of *P.emblica* extract-loaded BSA nanoparticles with different ratio between *P.emblica* 95% crude extract to BSA and *P.emblica* 95% crude extract and different concentrations.

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Literature Review

The findings of antioxidant and antimicrobial agents have drawn an enormous attention from the public and an extensive effort has been put into the studies to identify compounds that can act as antioxidant and antimicrobial agents to replace synthetic ones (Antara Sen and Amla Batra et al.,2012).,*Phyllanthus emblica* or Indian gooseberry, falls in the family of Euphorbiaceae , is a traditional medicine and its fruit is widely found and used in India and Southeast Asian countries. The fruit of *P.emblica* is globose, fleshy pale yellow with six obscure vertical furrows enclosing six trigonous seeds in two seeded three crustaceous cocci (Swetha Dasaroju et al., 2015).

Figure 1: The fruit of Phyllantusemblica.

The fruit of *P.emblica* has phytochemical compounds, including tannin, alkaloids, polyphenols, vitamins, and minerals. Gallic acid ellagic acid, emblicanin A and B, phyllembein, quercetin and ascorbic acid are found to be biologically active (Sabahat Saeed and Perween Tariq et al.,2007). Its fruit juice contains the highest concentration of vitamin -C (478.56mg/100mL) (Swetha Dasaroju et al., 2015). Its vitamin-C content level is more than those oranges, lemons and tangerines (Jain SK. et al., 2004;Scartezzini P. et al., 2006).Among many compounds, tannin, emblicanin A and B, and ascorbic acid have biochemical abilities to stop free

radical's activity which causes cancer and ageing. Moreover, emblicanin A and B are found to be actively inhibit the growth of a wide range of bacteria including *Staphylococcus aureus, Escherichia coli, Mycobacterium tuberculosis, S.typhosa* and *Candida albicans* (Swetha Dasaroju and Krishna Mohan Gottumukkala et al.,2006).The fruits and fruit extracts of *P.emblica* have proven protective effects on organs/tissues that caused from damages induced by chemicals, stresses, and aging in animal models. The fruits and fruit extracts have potential in inhibiting the growth of cancer cells and reducing DNA damage when exposed to harmful chemicals and radiation. Antioxidative activities are likely among the mechanisms of the biological activities and physiological effects (Yang B. et al., 2014)

Antioxidant Activity

P.emblica pulp's extraction has a high ascorbic acid content and some scientists believe that it is the main antioxidant substance that stops the activity of free radicals. Free radicals are the causes of cancer cells formation which make a high number of human lost their lives. However, there is a study reports that the strong antioxidant properties of Indian gooseberry fruit's extraction are not resulted from conjugated form of ascorbic acid but from two hydrolysable tannins which are emblicanin A (37%) and emblicanin B (33%) (Ghosal et. al., 1996). The hydrolysis of either one of emblicanins gives gallic acid, ellagic acid and glucose whereas the another hydrolyzed one gives ellagic acid and glucose (Kamal Rai Aneja, Radhika Joshi and Chetan Sharma et al.,2010). In addition, emblicanin A and B are potential substances for hepato-, cyto, and radio- protection stress (Juree Charoenteeraboon.et al., 2010). Another paper has indicated that emblicanin A and B has not only radical scavenging activity, but also an effective inhibitor against H_2O_2-induced cellular oxidative stress (Juree Charoenteeraboon, et al., 2010).

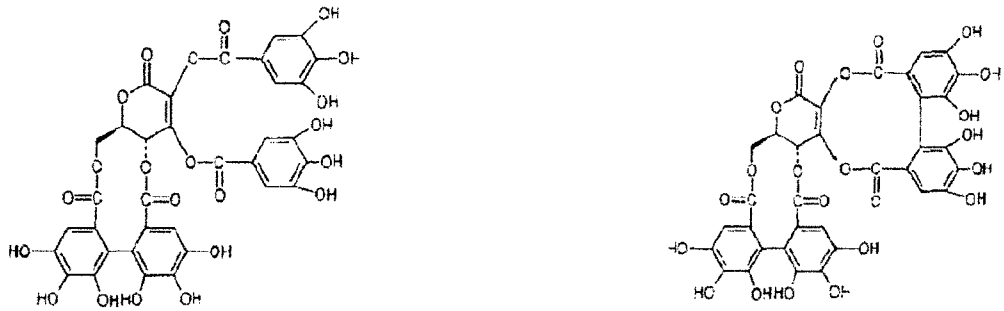

Figure 2: Active compounds of Phyllantusemblica.

Phytochemical Compound of *Phyllantus emblica* (Tannins)

Tannins were classified as another large group of phenolics that available in *P.emblica* and its constituents. These are generally subdivided into hydrolyzable and condensed tannins. Hydrolyzable tannins is composed with a central core of polyhydric alcohol such as glucose and hydroxyl groups, which are esterified either in small portion or the entire structure by gallotannins, ellagitannins.The tannins contain phenolic carboxylic acids such as gallic acid, ellagic acid, chebulic acid and gallotannins. Ellagitannins such as punacalagin, casurarinin, corilagin, terchebulin, chebulanin,neochebulinic acid, chebulagic acid and chebulinic acid have been reported in literature as antimicrobials(Kannan P . et al., 2009)

Antimicrobial activity

One study has suggested that antimicrobial properties of Indian gooseberry have high activity against the growth of *Staphylococcus aureus, Escherichia coli* and *S. haemolyticus*. The main reason behind the inhibition of bacteria growth is the presence of emblicanin A and B in Indian gooseberry (Kamal Rai Aneja, Radhika

Joshi and Chetan Sharma et al.,2010). These hydrolysable tannins (emblicanin A and B) stop bacteria from reproduction by invading a thick layer of peptidoglycan which is cell wall that surrounds inner parts of bacteria. Another possible reason for inhibition of bacteria growth is the effect of gallic acid and ellagic acid components that resulted from hydrolysis of emblicanin A and B.

Design of nanotechnology-based drug delivery system

Nanoparticles are used as a drug-delivery vehicles are generally in the size of less than100nm in at least one dimension, and consists variety of biodegradable materials such as natural or synthetic polymer, lipids, or metals. Nanoparticles are taken up by cells more efficiently than larger micromolecules and thus, it could be used as an efficient transport and delivery system. The aim of applying nanotechnology to plant extracts has been widely studied and used. It is because nanostructure systems could increase the power of plant extract's action. It releases bioactive compounds very sustainably, decreases the required dose of plant extracts and improve bioactivity (Ghosh et al., 2013; Rajendran et al., 2013)

Bovine Serum albumin (BSA) is a source for albumin protein and it is very versatile protein carrier for drug targeting. Albumin is the most abundant plasma protein with a molecular weight of 66.5 kDa (Kratz F. et al., 2008). It is acidic, very soluble protein and extremely robust. It is soluble in the range of pH 4-9, soluble in 40% ethanol and can be heated up to 60 °C without denaturing it.

Therefore, this study would be a possible alternative solution to improve bioavailability of *P.emblica*bioactive compound.

Materials and methods

Sample preparation

P. emblica was purchased approximately 4kg from local markets in Yangon, Myanmar and imported to Bangkok. Fresh *P. emblica* was washed with tap water. The fruit part of *P. emblica* was used and seeds were removed. It was chopped into small pieces for using.

Preparation of *P. emblica* Crude 95% Ethanol Extract

P. emblica was extracted with 95% ethanol by using 1:10 ratio (g/ml). *P.emblica* was soaked in 95% ethanol at room temperature(25°C) for 48 hours by using orbital shaker with the speed of 120rpm and then it was filtered using whatman filter paper no.4. The crude extract was concentrated using rotary evaporators at 45°C (BUCHI Rotavapor R-205) are then it was kept at -20°Cbefore using for preparation of *P. emblica* extract-loaded BSA.

Preparation of *P. emblica* Crude Extract-Loaded BSA Nanoparticles

PBNP is prepared by the desolvation method[2]. The 100 mg of BSA was dissolved in 1 ml of sodium chloride solution (10mM). Then, 4.0 ml of ethanol was added dropwise into the BSA solution with magnetic stirring (400 rpm) at room temperature. Additionally, the prepared BSA nanoparticles were formed cross-link with 0.2% glutaraldehyde (GA). Then, *P.emblica* crude ethanol extract was added into the solution and the solution was stirred for 24 hours at different ratio of *P. emblica* to BSA (1:2, 1:3, and 1:4) in the preparation of PBNP. The particles were centrifuged and washed with distilled water. The centrifuged particles were

resuspended and disperse in 2% mannitol, then freeze-dried for 24 hours. The dried nanopowder was kept at room temperature before using.

In-vitro Simulated Gastrointestinal Digestion (SGS) Release of *P. emblica* Crude Extract-Loaded BSA Nanoparticles (PBNP) and *P.emblica* 95% Crude Extract

To perform in-vitro simulated gastrointestinal digestion in the lab, in artificial mouth section, *P. emblica* crude 95% ethanol extract and PBNP are dissolved with distilled water and adjust pH to reach 6.9 by using 1mmol $NaHCO_3$. Then add mimicked saliva solution (α-amylase from *Apergillusoryzae* and 1mmol of $CaCl_2$) and shake it with shaking incubator at 200rpm for 2 minutes. The mixture is added with pepsin solution and adjust pH with 1mmol of HCl to reach 2.0 to imitate the environment in the stomach then shake at 130rpm. In artificial duodenum section, pancreatic and bile salt solution are added to the mixture and adjust pH to reach 5.0, shake for 20 minutes at 45rpm. Lastly, pH of the mixture is adjusted to slightly alkali condition (pH 6.5) in ileum section and shake for 90 minutes at 45rpm.

Antioxidant activity of In-vitro SGS Release by DPPH radical scavenging activity

The modified DPPH radical scavenging activity[6] was used for percentage DPPH radical scavenging determination. The samples from in-vitro simulated gastrointestinal digestion of *P. emblica* crude 95 % ethanol extract and PBNP are dissolved with methanol. 200 µl of sample is mixed with 1.8ml of DPPH solution. The mixture is shaken vigorously and stand in the dark condition for 30 minutes at room temperature. After 30 minutes, the optical density of the mixture is measured at 517nm. The results were interpreted as percentage reduction.

$$\textbf{Percentage reduction of DPPH} = \frac{(A_o - A_c)}{A_o} \; x \; 100$$

Where A_0 is the initial absorbance and A_c is the value for added sample concentration (c). All measurements three replications individually.

Antimicrobial activity

The modified agar well diffusion is used to perform antimicrobial activity[4]. The 100µl of pathogens (predictably 1.5 x10^8 CFU/ml) is swabbed on Mueller-Hinton agar(MHA) plate. The 100µl of *P.emblica* crude 95% ethanol extract and PBNP diluted with sterilized distilled water at three different concentration of 100, 200, and 300 µg/ml were used to test the antimicrobial activity against seven food borne pathogens ,namely, *Salmonella enterica* Typhimurium U302 (DT104b), *Salmonella enteric* Enteritidis (human), *Salmonella enterica* 4,5,12:i:-(human) US clone, *Bacillus cereus*, *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichai coli* ATCC25822. The 100 µl of penicillin G was as positive control. The inhibition zones were measured in centimeter to determine the effectiveness of antimicrobial activity of *P.emblica* 95% ethanol crude extract and PBNP against each pathogen. The experiment was done in duplicate and three replications independently.

Statistical analysis and Experimental Design

All experiments were conducted in three replications and statistical analysis was completed using ANOVA with Duncan's multiple range tests ($p<0.05$) by SAS software version 9.3

Result and Discussion

Antioxidant activity of In-Vitro Simulated Gastrointestinal Digestive System or SGS

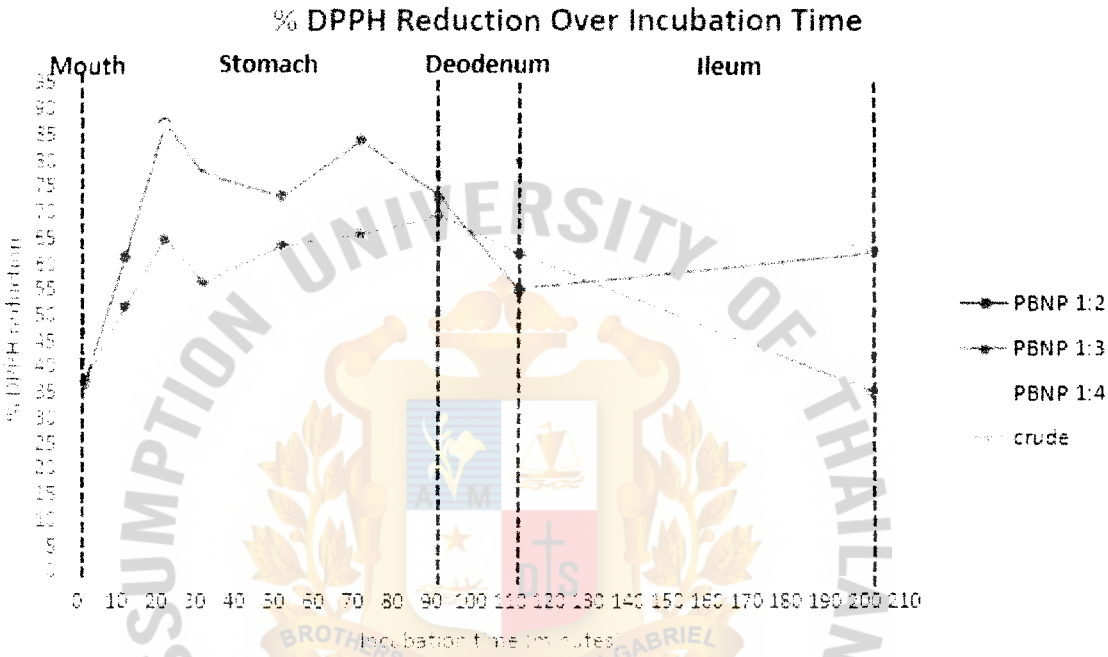

Figure 3: The release of PBNP in in-vitro simulated gastrointestinal digestive system at 37°C over the period of 3.6 hour.

The cumulative percentage of released *P. emblica* is shown in figure 1 .According to the figure1, it can be clearly observed that the trend lines of *P. emblica* crude 95% ethanol extract and PBNPs peaked in different levels after 22 minutes of incubation time. Once the condition reached pH4.6, which was artificial environment in stomach, the major parts of BSA have been digested by protease and unfold that resulted in releasing of bioactive active compounds of *P. emblica.* The majority of bioactive compounds of *P.emblica* are hydrolysable tannins, emblicanin A and B, and ascorbic acid[7]. Figure 1 showed that the release of bioactive compounds tended to be higher in stomach than in any other section in digestive system. However, the trendlines of *P. emblica* crude 95% ethanol extract and PBNPs fluctuated for several times before reaching the artificial

environment in duodenum. When it reached pH 5.0, only *P. emblica* crude 95% ethanol extract showed the activity of DPPH radical scavenging. At this stage, the proteins of PBNPs' particles were likely to fold back and stopped the releasing of bioactive compounds. BSA are most stable at pH 7 but rapidly degraded by aggregation and hydrolyzed at pH 2[8]. Only PBNP using ratio 1:2 released highest in artificial environment in ileum compared PBNP using ration 1:3, 1:4 and crude extract. To summarize the result of the graph, *P. emblica* crude 95% ethanol extract was likely to be more stable than PBNP even though it was exposed to the pH changes in the artificial physiological conditions of in-vitro simulated gastrointestinal digestive system. It might possible that the tannins (emblicanin A and B) are important bioactive compounds in*P. emblica* are highly stable and active.

Table 1: The Percentage of DPPH reduction of PBNP and P.emblica 95% ethanol crude extract in SGS.

SGS sections	pH	PBNP 1:2	PBNP 1:3	PBNP 1:4	Crude Extract
Mouth	6.9	$37.05\pm9.36^{D,a}$	$37.28\pm1.19^{BC,a}$	$41.74\pm1.57^{AB,a}$	$47.70\pm3.52^{B,a}$
Esophagus	5.5	$61.52\pm6.16^{BC,b}$	$51.88\pm9.6^{ABC,bc}$	$38.93\pm5.25^{B,c}$	$86.4\pm2.34^{A,a}$
	4.6	$87.65\pm4.22^{A,a}$	$65.20\pm3.62^{A,b}$	$53.35\pm4.81^{AB,b}$	$86.87\pm5.66^{A,a}$
	3.8	$77.59\pm3.14^{AB,a}$	$56.61\pm11.03^{ABC,ab}$	$36.09\pm3.41^{B,b}$	$76.77\pm14.66^{A,a}$
	2.8	$73.46\pm2.44^{ABC,a}$	$64\pm8.44^{A,a}$	$42.1\pm6.91^{AB,a}$	$83.43\pm4.80^{A,b}$
	2.3	$84.39\pm1.13^{A,a}$	$66.12\pm11.18^{A,a}$	$64.05\pm13.33^{A,a}$	$80.72\pm6.25^{A,a}$
	2.0	$73.14\pm2.07^{ABC,a}$	$69.7\pm1.79^{A,a}$	$50.51\pm9.05^{AB,b}$	$77.82\pm4.24^{A,a}$
Deodenum	5.0	$55.46\pm7.60^{C,ab}$	$62.35\pm9.34^{AB,ab}$	$49.17\pm5.59^{AB,b}$	$80.76\pm10.42^{A,a}$
Ileum	6.5	$62.49\pm8.12^{BC,a}$	$35.35\pm7.29^{C,a}$	$42.75\pm4.33^{AB,a}$	$62.86\pm15.15^{AB,a}$

Note: Different capital letters (A,B,C) show significantly different in column ($p<0.05$).Different small letters (a,b,c) showed significantly different in row ($p<0.05$).

Antimicrobial activity

PBNP was prepared by a desolvation method[2].The antimicrobial activity of PBNP and *P.emblica*95% ethanol crude extractwas evaluated by using well agar diffusion method. The results of PBNP and *P.emblica* 95% ethanol crude extract were interpreted by using Randomized Complete Block Design (RCBD) with Duncan's multiple range tests in SAS program version 9.3 as shown in Table 1.

It has been observed that CBNP and *P. emblica* crude extract have higher effectiveness on inhibiting the growth of gram positive bacteria,(*Bacillus cereus* and *Bacillus subtilis)*than gram negative bacteria, (*Salmonella enterica* Typhimurium U302 (DT104b), *Salmonella enteric* Enteritidis (human), *Salmonella enterica* 4,5,12:i:-(human) US clone, *Escherichai coli* ATCC25822).The highest antimicrobial activity of PBNP was 0.50±00 cm in diameter.But, the antimicrobial activity of PBNP between three different ratio of *P.emblica* 95% ethanol crude extract:BSA (1:2, 1:3, 1:4) with three concentrations showed no significant difference from each other on inhibition of seven food borne pathogens. Bovine serum albumin (BSA) of PBNP is a protein that can withdraw macromolecular and serves as a carrier for the variety of active compound molecules[14] and can readily bind and release small molecules[14]. In addition, *P.emblica* 95% ethanol crude extracthas greater effectiveness of inhibiting against gram positive bacteria than PBNP. *P.emblica* 95% ethanol crude extract is obtained by 95% and macerated at room temperature (24°C), 200rpm for 48 hours using shaking incubator. Ethanol is a very polar organic solvent and it is possible that it attracts polar side of bioactive compounds of *P.emblica*. Another possible reason for the antibacterial activity of PBNP and *P.emblica* crude extract might be due to the tannins present in its fruits. The fruits have 28% of the total tannins distributed in the whole plant. The fruit contains two hydrolysable tannins Emblicanin A and B, which has antioxidant activity, one on hydrolysis contributes to giving gallic acid, ellagic acid and glucose wherein the other gives ellagic acid and glucose. The fruit also contains Phyllemblin(Wealth of Asia, 1998; Ghosal, 1996; Dictionary of Indian Medicinal Plants, 1988).Therefore, it probably indicated that polar side of bioactive compounds and emblicanin A and B have the higher chance to permeate through the thick layer of peptidoglycan in bacterial cell wall.

Microorganisms	Inhibition zone of PBNP with different ratio between crude:BSA and crude extract (cm)											
	1:2			1:3			1:4			Crude extract		
	100 µg/ml	200 µg/ml	300 µg/ml	100 µg/ml	200 µg/ml	300 µg/ml	100 µg/ml	200 µg/ml	300 µg/ml	100 µg/ml	200 µg/ml	300 µg/ml
***Salmonella enterica*Typhimurium**	0.37± 0.19^{A,,a}	0.42± 0.12^{A,ab}	0.42± 0.12^{A,a}	0.43± 0.12^{A,a}	0.49± 0.03^{A,,a}	0.50± 0.00^{A,,a}	0.41± 0.14^{A,,a}	0.49± 0.03^{A,a}	0.44± 0.07^{A,a}	0.50± 0.00^{A,a}	0.37± 0.20^{A,a}	0.45± 0.09^{A,a}
***Salmonella enterica*Enteritidis(human)**	0.33± 0.20^{A,,a}	0.41± 0.15^{A,ab}	0.31± 0.19^{A,a}	0.46± 0.25^{A,a}	0.46± 0.09^{A,,a}	0.49± 0.03^{A,,a}	0.34± 0.17^{A,,a}	0.38± 0.19^{A,ab}	0.31± 0.19^{A,a}	0.30± 0.25^{A,b}	0.42± 0.19^{A,a}	0.33± 0.26^{A,a}
***Salmonella enterica* 4,5,12:i:-(human) US clone**	0.43± 0.12^{AB,a}	0.37± 0.21^{AB,ab}	0.29± 0.22^{B,a}	0.37± 0.12^{AB,a}	0.48± 0.03^{A,,a}	0.50± 0.00^{A,a}	0.47± 0.07^{A,,a}	0.42± 0.20^{AB,ab}	0.34± 0.19^{AB,a}	0.50± 0.00^{A,a}	0.50± 0.00^{A,a}	0.45± 0.08^{AB,a}
***Escherichai coli* ATCC25822**	0.29± 0.16^{A,,a}	0.25± 0.23^{A,bc}	0.33± 0.23^{A,,a}	0.30± 0.25^{A,a}	0.27± 0.26^{A,,b}	0.23± 0.14^{A,,b}	0.31± 0.20^{A,a}	0.42± 0.20^{A,,ab}	0.37± 0.21^{A,a}	0.42± 0.12^{A,a}	0.40± 0.20^{A,a}	0.33± 0.26^{A,a}
Bacillus cereus	0.47± 0.07^{A,,a}	0.50± 0.01^{A,a}	0.48± 0.05^{A,,a}	0.46± 0.08^{A,a}	0.50± 0.00^{A,,a}	0.49± 0.03^{A,a}	0.49± 0.03^{A,a}	0.5± 0.00^{A,a}	0.46± 0.07^{A,a}	0.50± 0.00^{A,a}	0.50± 0.00^{A,a}	0.50± 0.00^{A,a}
Bacillus subtilis	0.45± 0.08^{A,,a}	0.40± 0.20^{A,ab}	0.47± 0.14^{A,,a}	0.37± 0.21^{A,a}	0.41± 0.20^{A,,ab}	0.50± 0.00^{A,,a}	0.42± 0.20^{A,a}	0.42± 0.20^{A,ab}	0.32± 0.25^{A,a}	0.50± 0.00^{A,a}	0.50± 0.00^{A,a}	0.43± 0.18^{A,a}
Staphylococcus aureus	0.30± 0.22^{AB,a}	0.14± 0.21^{B,c}	0.36± 0.20^{AB,a}	0.34± 0.21^{AB,a}	0.34± 0.20^{AB,ab}	0.37± 0.22^{AB,a}	0.38± 0.09^{AB,a}	0.27± 0.23^{AB,b}	0.33± 0.22^{AB,a}	0.43± 0.10^{A,a}	0.50± 0.00^{A,a}	0.43± 0.16^{A,a}

Note: Different capital letters (A,B,C) within a row showed significantly different (P<0.05). Different small letters (a,b,c) with a column showed significantly different (p<0.05)

Conclusion

The study shows that the release of PBNP is affect by the acidic conditions in-vitro gastrointestinal digestive system over the period of incubation time. Moreover, BSA is rapidly degraded by aggregation and hydrolysis at pH 2[15].The transitional conformation of BSA begins at the acidic condition[9] and the structure will recovery when it reaches alkali condition. Moreover, *P.emblica* 95% ethanol crude extract has higher stability than PBNP when it was exposed to different changes of pH in in-vitro simulated gastrointestinal digestive system. Antimicrobial activity testing showed that PBNP and *P.emblica*95% ethanol crude extract have higher effective inhibition against gram positive bacteria than gram negative bacteria. However, three ratios of PBNP between *P.emblica*95% ethanol crude extract:BSA with three different concentrations did not show significantly different from each other.

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Appendix

Appendix A

Solution preparation

DPPH (50µM)

DPPH	0.0197g
Distilled water	1L

Preparation: Weight 0.0197 g of DPPH and add up distilled water to 1L in volumetric flask.

Diluted NaOH solution (0.1M)

NaOH	0.4000g
Distilled water	100ml

Preparation: Weight 0.4000 g of NaOH solid and add up distilled water to 100ml in volumetric flask.

Diluted HCl solution (0.1M)

1M of HCl	10ml
Distilled water	100ml

Preparation: Pour distilled water up to 100ml in cylinder. Transfer 100ml of distilled water into 250mL of Erlenmeyer flask and add 10ml of 1M of HCl.

Media Composition and Preparation

1. Mueller Hinton Broth

Composition per liter:

Beef Infusion form	3000.0g
Casien hydrolysate (acid)	17.5g
Starch	1.5g
Distilled water	1L

pH7.4±0.2 at 25°C

Source: Medium is available as a premixed powder from Titan Biotech Ltd.

Preparation: Dissolve 21 grams of premixed medium powder in 1000ml of distilled water. Gently heat if required to dissolve medium completely. Distribute into tube or flask and sterilize by autoclaving at 15 psi (121°C) for 15 minutes. Cool to room temperature prior to dispense.

2. Mueller Hinton Agar

Composition per liter:

Beef Infusion form	3000.0g
Casien hydrolysate (acid)	17.5g
Starch	1.5g
Distilled water	1L
Agar	15g

pH7.4±0.2 at 25°C

Source: This medium is available as a premixed powder from Titan Biotech Ltd as broth form. Add 15 g of agar powder to make broth into agar form.

Preparation: Dissolve 21 grams of premixed medium powder in 1000ml of distilled water. Add 15 g of agar powder into the previous medium broth. Gently heat if it is required to dissolve medium completely.
Distribute into tube or flask and sterilize by autoclaving at 15 psi (121°C) for 15 minutes. Cool to room temperature prior to dispense.

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Appendix B

1. Procedure:

1.1. In-vitro Simulated Gastrointestinal Digestion Release of *P. emblica* Crude Extract-Loaded BSA Nanoparticles (PBNP) and *P.emblica* 95% Crude Extract

To perform in-vitro simulated gastrointestinal digestion in the lab, in artificial mouth section, *P. emblica* crude 95% ethanol extract and PBNP are dissolved with distilled water and adjust pH to reach 6.9 by using 1mmol $NaHCO_3$. Then add mimicked saliva solution (α-amylase from *Apergillusoryzae* and 1mmol of $CaCl_2$) and shake it with shaking incubator at 200rpm for 2 minutes. The mixture is added with pepsin solution and adjust pH with 1mmol of HCl to reach 2.0 to imitate the environment in the stomach then shake at 130rpm. In artificial duodenum section, pancreatic and bile salt solution are added to the mixture and adjust pH to reach 5.0, shake for 20 minutes at 45rpm. Lastly, pH of the mixture is adjusted to slightly alkali condition (pH 6.5) in ileum section and shake for 90 minutes at 45rpm.

1.2. Antioxidant activity of In-vitro SGS Release by DPPH radical scavenging activity

The modified DPPH radical scavenging activity[6] was used for percentage DPPH radical scavenging determination. The samples from in-vitro simulated gastrointestinal digestion of *P. emblica* crude 95 % ethanol extract and PBNP are dissolved with methanol. 200 µl of sample is mixed with 1.8ml of DPPH solution. The mixture is shaken vigorously and stand in the dark condition for 30 minutes at room temperature. After 30 minutes, the optical density of the mixture is measured at 517nm. The results were interpreted as percentage reduction.

$$\textbf{Percentage reduction of DPPH} = \frac{(A_o - A_c)}{A_o} \; x \; 100$$

Where A_0 is the initial absorbance and A_c is the value for added sample concentration (c). All measurements three replications invidually.

Rawdata

Table 2: The outcome of DPPH as absorbance 517nm at pH6.9 in in-vitro SGS release

Sample	Replication		
	1	2	3
1:2	0.326	0.491	0.554
1:3	0.472	0.443	0.451
1:4	0.439	0.401	0.429
Crude	0.362	0.43	0.347

Table 3: The outcome of DPPH as absorbance 517nm at pH5.5 in-vitro SGS release

Sample	Replication		
	1	2	3
1:2	0.27	0.361	0.207
1:3	0.234	0.339	0.475
1:4	0.508	0.376	0.446
Crude	0.132	0.0883	0.076

Table 4:The outcome of DPPH as absorbance 517nm at pH4.6 in-vitro SGS release

Sample	Replication		
	1	2	3
1:2	0.15	0.05	0.069
1:3	0.201	0.27	0.287
1:4	0.384	0.27	0.362
Crude	0.046	0.177	0.063

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Table 5:The outcome of DPPH as absorbance 517nm at pH2.8 in-vitro SGS release

Sample	Replication		
	1	2	3
1:2	0.225	0.164	0.189
1:3	0.14	0.307	0.337
1:4	0.387	0.355	0.519
Crude	0.19	0.084	0.087

Table 6:The outcome of DPPH as absorbance 517nm at pH3.8 in-vitro SGS release

Sample	Replication		
	1	2	3
1:2	0.131	0.15	0.19
1:3	0.24	0.23	0.022
1:4	0.513	0.433	0.424
Crude	0.381	0.049	0.129

Table 7: The outcome of DPPH as absorbance 517nm at pH2.3 in-vitro SGS release

Sample	Replication		
	1	2	3
1:2	0.098	0.126	0.116
1:3	0.406	0.19	0.142
1:4	0.433	0.098	0.252
Crude	0.23	0.085	0.105

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Table 8:The outcome of DPPH as absorbance 517nm at pH2.0 in-vitro SGS release

Sample	Replication		
	1	2	3
1:2	0.18	0.18	0.225
1:3	0.194	0.233	0.233
1:4	0.451	0.232	0.395
Crude	0.199	0.1	0.184

Table 9: The outcome of DPPH as absorbance 517nm at pH5.0 in-vitro SGS release

Sample	Replication		
	1	2	3
1:2	0.215	0.359	0.396
1:3	0.22	0.192	0.408
1:4	0.334	0.323	0.45
Crude	0.291	0.062	0.066

Table 10: The outcome of DPPH as absorbance 517nm at pH6.5 in-vitro SGS release

Sample	Replication		
	1	2	3
1:2	0.246	0.186	0.385
1:3	0.531	0.513	0.364
1:4	0.442	0.452	0.353
Crude	0.489	0.145	0.175

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Appendix C

SAS output

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The SAS System

The ANOVA Procedure

Class Level Information		
Class	**Levels**	**Values**
Treatment	7	bac1 bac2 bac3 bac4 bac5 bac6 bac7
rep	3	1 2 3

Number of Observations Read	43
Number of Observations Used	42

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The ANOVA Procedure

Dependent Variable: Inhibition

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	8	0.15804286	0.01975536	1.22	0.3195
Error	33	0.53539762	0.01622417		
Corrected Total	41	0.69344048			

R-Square	Coeff Var	Root MSE	Inhibition Mean
0.227911	27.96505	0.127374	0.455476

Source	DF	Anova SS	Mean Square	F Value	Pr > F
Treatment	6	0.11852381	0.01975397	1.22	0.3222
rep	2	0.03951905	0.01975952	1.22	0.3088

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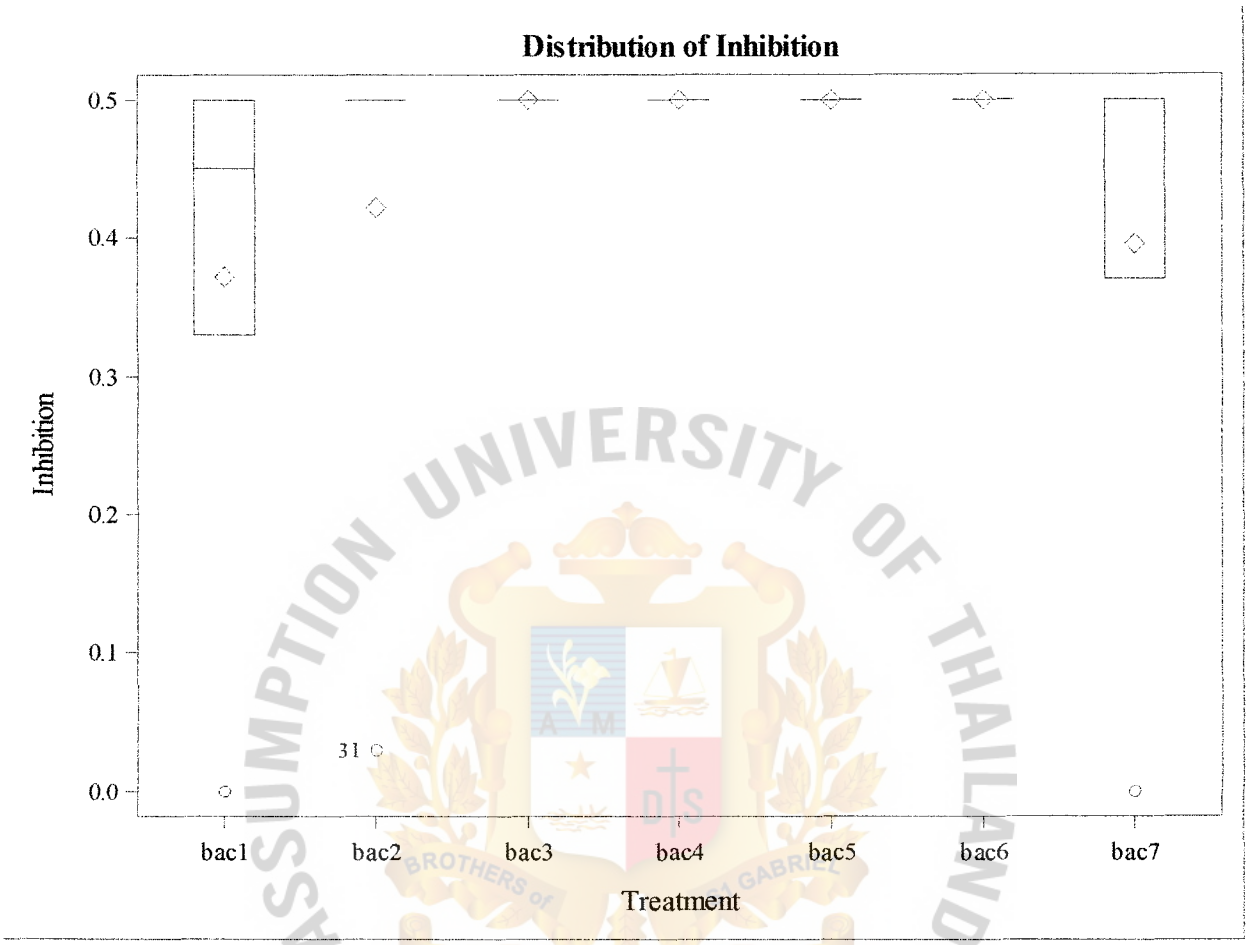
The ANOVA Procedure



Level of Treatment	N	Inhibition Mean	Inhibition Std Dev
bac1	6	0.37166667	0.19497863
bac2	6	0.42166667	0.19187670
bac3	6	0.50000000	0.00000000
bac4	6	0.50000000	0.00000000
bac5	6	0.50000000	0.00000000
bac6	6	0.50000000	0.00000000
bac7	6	0.39500000	0.20037465

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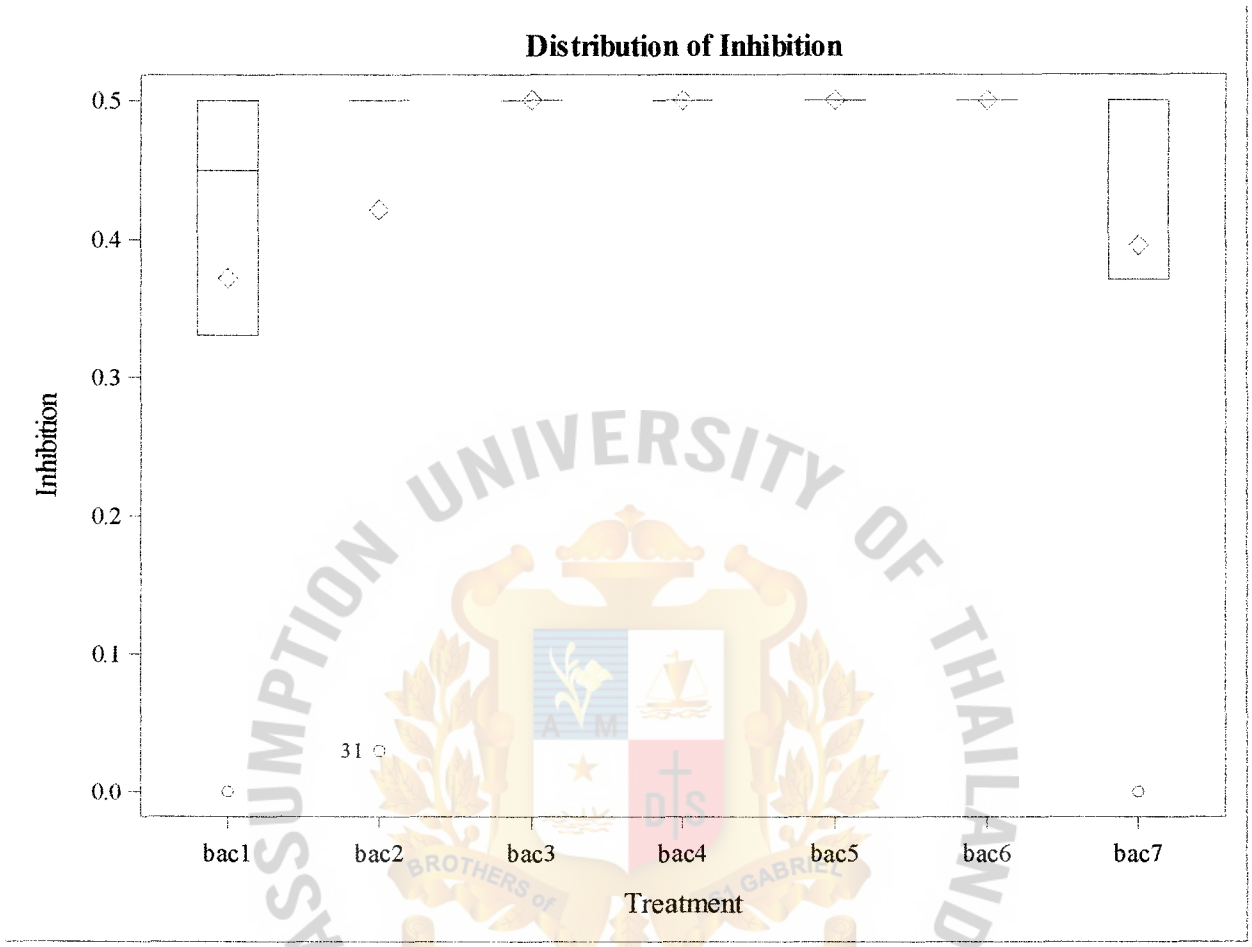

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The SAS System

The ANOVA Procedure

Duncan's Multiple Range Test for Inhibition

Note: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	33
Error Mean Square	0.016224

Number of Means	**2**	**3**	**4**	**5**	**6**	**7**
Critical Range	.1496	.1573	.1622	.1658	.1685	.1706

Means with the same letter are not significantly different.			
Duncan Grouping	**Mean**	**N**	**Treatment**
A	0.50000	6	bac5
A			
A	0.50000	6	bac6
A			
A	0.50000	6	bac3
A			
A	0.50000	6	bac4
A			
A	0.42167	6	bac2
A			
A	0.39500	6	bac7
A			
A	0.37167	6	bac1

The SAS System

The ANOVA Procedure

Class Level Information		
Class	**Levels**	**Values**
Treatment	7	bac1 bac2 bac3 bac4 bac5 bac6 bac7
rep	3	1 2 3

Number of Observations Read	43
Number of Observations Used	42

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The ANOVA Procedure

Dependent Variable: Inhibition

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	8	0.29700476	0.03712560	1.42	0.2268
Error	33	0.86555714	0.02622900		
Corrected Total	41	1.16256190			

R-Square	Coeff Var	Root MSE	Inhibition Mean
0.255474	38.78025	0.161954	0.417619

Source	DF	Anova SS	Mean Square	F Value	Pr > F
Treatment	6	0.13892857	0.02315476	0.88	0.5183
rep	2	0.15807619	0.07903810	3.01	0.0628

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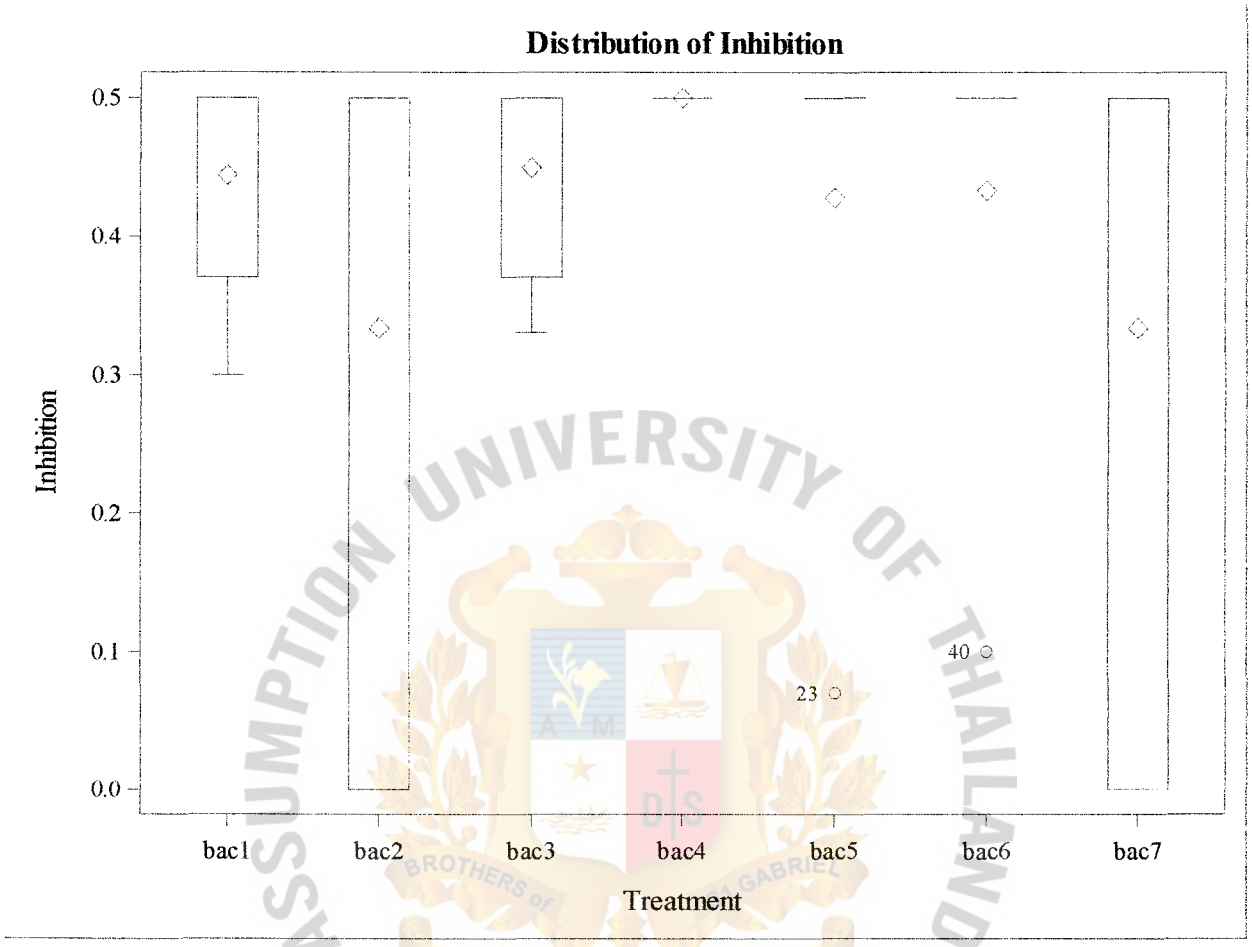
The ANOVA Procedure



Level of Treatment	N	Inhibition Mean	Inhibition Std Dev
bac1	6	0.44500000	0.08803408
bac2	6	0.33333333	0.25819889
bac3	6	0.45000000	0.07848567
bac4	6	0.50000000	0.00000000
bac5	6	0.42833333	0.17554676
bac6	6	0.43333333	0.16329932
bac7	6	0.33333333	0.25819889

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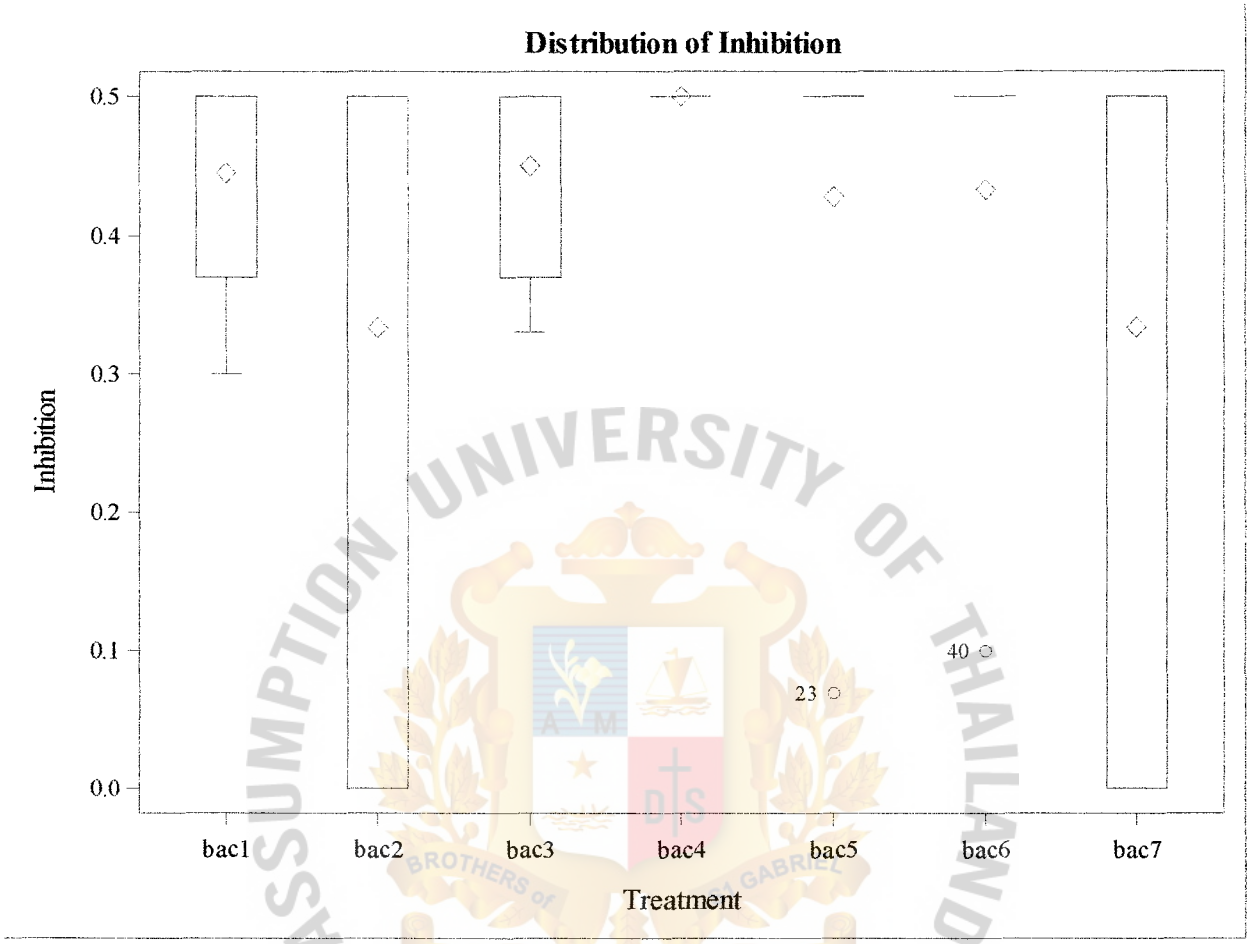
The ANOVA Procedure



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The ANOVA Procedure

Duncan's Multiple Range Test for Inhibition

Note: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	33
Error Mean Square	0.026229

Number of Means	**2**	**3**	**4**	**5**	**6**	**7**
Critical Range	.1902	.2000	.2063	.2108	.2142	.2169

Means with the same letter are not significantly different.			
Duncan Grouping	**Mean**	**N**	**Treatment**
A	0.50000	6	bac4
A			
A	0.45000	6	bac3
A			
A	0.44500	6	bac1
A			
A	0.43333	6	bac6
A			
A	0.42833	6	bac5
A			
A	0.33333	6	bac2
A			
A	0.33333	6	bac7

The SAS System

The ANOVA Procedure

Class Level Information		
Class	**Levels**	**Values**
Treatment	7	bac1 bac2 bac3 bac4 bac5 bac6 bac7
rep	3	1 2 3

Number of Observations Read	43
Number of Observations Used	42

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The SAS System

The ANOVA Procedure

Dependent Variable: Inhibition

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	8	0.25958095	0.03244762	1.29	0.2814
Error	33	0.82878333	0.02511465		
Corrected Total	41	1.08836429			

R-Square	Coeff Var	Root MSE	Inhibition Mean
0.238506	42.09989	0.158476	0.376429

Source	DF	Anova SS	Mean Square	F Value	Pr > F
Treatment	6	0.20168095	0.03361349	1.34	0.2683
rep	2	0.05790000	0.02895000	1.15	0.3282

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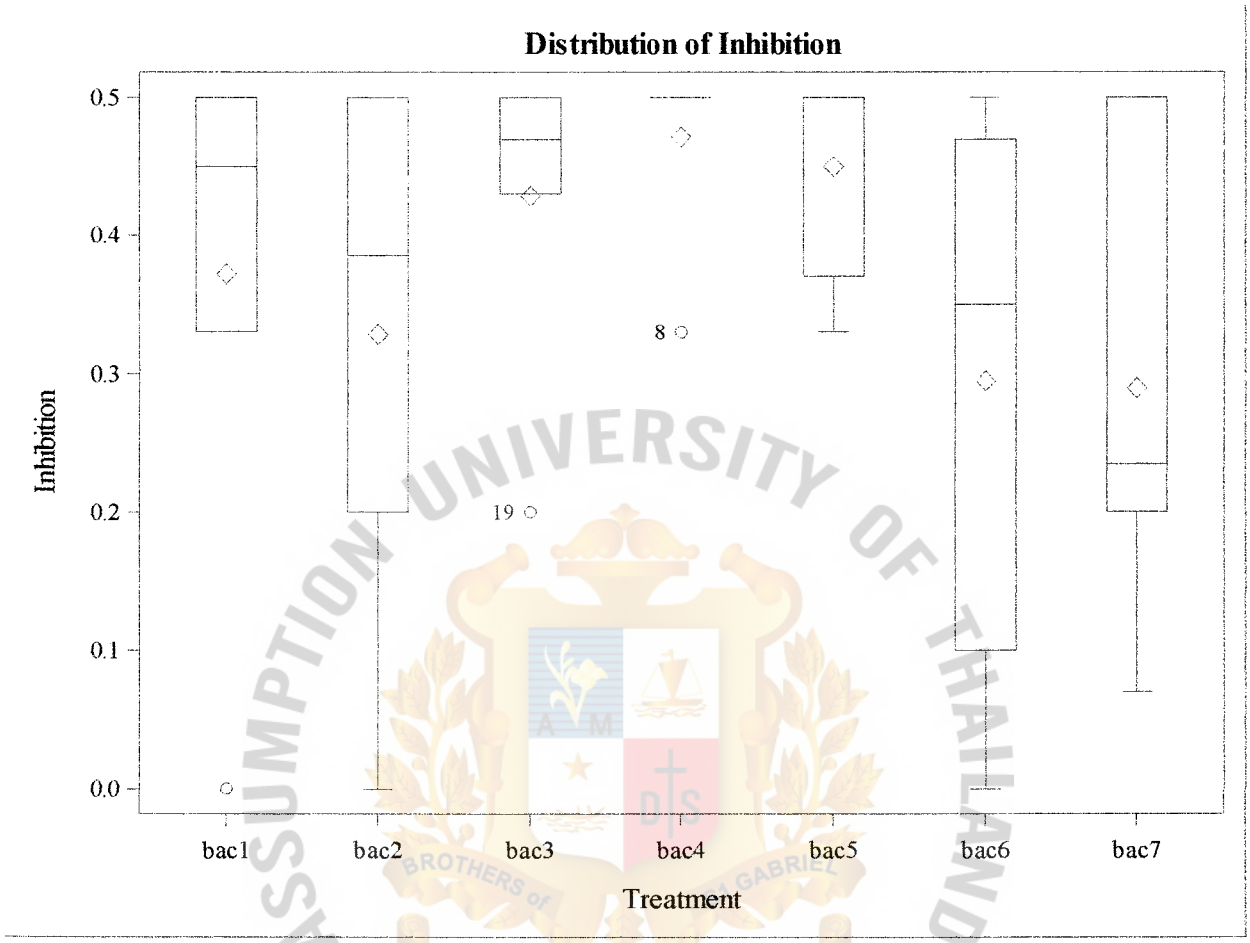
The SAS System

The ANOVA Procedure



Level of Treatment	N	Inhibition Mean	Inhibition Std Dev
bac1	6	0.37166667	0.19497863
bac2	6	0.32833333	0.19497863
bac3	6	0.42833333	0.11478966
bac4	6	0.47166667	0.06940221
bac5	6	0.45000000	0.07848567
bac6	6	0.29500000	0.21566177
bac7	6	0.29000000	0.17504285

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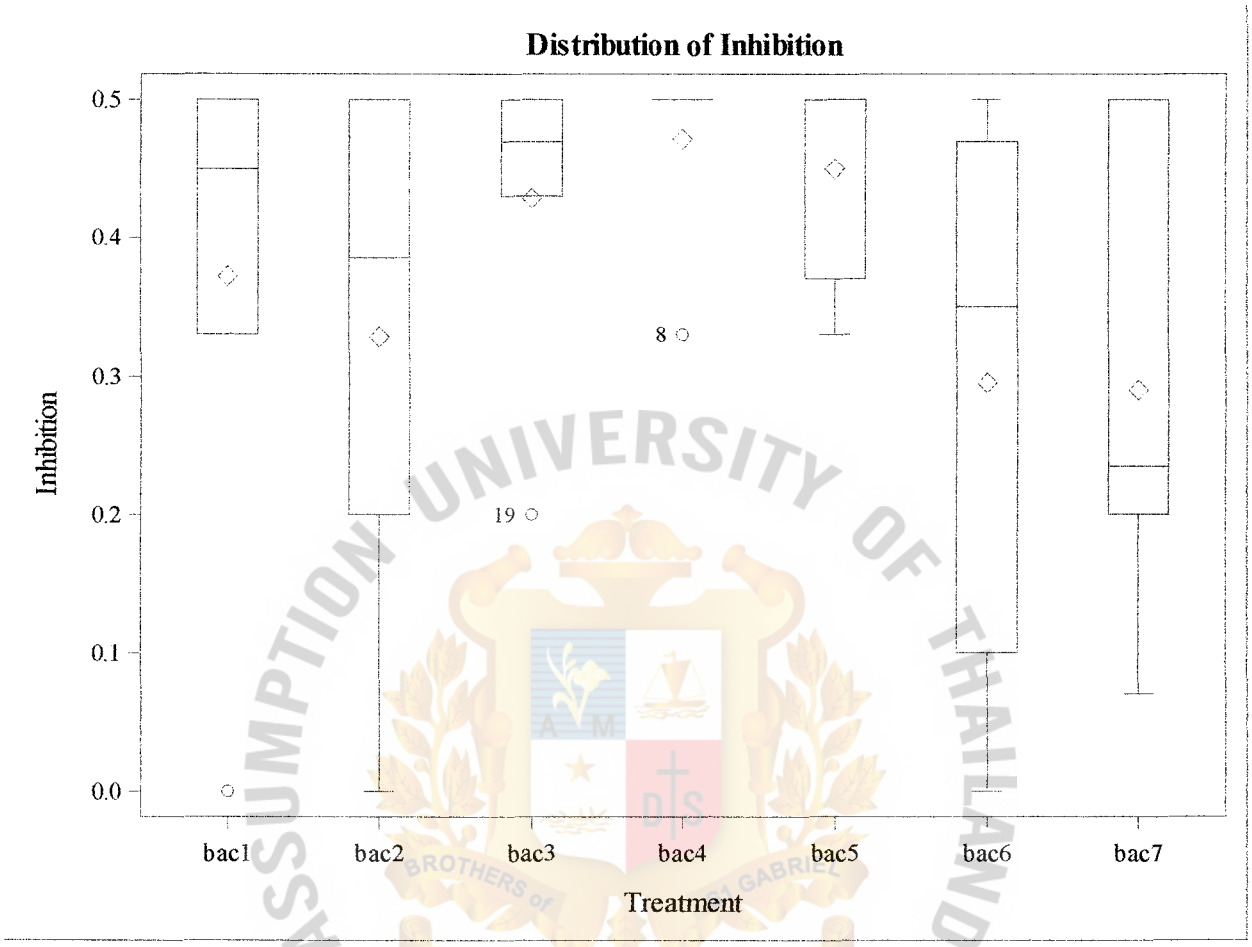

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The ANOVA Procedure

Duncan's Multiple Range Test for Inhibition

Note: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	33
Error Mean Square	0.025115

Number of Means	**2**	**3**	**4**	**5**	**6**	**7**
Critical Range	.1861	.1957	.2019	.2063	.2096	.2123

Means with the same letter are not significantly different.			
Duncan Grouping	**Mean**	**N**	**Treatment**
A	0.47167	6	bac4
A			
A	0.45000	6	bac5
A			
A	0.42833	6	bac3
A			
A	0.37167	6	bac1
A			
A	0.32833	6	bac2
A			
A	0.29500	6	bac6
A			
A	0.29000	6	bac7

The SAS System

The ANOVA Procedure

Class Level Information		
Class	**Levels**	**Values**
Treatment	7	bac1 bac2 bac3 bac4 bac5 bac6 bac7
rep	3	1 2 3

Number of Observations Read	43
Number of Observations Used	42

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The ANOVA Procedure

Dependent Variable: Inhibition

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	8	0.51848571	0.06481071	2.01	0.0759
Error	33	1.06314286	0.03221645		
Corrected Total	41	1.58162857			

R-Square	Coeff Var	Root MSE	Inhibition Mean
0.327818	50.66233	0.179489	0.354286

Source	DF	Anova SS	Mean Square	F Value	Pr > F
Treatment	6	0.51372857	0.08562143	2.66	0.0325
rep	2	0.00475714	0.00237857	0.07	0.9290

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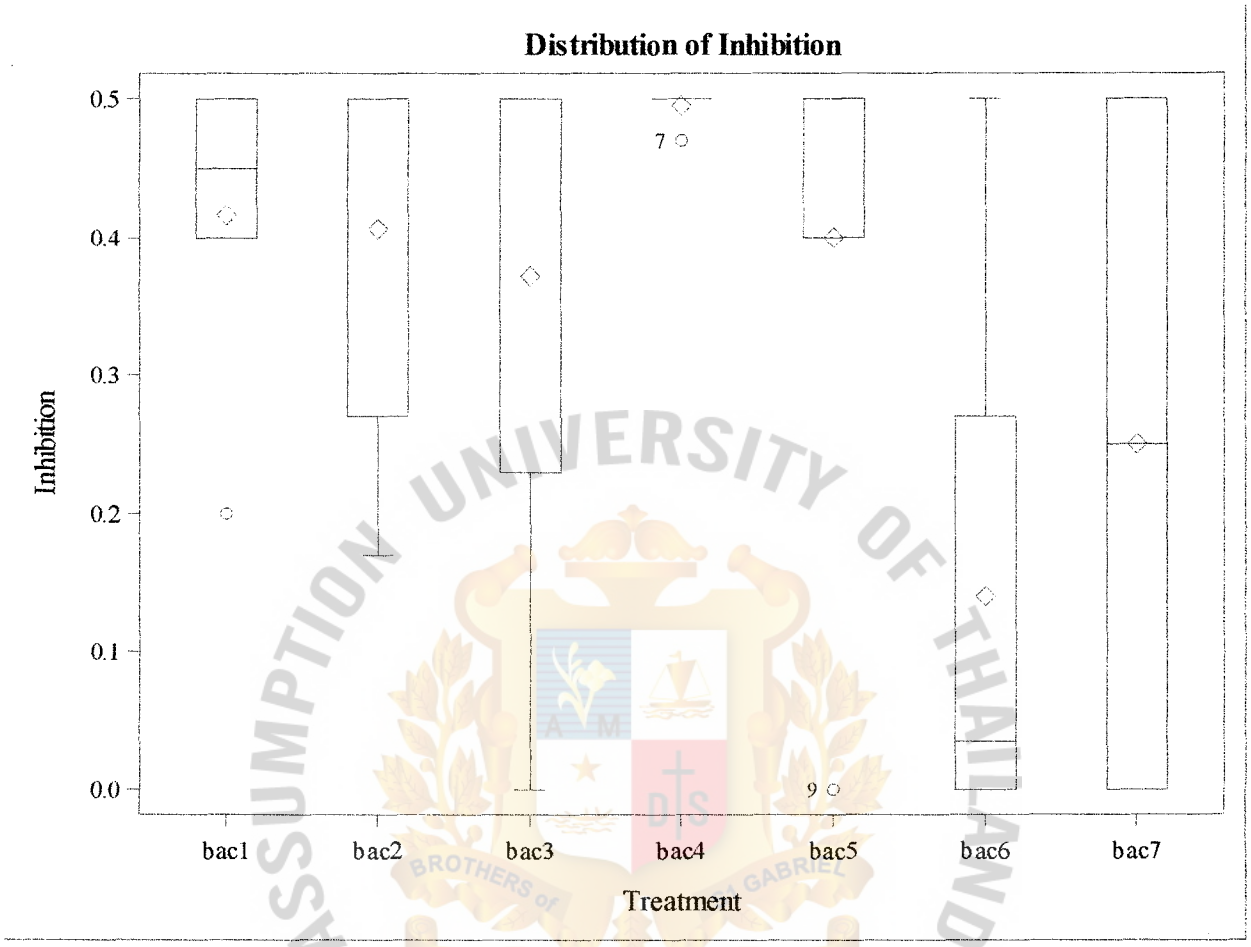

Level of Treatment	N	Inhibition Mean	Inhibition Std Dev
bac1	6	0.41666667	0.11690452
bac2	6	0.40666667	0.14800901
bac3	6	0.37166667	0.21169947
bac4	6	0.49500000	0.01224745
bac5	6	0.40000000	0.20000000
bac6	6	0.14000000	0.20503658
bac7	6	0.25000000	0.22583180

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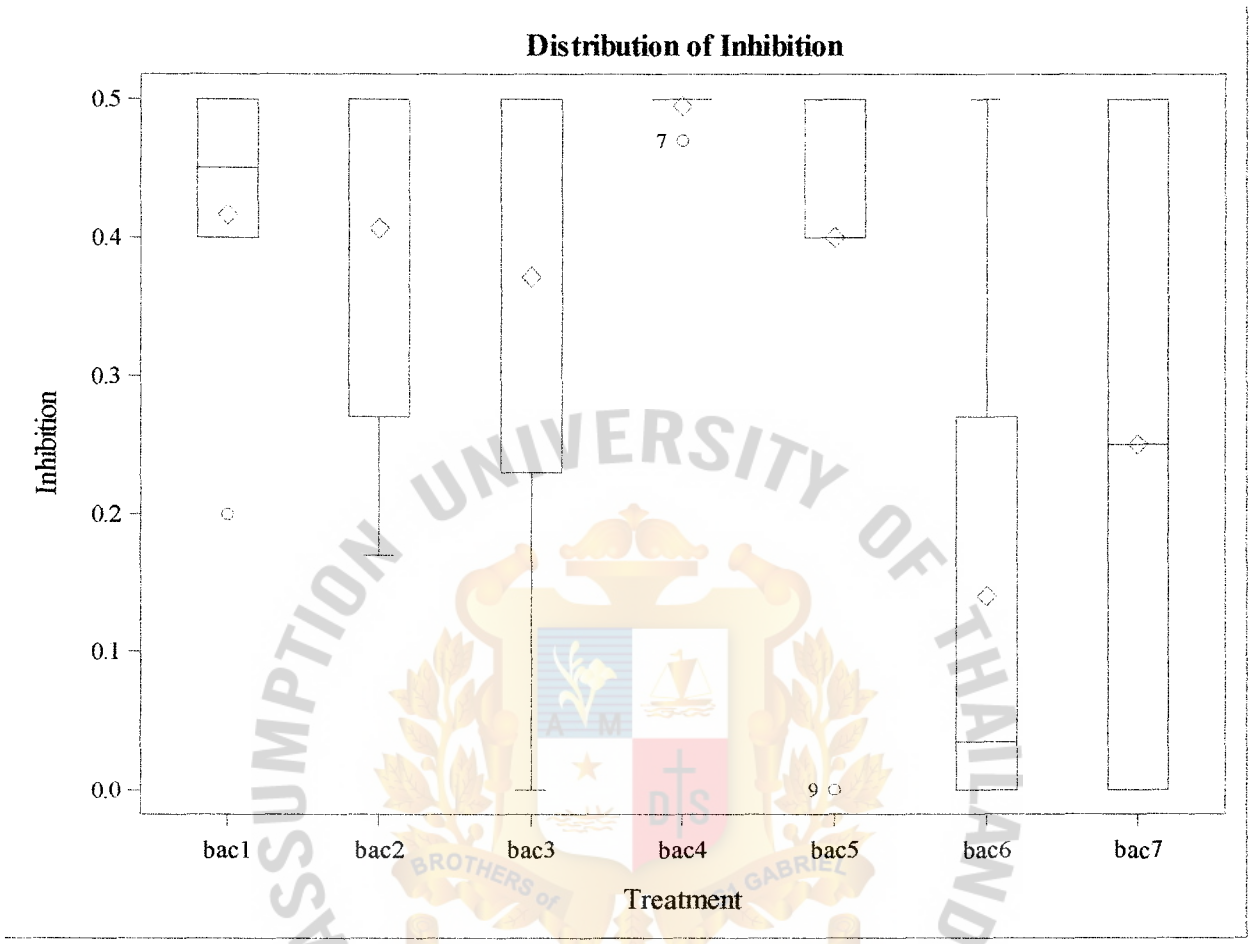

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The ANOVA Procedure

Duncan's Multiple Range Test for Inhibition

Note: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	33
Error Mean Square	0.032216

Number of Means	**2**	**3**	**4**	**5**	**6**	**7**
Critical Range	.2108	.2216	.2286	.2336	.2374	.2404

Means with the same letter are not significantly different.				
Duncan Grouping		**Mean**	**N**	**Treatment**
	A	0.4950	6	bac4
	A			
B	A	0.4167	6	bac1
B	A			
B	A	0.4067	6	bac2
B	A			
B	A	0.4000	6	bac5
B	A			
B	A	0.3717	6	bac3
B				
B	C	0.2500	6	bac7
	C			
	C	0.1400	6	bac6

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The ANOVA Procedure

Class Level Information		
Class	**Levels**	**Values**
Treatment	7	bac1 bac2 bac3 bac4 bac5 bac6 bac7
rep	3	1 2 3

Number of Observations Read	43
Number of Observations Used	42

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The ANOVA Procedure

Dependent Variable: Inhibition

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	8	0.23178571	0.02897321	0.92	0.5139
Error	33	1.04107143	0.03154762		
Corrected Total	41	1.27285714			

R-Square	Coeff Var	Root MSE	Inhibition Mean
0.182099	47.09528	0.177616	0.377143

Source	DF	Anova SS	Mean Square	F Value	Pr > F
Treatment	6	0.21325714	0.03554286	1.13	0.3686
rep	2	0.01852857	0.00926429	0.29	0.7475

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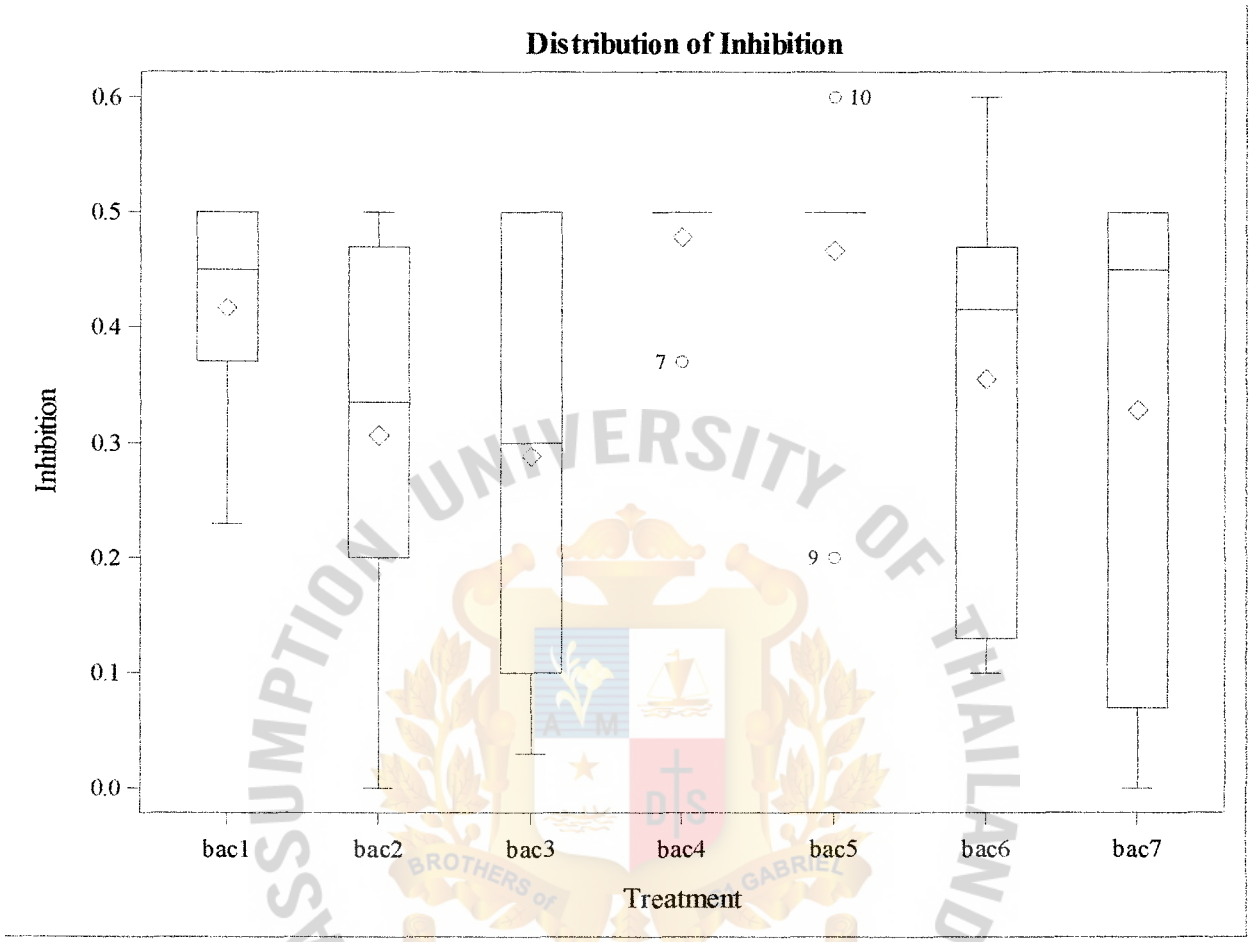

Level of Treatment	N	Inhibition Mean	Inhibition Std Dev
bac1	6	0.41666667	0.10782702
bac2	6	0.30666667	0.18949055
bac3	6	0.28833333	0.22355462
bac4	6	0.47833333	0.05307228
bac5	6	0.46666667	0.13662601
bac6	6	0.35500000	0.19826750
bac7	6	0.32833333	0.23155273

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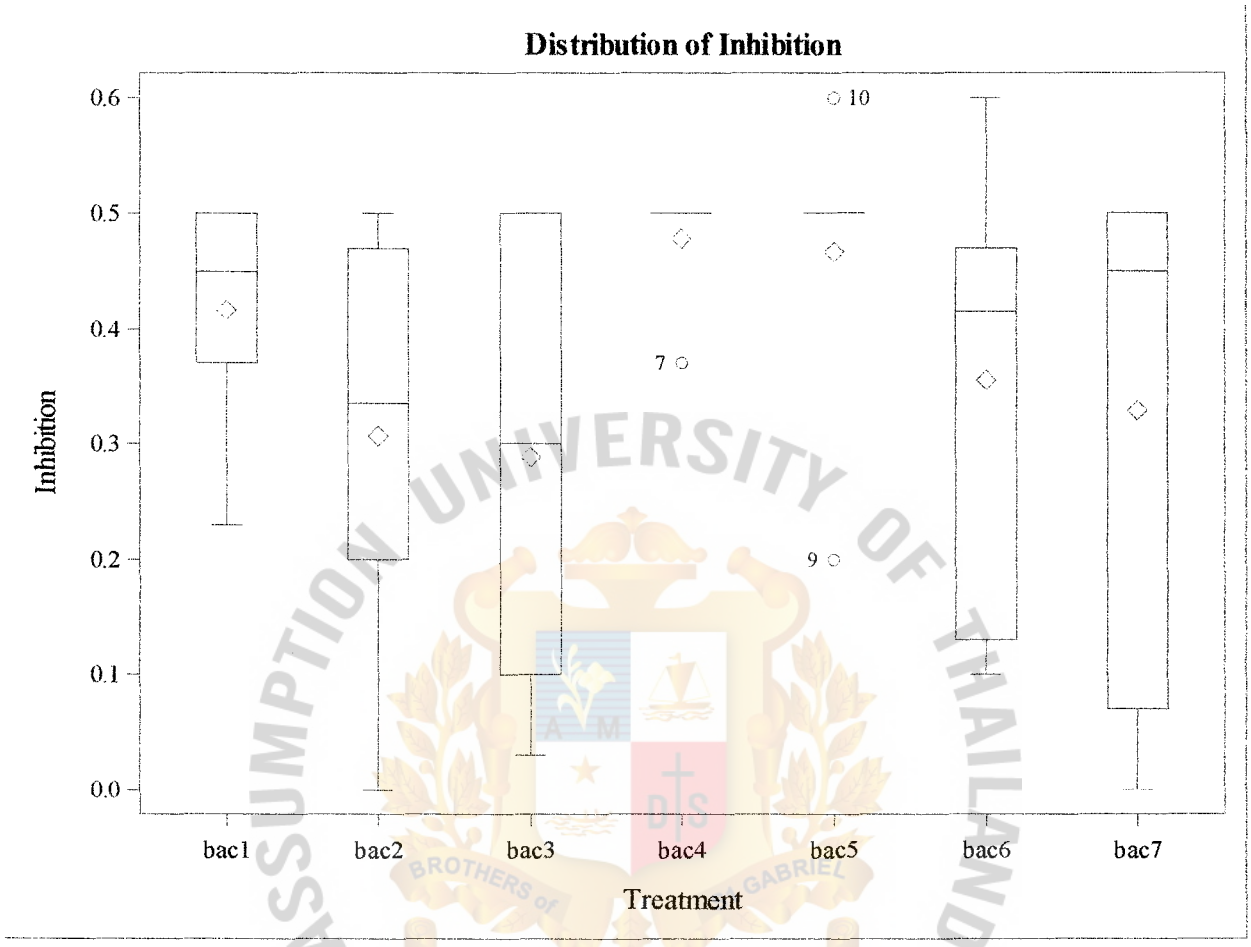

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The ANOVA Procedure

Duncan's Multiple Range Test for Inhibition

Note: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	33
Error Mean Square	0.031548

Number of Means	**2**	**3**	**4**	**5**	**6**	**7**
Critical Range	.2086	.2193	.2262	.2312	.2350	.2379

Means with the same letter are not significantly different.			
Duncan Grouping	**Mean**	**N**	**Treatment**
A	0.4783	6	bac4
A			
A	0.4667	6	bac5
A			
A	0.4167	6	bac1
A			
A	0.3550	6	bac6
A			
A	0.3283	6	bac7
A			
A	0.3067	6	bac2
A			
A	0.2883	6	bac3

The SAS System

The ANOVA Procedure

Class Level Information		
Class	**Levels**	**Values**
Treatment	7	bac1 bac2 bac3 bac4 bac5 bac6 bac7
rep	3	1 2 3

Number of Observations Read	43
Number of Observations Used	42

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The ANOVA Procedure

Dependent Variable: Inhibition

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	8	0.56308095	0.07038512	2.87	0.0154
Error	33	0.80979762	0.02453932		
Corrected Total	41	1.37287857			

R-Square	Coeff Var	Root MSE	Inhibition Mean
0.410146	40.09332	0.156650	0.390714

Source	DF	Anova SS	Mean Square	F Value	Pr > F
Treatment	6	0.13309524	0.02218254	0.90	0.5039
rep	2	0.42998571	0.21499286	8.76	0.0009

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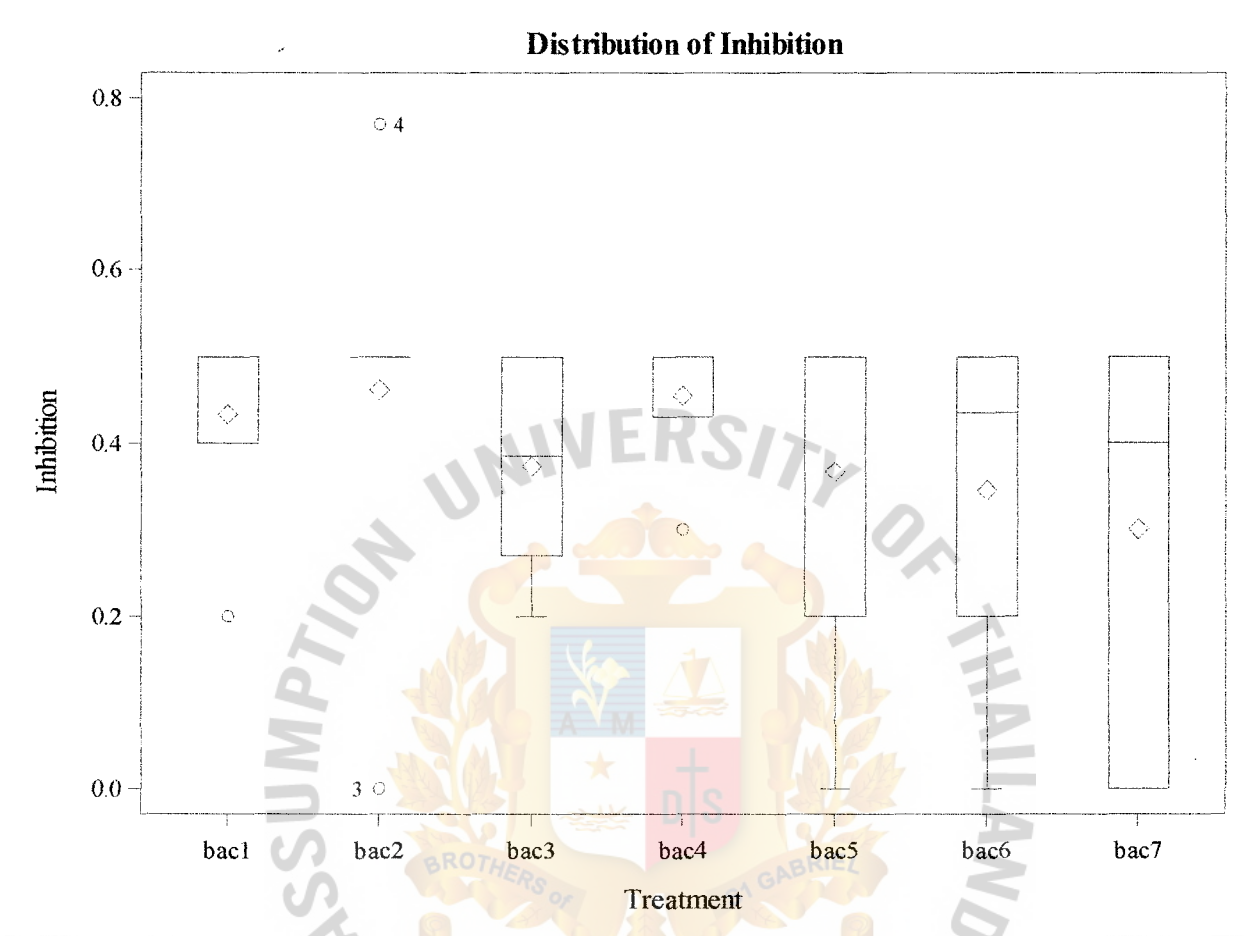
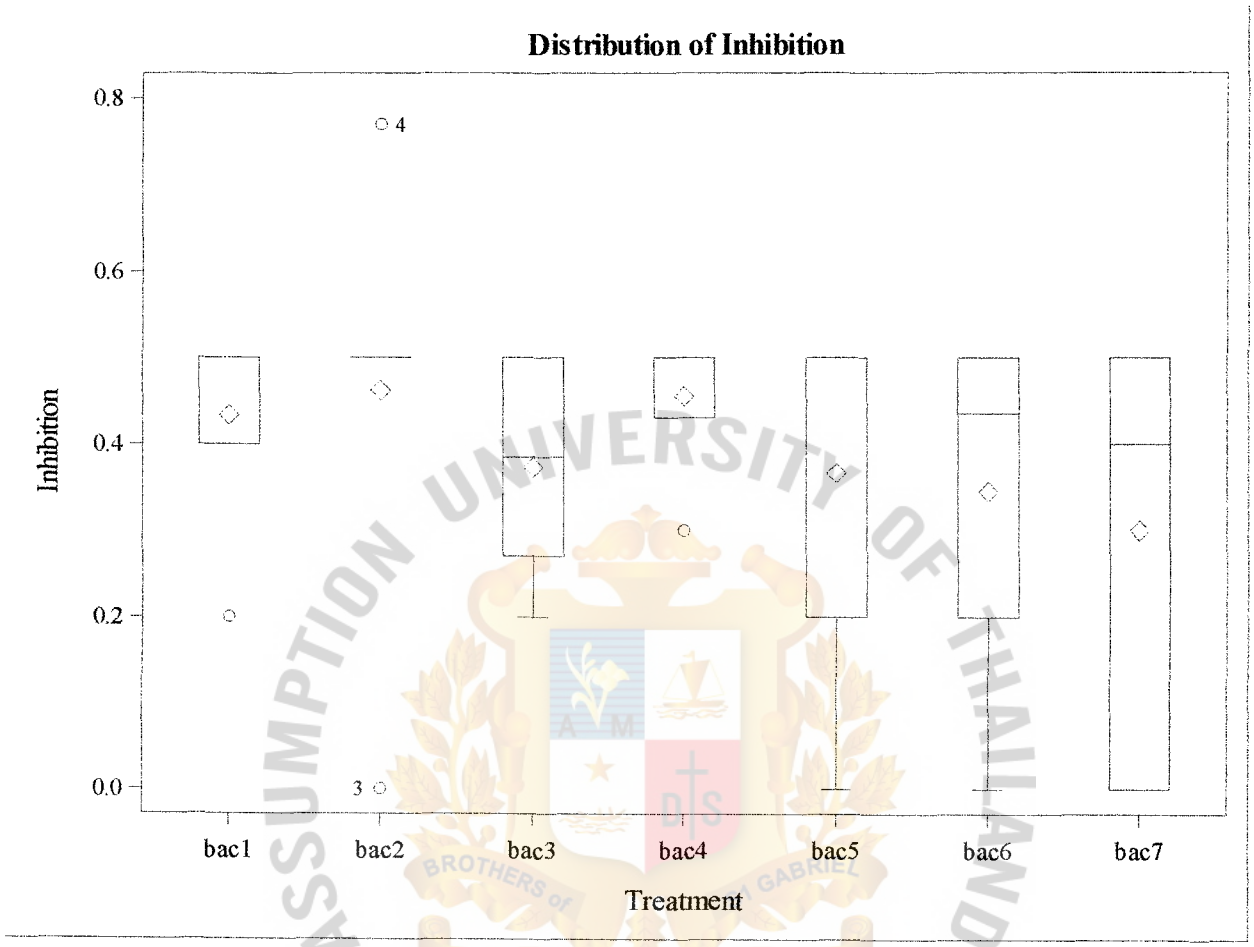


Level of Treatment	N	Inhibition Mean	Inhibition Std Dev
bac1	6	0.43333333	0.12110601
bac2	6	0.46166667	0.25063253
bac3	6	0.37333333	0.12127105
bac4	6	0.45500000	0.08093207
bac5	6	0.36666667	0.21602469
bac6	6	0.34500000	0.20627651
bac7	6	0.30000000	0.24494897

The SAS System

The ANOVA Procedure



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The ANOVA Procedure

Duncan's Multiple Range Test for Inhibition

Note: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	33
Error Mean Square	0.024539

Number of Means	**2**	**3**	**4**	**5**	**6**	**7**
Critical Range	.1840	.1934	.1995	.2039	.2072	.2098

Means with the same letter are not significantly different.			
Duncan Grouping	**Mean**	**N**	**Treatment**
A	0.46167	6	bac2
A			
A	0.45500	6	bac4
A			
A	0.43333	6	bac1
A			
A	0.37333	6	bac3
A			
A	0.36667	6	bac5
A			
A	0.34500	6	bac6
A			
A	0.30000	6	bac7

The SAS System

The ANOVA Procedure

Class Level Information		
Class	**Levels**	**Values**
Treatment	7	bac1 bac2 bac3 bac4 bac5 bac6 bac7
rep	3	1 2 3

Number of Observations Read	43
Number of Observations Used	42

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The ANOVA Procedure

Dependent Variable: Inhibition

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	8	0.53551429	0.06693929	4.33	0.0012
Error	33	0.51038333	0.01546616		
Corrected Total	41	1.04589762			

R-Square	Coeff Var	Root MSE	Inhibition Mean
0.512014	29.39362	0.124363	0.423095

Source	DF	Anova SS	Mean Square	F Value	Pr > F
Treatment	6	0.27528095	0.04588016	2.97	0.0199
rep	2	0.26023333	0.13011667	8.41	0.0011

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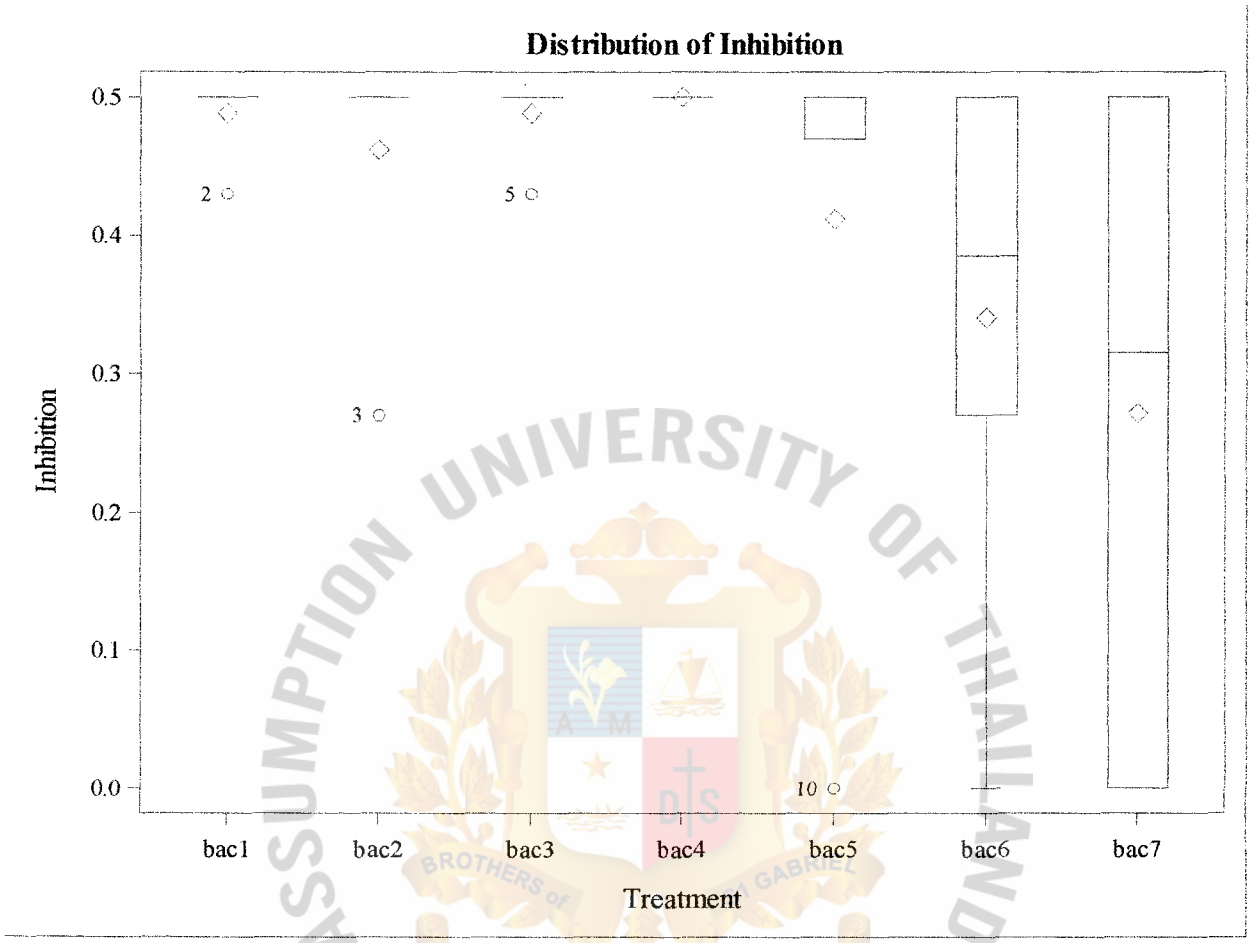
The ANOVA Procedure



Level of Treatment	N	Inhibition Mean	Inhibition Std Dev
bac1	6	0.48833333	0.02857738
bac2	6	0.46166667	0.09389711
bac3	6	0.48833333	0.02857738
bac4	6	0.50000000	0.00000000
bac5	6	0.41166667	0.20203135
bac6	6	0.34000000	0.19503846
bac7	6	0.27166667	0.25459118

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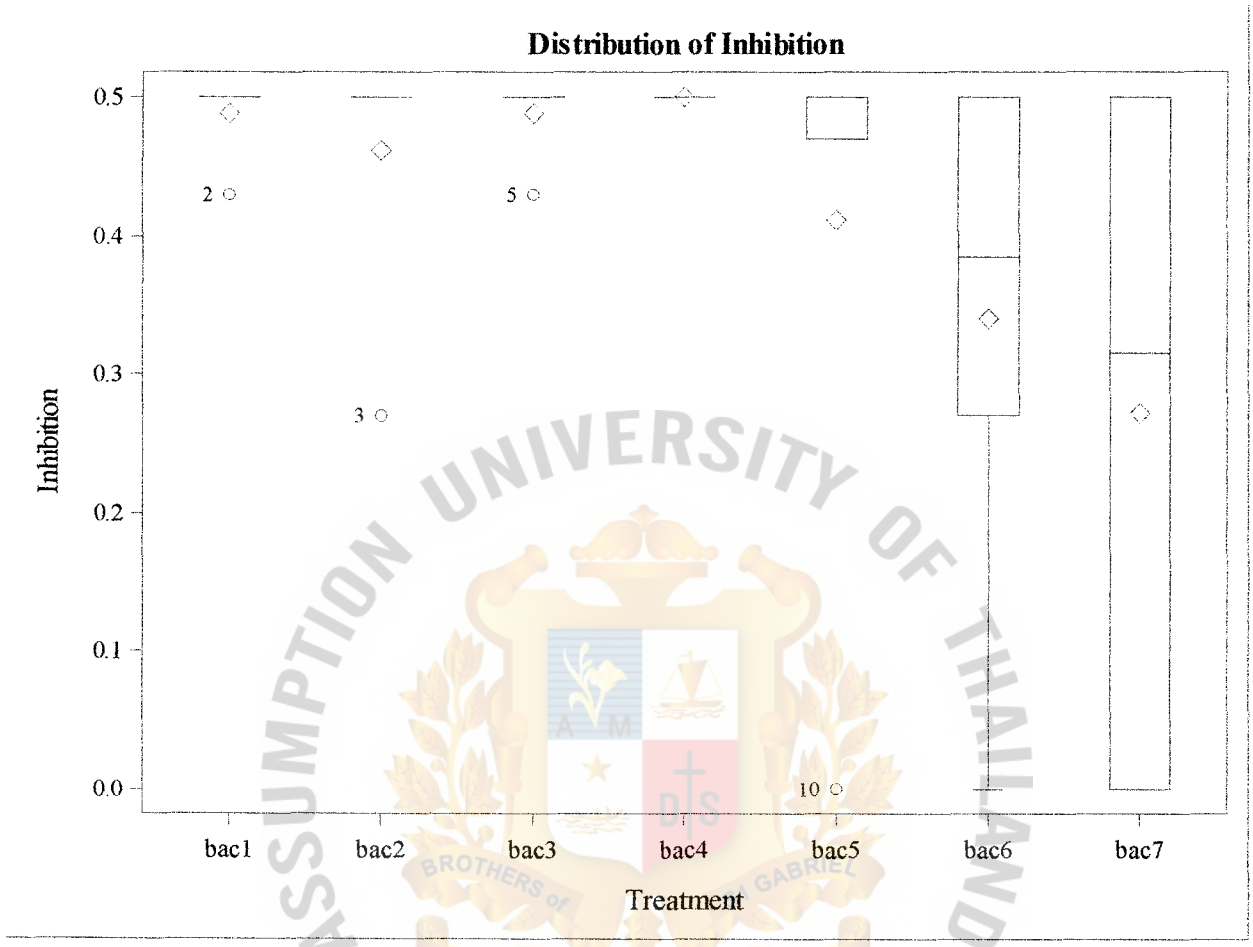
The SAS System

The ANOVA Procedure



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The SAS System

The ANOVA Procedure

Duncan's Multiple Range Test for Inhibition

Note: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	33
Error Mean Square	0.015466

Number of Means	**2**	**3**	**4**	**5**	**6**	**7**
Critical Range	.1461	.1535	.1584	.1619	.1645	.1666

Means with the same letter are not significantly different.				
Duncan Grouping		**Mean**	**N**	**Treatment**
	A	0.50000	6	bac4
	A			
	A	0.48833	6	bac1
	A			
	A	0.48833	6	bac3
	A			
	A	0.46167	6	bac2
	A			
B	A	0.41167	6	bac5
B	A			
B	A	0.34000	6	bac6
B				
B		0.27167	6	bac7

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The SAS System

The ANOVA Procedure

Class Level Information		
Class	**Levels**	**Values**
Treatment	7	bac1 bac2 bac3 bac4 bac5 bac6 bac7
rep	3	1 2 3

Number of Observations Read	43
Number of Observations Used	42

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The ANOVA Procedure

Dependent Variable: Inhibition

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	8	0.38487619	0.04810952	4.68	0.0007
Error	33	0.33911429	0.01027619		
Corrected Total	41	0.72399048			

R-Square	Coeff Var	Root MSE	Inhibition Mean
0.531604	23.06395	0.101372	0.439524

Source	DF	Anova SS	Mean Square	F Value	Pr > F
Treatment	6	0.38135714	0.06355952	6.19	0.0002
rep	2	0.00351905	0.00175952	0.17	0.8434

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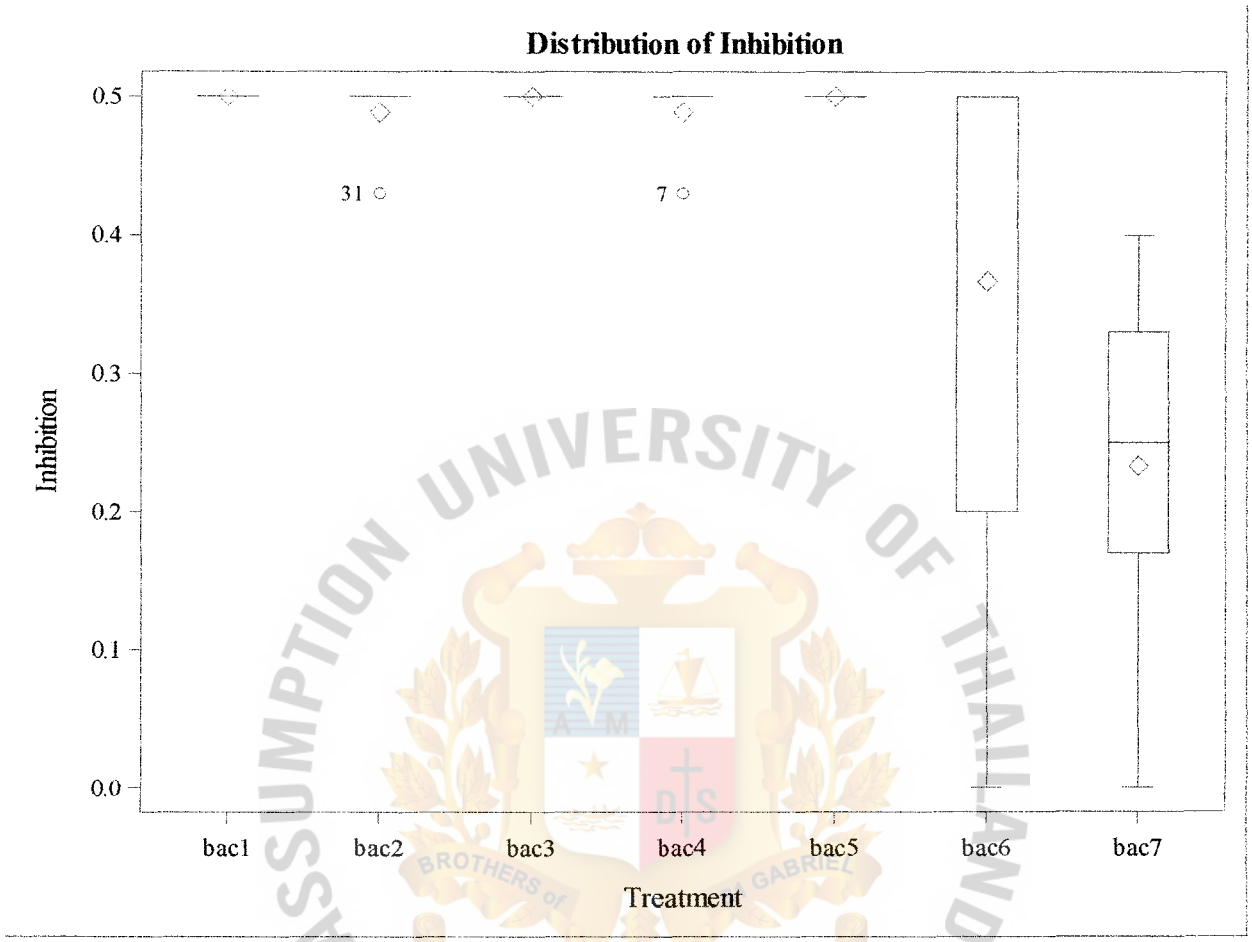
The ANOVA Procedure



Level of Treatment	N	Inhibition Mean	Inhibition Std Dev
bac1	6	0.50000000	0.00000000
bac2	6	0.48833333	0.02857738
bac3	6	0.50000000	0.00000000
bac4	6	0.48833333	0.02857738
bac5	6	0.50000000	0.00000000
bac6	6	0.36666667	0.21602469
bac7	6	0.23333333	0.14222049

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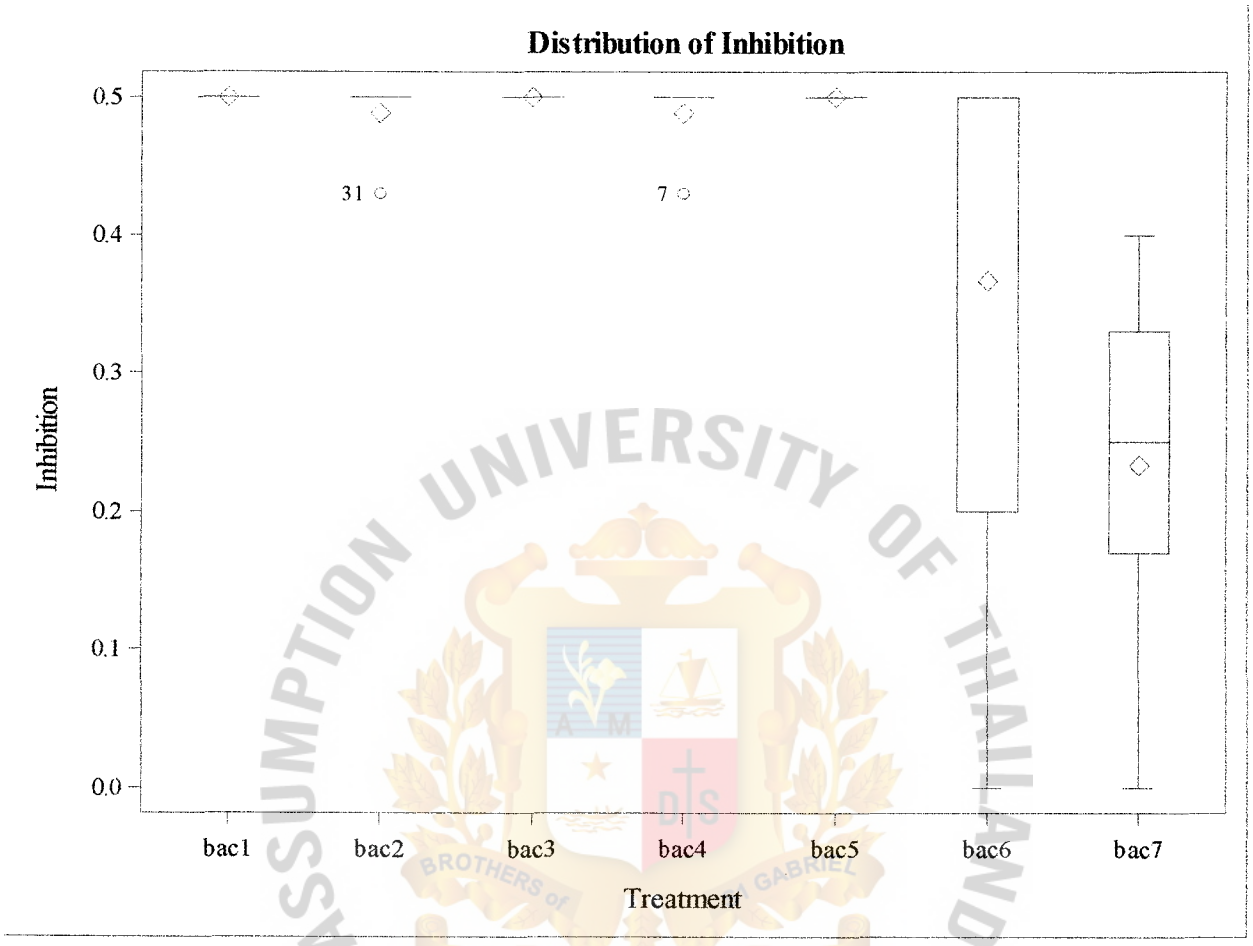
The ANOVA Procedure



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The SAS System

The ANOVA Procedure

Duncan's Multiple Range Test for Inhibition

Note: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	33
Error Mean Square	0.010276

Number of Means	**2**	**3**	**4**	**5**	**6**	**7**
Critical Range	.1191	.1252	.1291	.1320	.1341	.1358

Means with the same letter are not significantly different.			
Duncan Grouping	**Mean**	**N**	**Treatment**
A	0.50000	6	bac1
A			
A	0.50000	6	bac3
A			
A	0.50000	6	bac5
A			
A	0.48833	6	bac2
A			
A	0.48833	6	bac4
A			
A	0.36667	6	bac6
B	0.23333	6	bac7

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The SAS System

The ANOVA Procedure

Class Level Information		
Class	**Levels**	**Values**
Treatment	7	bac1 bac2 bac3 bac4 bac5 bac6 bac7
rep	3	1 2 3

Number of Observations Read	43
Number of Observations Used	42

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The ANOVA Procedure

Dependent Variable: Inhibition

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	8	0.15836190	0.01979524	0.90	0.5284
Error	33	0.72640000	0.02201212		
Corrected Total	41	0.88476190			

R-Square	Coeff Var	Root MSE	Inhibition Mean
0.178988	36.87173	0.148365	0.402381

Source	DF	Anova SS	Mean Square	F Value	Pr > F
Treatment	6	0.15422857	0.02570476	1.17	0.3470
rep	2	0.00413333	0.00206667	0.09	0.9106

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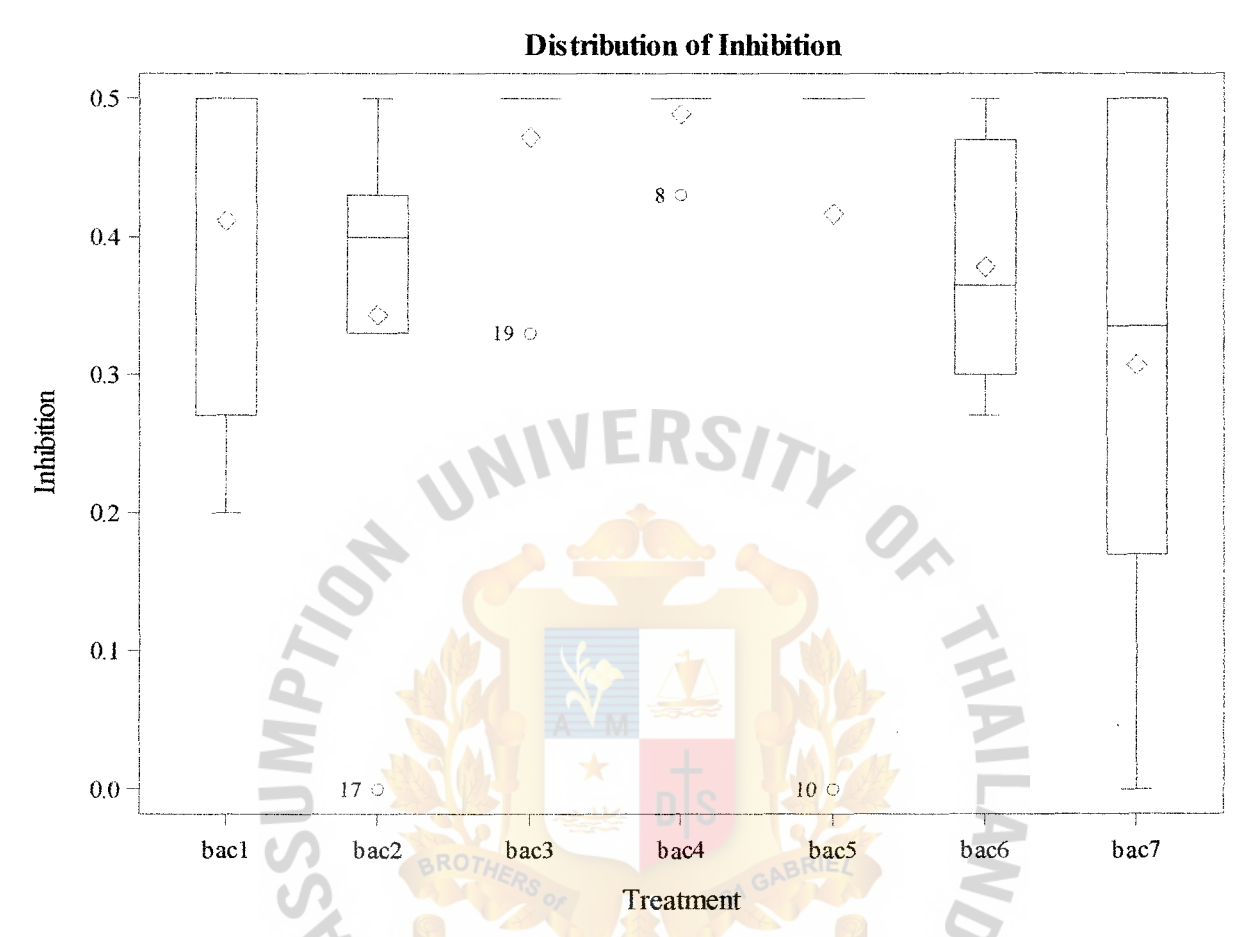
The SAS System

The ANOVA Procedure



Level of Treatment	N	Inhibition Mean	Inhibition Std Dev
bac1	6	0.41166667	0.13862419
bac2	6	0.34333333	0.17693690
bac3	6	0.47166667	0.06940221
bac4	6	0.48833333	0.02857738
bac5	6	0.41666667	0.20412415
bac6	6	0.37833333	0.09368387
bac7	6	0.30666667	0.19876284

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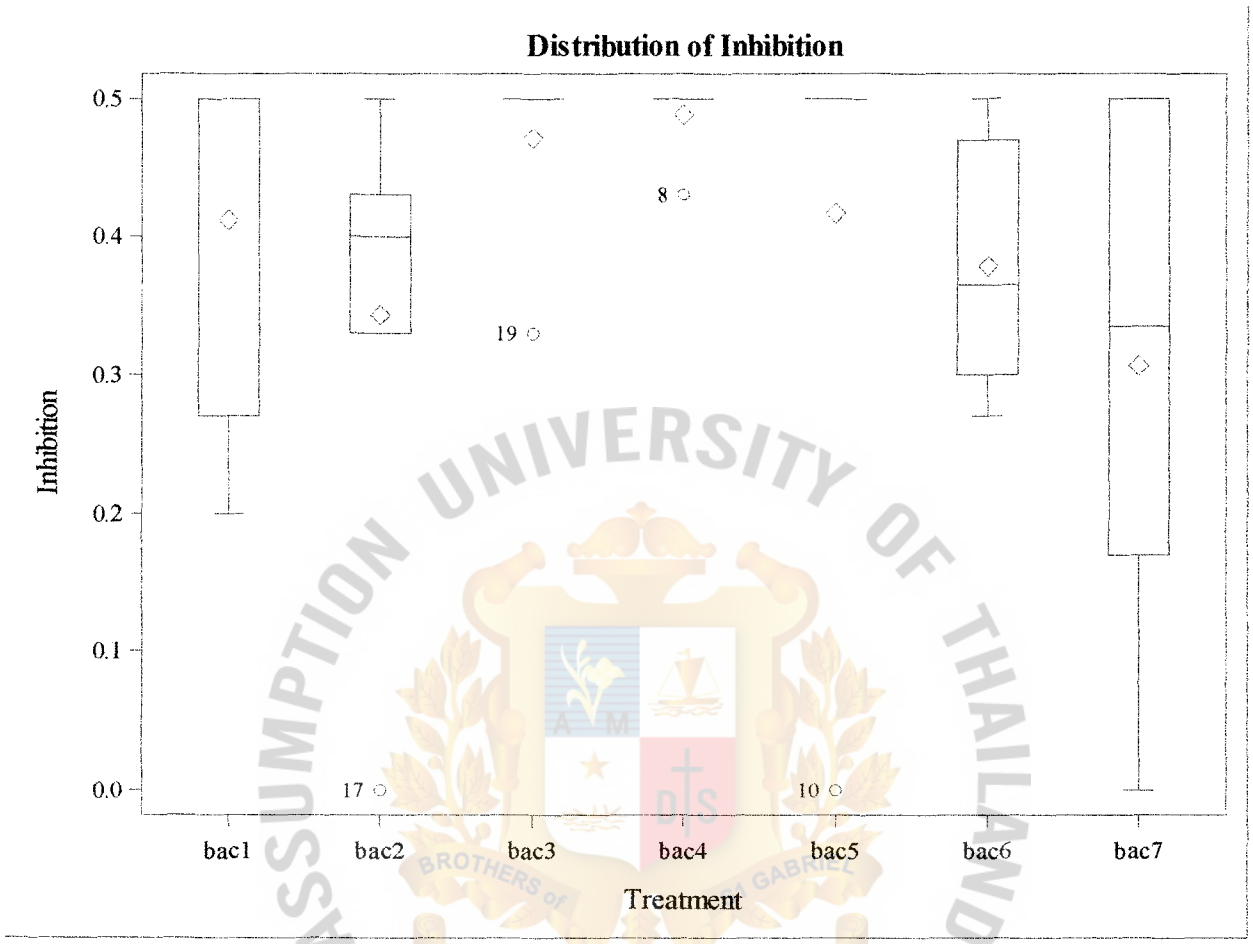
The ANOVA Procedure



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The SAS System

The ANOVA Procedure

Duncan's Multiple Range Test for Inhibition

Note: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	33
Error Mean Square	0.022012

Number of Means	**2**	**3**	**4**	**5**	**6**	**7**
Critical Range	.1743	.1832	.1890	.1931	.1963	.1987

Means with the same letter are not significantly different.			
Duncan Grouping	**Mean**	**N**	**Treatment**
A	0.48833	6	bac4
A			
A	0.47167	6	bac3
A			
A	0.41667	6	bac5
A			
A	0.41167	6	bac1
A			
A	0.37833	6	bac6
A			
A	0.34333	6	bac2
A			
A	0.30667	6	bac7

The SAS System

The ANOVA Procedure

Class Level Information		
Class	**Levels**	**Values**
Treatment	7	bac1 bac2 bac3 bac4 bac5 bac6 bac7
rep	3	1 2 3

Number of Observations Read	43
Number of Observations Used	42

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The SAS System

The ANOVA Procedure

Dependent Variable: Inhibition

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	8	0.51439524	0.06429940	2.74	0.0194
Error	33	0.77325238	0.02343189		
Corrected Total	41	1.28764762			

R-Square	Coeff Var	Root MSE	Inhibition Mean
0.399484	37.16266	0.153075	0.411905

Source	DF	Anova SS	Mean Square	F Value	Pr > F
Treatment	6	0.21534762	0.03589127	1.53	0.1986
rep	2	0.29904762	0.14952381	6.38	0.0045

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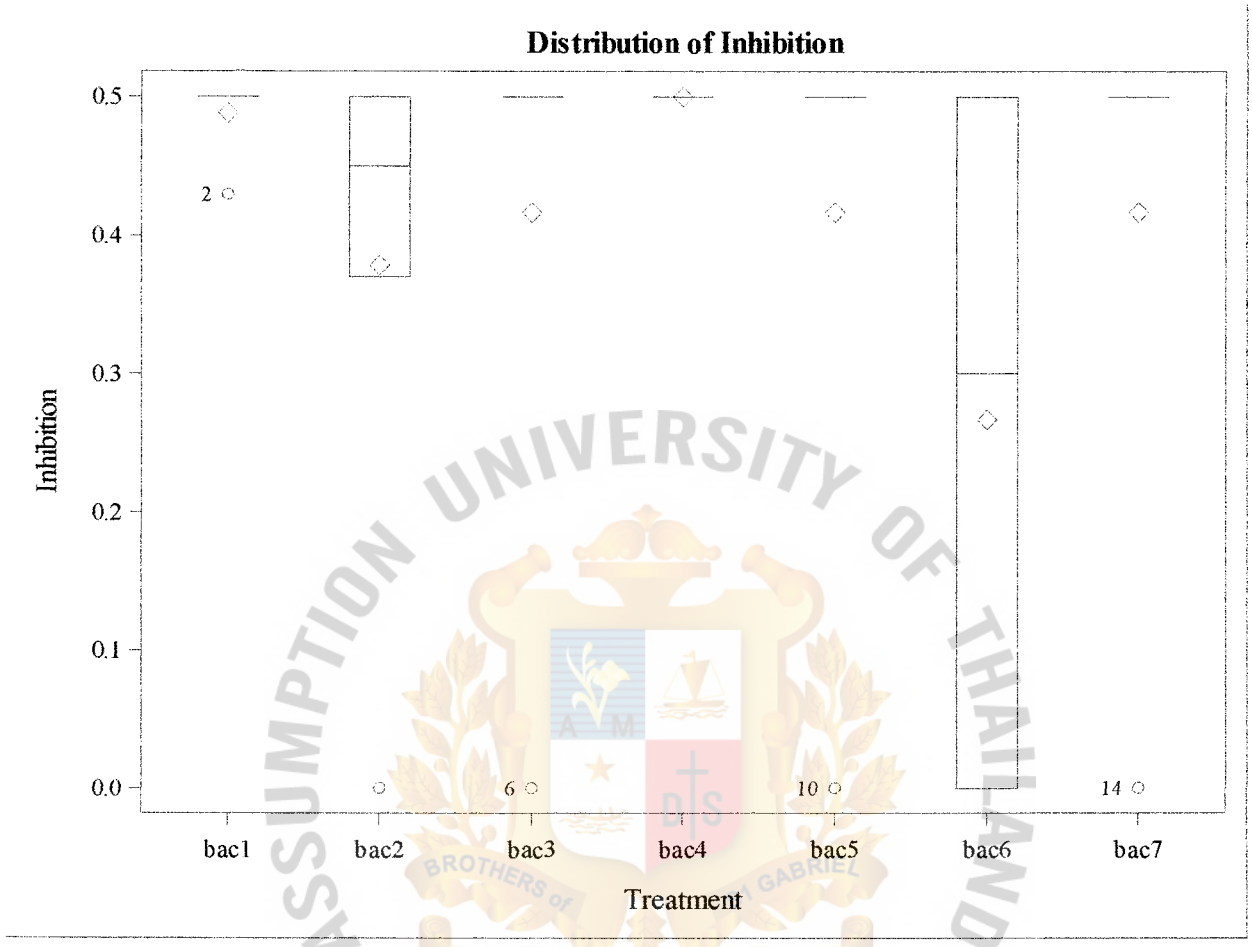
The ANOVA Procedure



Level of Treatment	N	Inhibition	
		Mean	Std Dev
bac1	6	0.48833333	0.02857738
bac2	6	0.37833333	0.19395017
bac3	6	0.41666667	0.20412415
bac4	6	0.50000000	0.00000000
bac5	6	0.41666667	0.20412415
bac6	6	0.26666667	0.22589083
bac7	6	0.41666667	0.20412415

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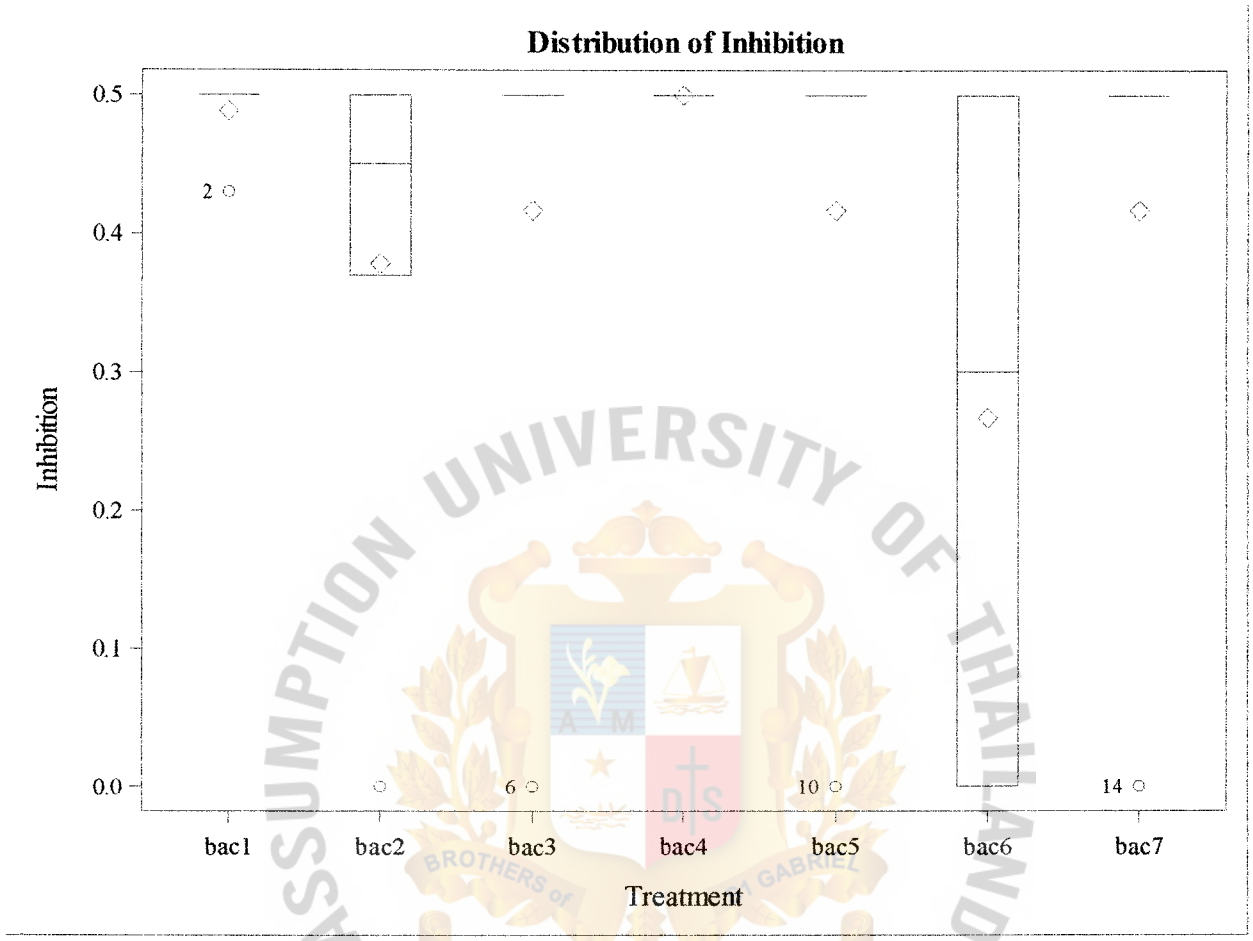
The ANOVA Procedure



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The SAS System

The ANOVA Procedure

Duncan's Multiple Range Test for Inhibition

Note: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	33
Error Mean Square	0.023432

Number of Means	**2**	**3**	**4**	**5**	**6**	**7**
Critical Range	.1798	.1890	.1950	.1993	.2025	.2051

Means with the same letter are not significantly different.				
Duncan Grouping		**Mean**	**N**	**Treatment**
	A	0.50000	6	bac4
	A			
	A	0.48833	6	bac1
	A			
B	A	0.41667	6	bac3
B	A			
B	A	0.41667	6	bac5
B	A			
B	A	0.41667	6	bac7
B	A			
B	A	0.37833	6	bac2
B				
B		0.26667	6	bac6

The SAS System

The ANOVA Procedure

Class Level Information		
Class	**Levels**	**Values**
Treatment	7	bac1 bac2 bac3 bac4 bac5 bac6 bac7
rep	3	1 2 3

Number of Observations Read	43
Number of Observations Used	42

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The SAS System

The ANOVA Procedure

Dependent Variable: Inhibition

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	8	0.20934286	0.02616786	0.78	0.6210
Error	33	1.10342143	0.03343701		
Corrected Total	41	1.31276429			

R-Square	Coeff Var	Root MSE	Inhibition Mean
0.159467	49.90274	0.182858	0.366429

Source	DF	Anova SS	Mean Square	F Value	Pr > F
Treatment	6	0.12301429	0.02050238	0.61	0.7181
rep	2	0.08632857	0.04316429	1.29	0.2885

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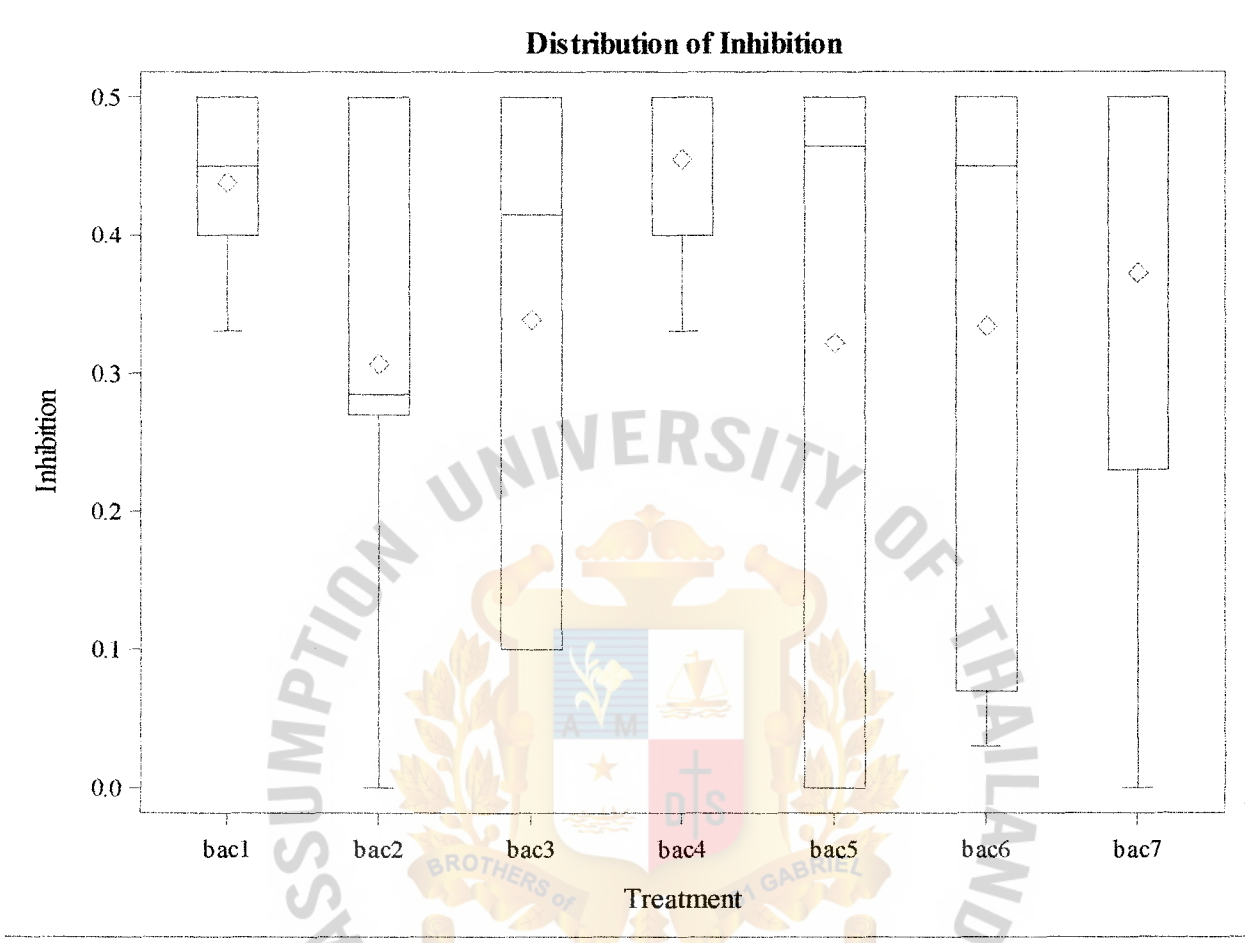
The ANOVA Procedure



Level of Treatment	N	Inhibition	
		Mean	Std Dev
bac1	6	0.43833333	0.07222650
bac2	6	0.30666667	0.18522059
bac3	6	0.33833333	0.18872378
bac4	6	0.45500000	0.07314369
bac5	6	0.32166667	0.25063253
bac6	6	0.33333333	0.22321888
bac7	6	0.37166667	0.21169947

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The ANOVA Procedure

Class Level Information		
Class	**Levels**	**Values**
Treatment	12	C100 C200 C300 R12C100 R12C200 R12C300 R13C100 R13C200 R13C300 R14C100 R14C200 R14C300
rep	3	1 2 3

Number of Observations Read	73
Number of Observations Used	72

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The ANOVA Procedure

Dependent Variable: Inhibition

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	13	0.15493472	0.01191806	0.94	0.5165
Error	58	0.73316389	0.01264076		
Corrected Total	71	0.88809861			

R-Square	Coeff Var	Root MSE	Inhibition Mean
0.174457	25.54446	0.112431	0.440139

Source	DF	Anova SS	Mean Square	F Value	Pr > F
Treatment	11	0.13904861	0.01264078	1.00	0.4577
rep	2	0.01588611	0.00794306	0.63	0.5371

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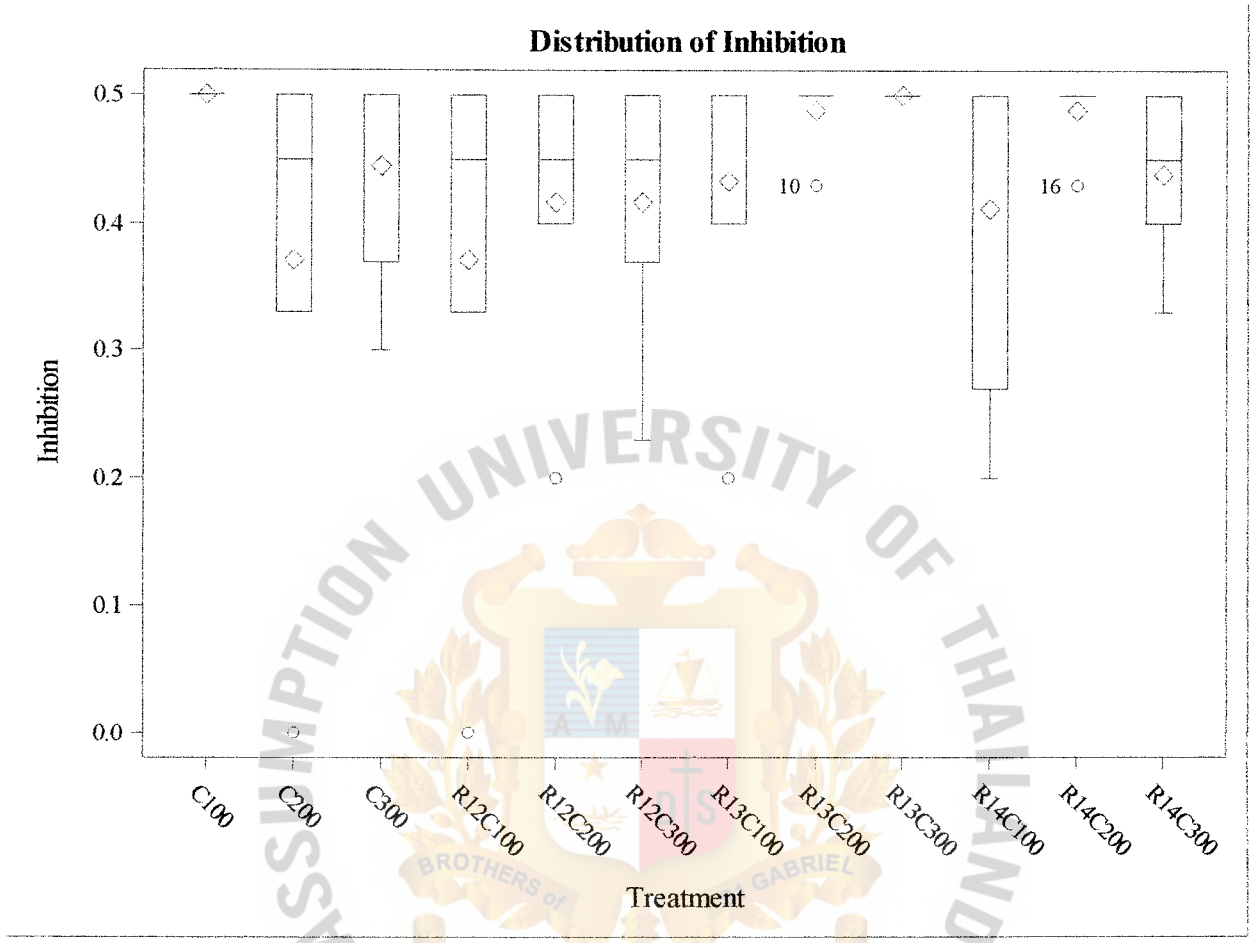
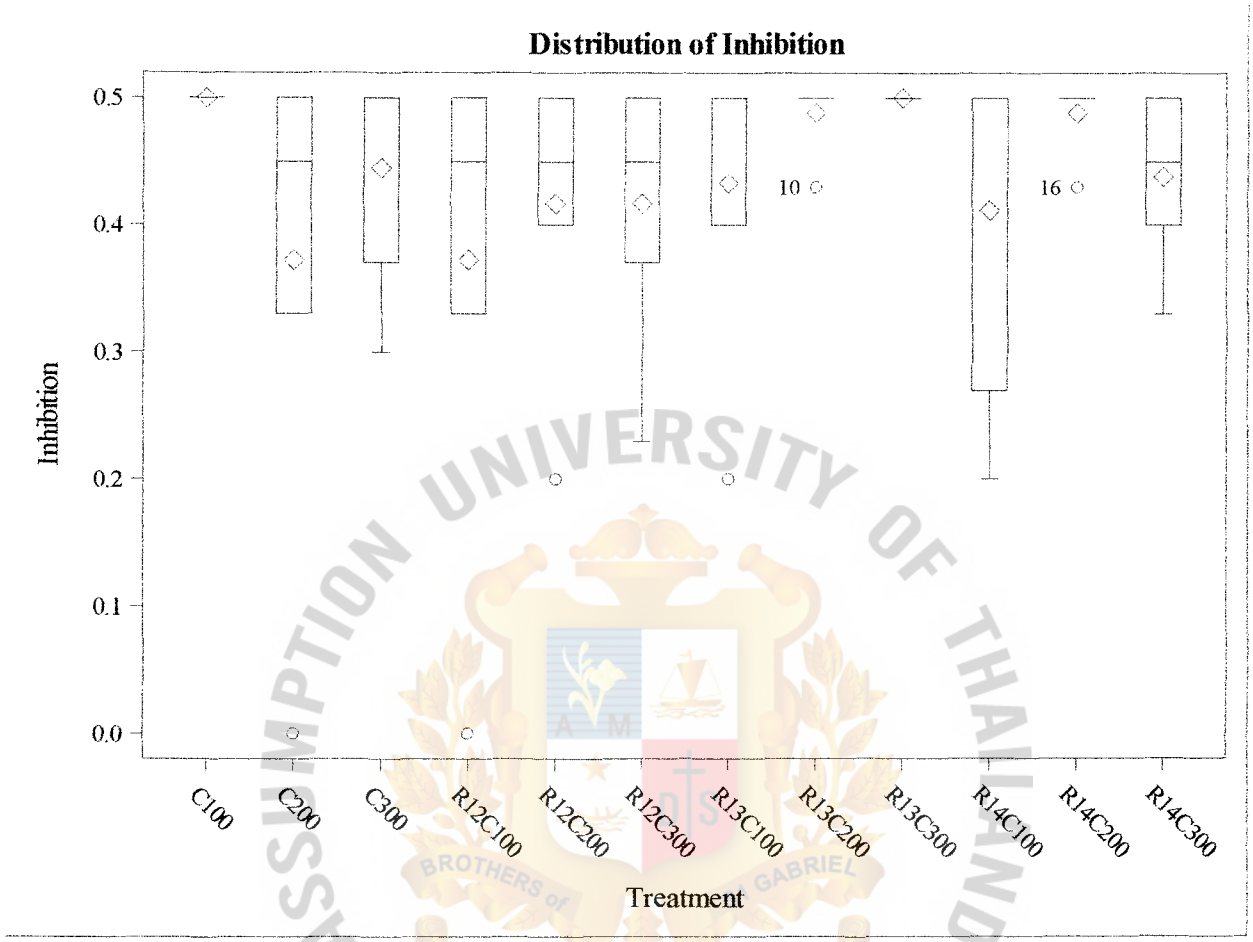
The ANOVA Procedure



Level of Treatment	N	Inhibition Mean	Inhibition Std Dev
C100	6	0.50000000	0.00000000
C200	6	0.37166667	0.19497863
C300	6	0.44500000	0.08803408
R12C100	6	0.37166667	0.19497863
R12C200	6	0.41666667	0.11690452
R12C300	6	0.41666667	0.10782702
R13C100	6	0.43333333	0.12110601
R13C200	6	0.48833333	0.02857738
R13C300	6	0.50000000	0.00000000
R14C100	6	0.41166667	0.13862419
R14C200	6	0.48833333	0.02857738
R14C300	6	0.43833333	0.07222650

The SAS System

The ANOVA Procedure



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The SAS System

The ANOVA Procedure

Duncan's Multiple Range Test for Inhibition

Note: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	58
Error Mean Square	0.012641

Number of Means	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Critical Range	.1299	.1367	.1411	.1444	.1469	.1489	.1505	.1519	.1530	.1540	.1549

Means with the same letter are not significantly different.			
Duncan Grouping	**Mean**	**N**	**Treatment**
A	0.50000	6	C100
A			
A	0.50000	6	R13C300
A			
A	0.48833	6	R13C200
A			
A	0.48833	6	R14C200
A			
A	0.44500	6	C300
A			
A	0.43833	6	R14C300
A			
A	0.43333	6	R13C100
A			
A	0.41667	6	R12C300
A			
A	0.41667	6	R12C200
A			
A	0.41167	6	R14C100
A			
A	0.37167	6	C200

The SAS System

The ANOVA Procedure

Duncan's Multiple Range Test for Inhibition

Means with the same letter are not significantly different.			
Duncan Grouping	**Mean**	**N**	**Treatment**
A			
A	0.37167	6	R12C100

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The ANOVA Procedure

Class Level Information		
Class	**Levels**	**Values**
Treatment	12	C100 C200 C300 R12C100 R12C200 R12C300 R13C100 R13C200 R13C300 R14C100 R14C200 R14C300
rep	3	1 2 3

Number of Observations Read	73
Number of Observations Used	72

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The SAS System

The ANOVA Procedure

Dependent Variable: Inhibition

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	13	0.34621389	0.02663184	0.72	0.7325
Error	58	2.13371389	0.03678817		
Corrected Total	71	2.47992778			

R-Square	Coeff Var	Root MSE	Inhibition Mean
0.139606	50.73393	0.191802	0.378056

Source	DF	Anova SS	Mean Square	F Value	Pr > F
Treatment	11	0.30496111	0.02772374	0.75	0.6833
rep	2	0.04125278	0.02062639	0.56	0.5739

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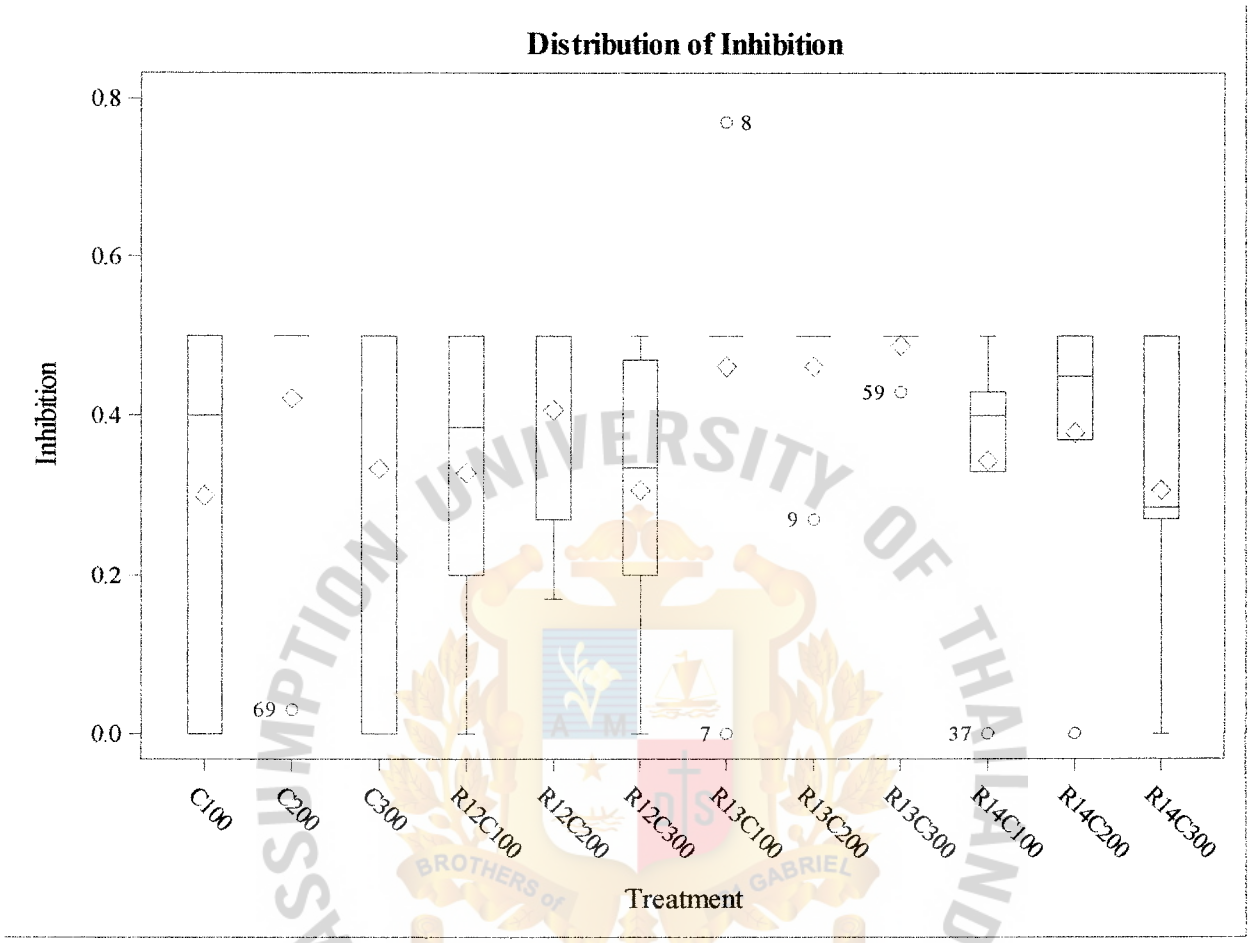
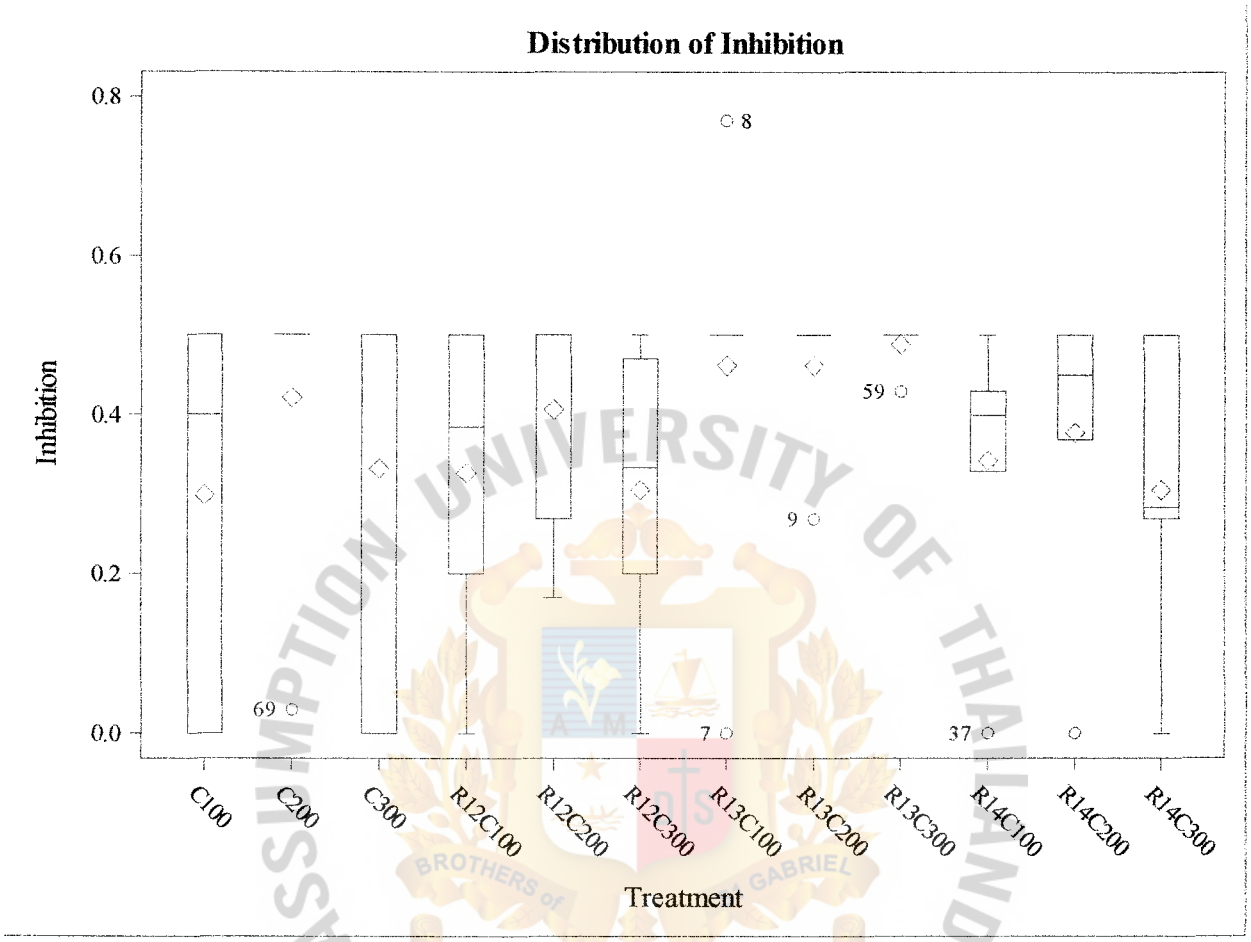
The ANOVA Procedure



Level of Treatment	N	Inhibition Mean	Inhibition Std Dev
C100	6	0.30000000	0.24494897
C200	6	0.42166667	0.19187670
C300	6	0.33333333	0.25819889
R12C100	6	0.32833333	0.19497863
R12C200	6	0.40666667	0.14800901
R12C300	6	0.30666667	0.18949055
R13C100	6	0.46166667	0.25063253
R13C200	6	0.46166667	0.09389711
R13C300	6	0.48833333	0.02857738
R14C100	6	0.34333333	0.17693690
R14C200	6	0.37833333	0.19395017
R14C300	6	0.30666667	0.18522059

The SAS System

The ANOVA Procedure



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The SAS System

The ANOVA Procedure

Duncan's Multiple Range Test for Inhibition

Note: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	58
Error Mean Square	0.036788

Number of Means	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Critical Range	.2217	.2332	.2408	.2463	.2505	.2539	.2567	.2591	.2611	.2628	.2643

Means with the same letter are not significantly different.			
Duncan Grouping	**Mean**	**N**	**Treatment**
A	0.4883	6	R13C300
A			
A	0.4617	6	R13C200
A			
A	0.4617	6	R13C100
A			
A	0.4217	6	C200
A			
A	0.4067	6	R12C200
A			
A	0.3783	6	R14C200
A			
A	0.3433	6	R14C100
A			
A	0.3333	6	C300
A			
A	0.3283	6	R12C100
A			
A	0.3067	6	R12C300
A			
A	0.3067	6	R14C300

The SAS System

The ANOVA Procedure

Duncan's Multiple Range Test for Inhibition

Means with the same letter are not significantly different.			
Duncan Grouping	**Mean**	**N**	**Treatment**
A			
A	0.3000	6	C100

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The SAS System

The ANOVA Procedure

Class Level Information		
Class	**Levels**	**Values**
Treatment	12	C100 C200 C300 R12C100 R12C200 R12C300 R13C100 R13C200 R13C300 R14C100 R14C200 R14C300
rep	3	1 2 3

Number of Observations Read	73
Number of Observations Used	72

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The SAS System

The ANOVA Procedure

Dependent Variable: Inhibition

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	13	0.36302222	0.02792479	1.58	0.1193
Error	58	1.02822222	0.01772797		
Corrected Total	71	1.39124444			

R-Square	Coeff Var	Root MSE	Inhibition Mean
0.260933	31.16561	0.133146	0.427222

Source	DF	Anova SS	Mean Square	F Value	Pr > F
Treatment	11	0.33247778	0.03022525	1.70	0.0950
rep	2	0.03054444	0.01527222	0.86	0.4279

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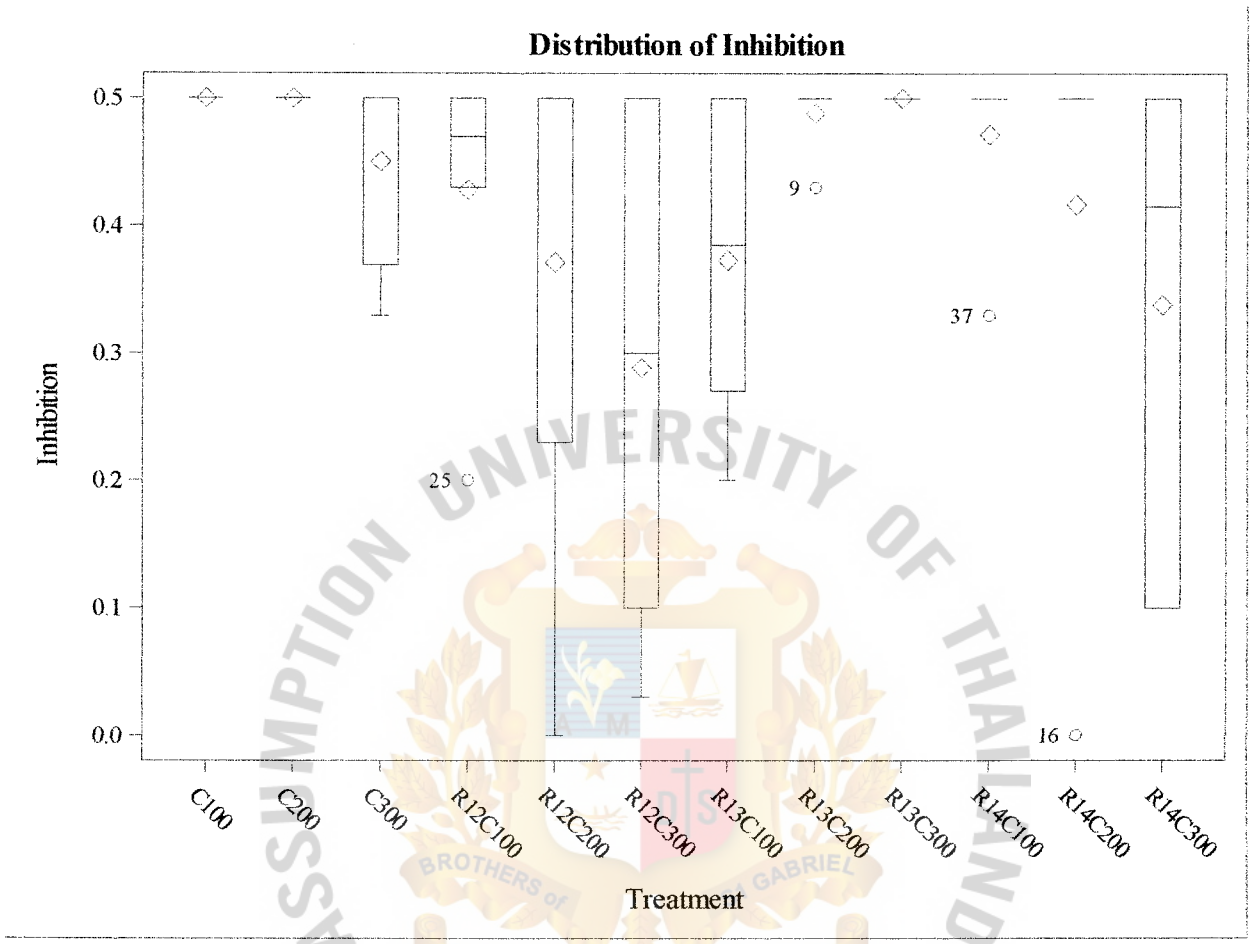
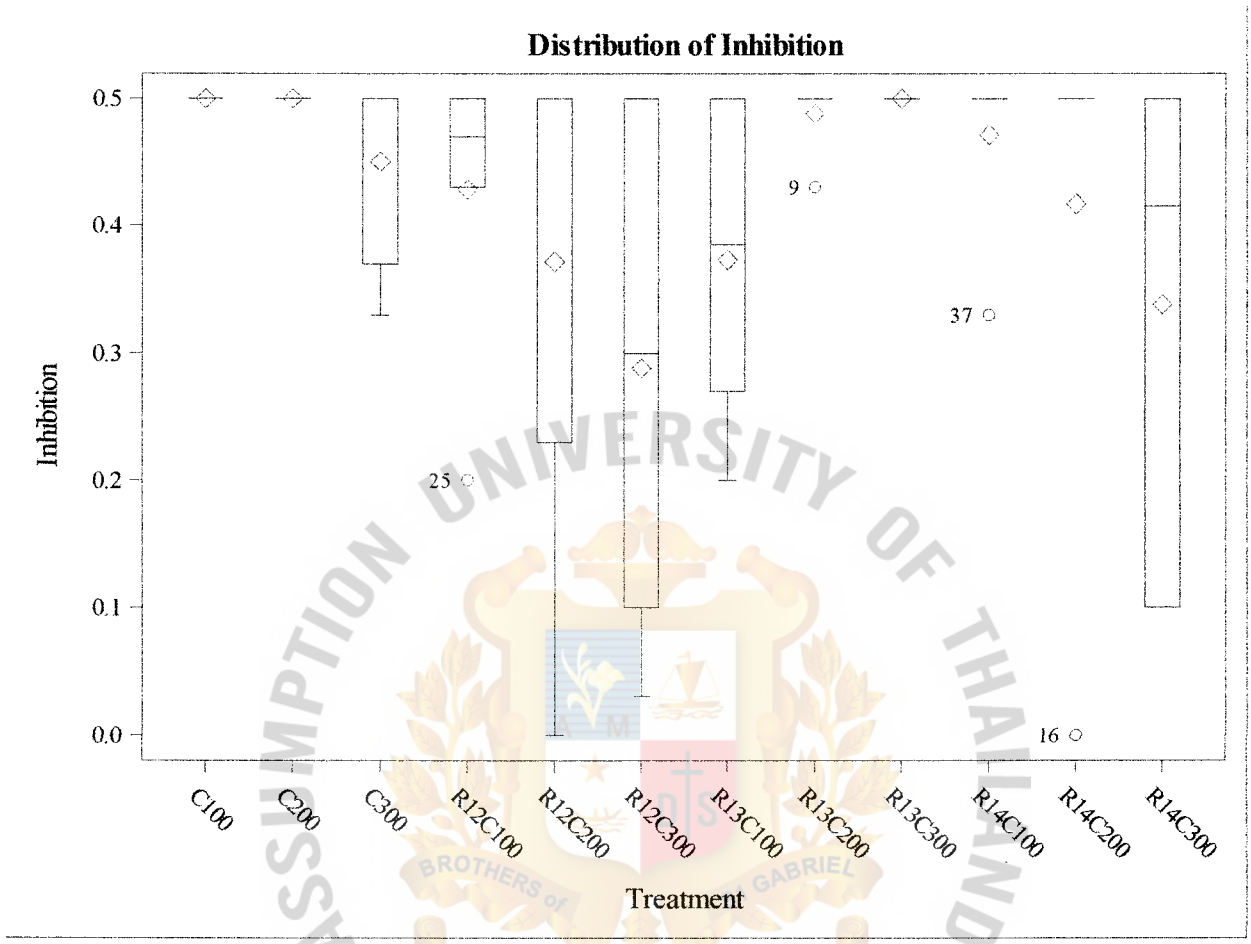
The ANOVA Procedure



Level of Treatment	N	Inhibition Mean	Inhibition Std Dev
C100	6	0.50000000	0.00000000
C200	6	0.50000000	0.00000000
C300	6	0.45000000	0.07848567
R12C100	6	0.42833333	0.11478966
R12C200	6	0.37166667	0.21169947
R12C300	6	0.28833333	0.22355462
R13C100	6	0.37333333	0.12127105
R13C200	6	0.48833333	0.02857738
R13C300	6	0.50000000	0.00000000
R14C100	6	0.47166667	0.06940221
R14C200	6	0.41666667	0.20412415
R14C300	6	0.33833333	0.18872378

The SAS System

The ANOVA Procedure



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The SAS System

The ANOVA Procedure

Duncan's Multiple Range Test for Inhibition

Note: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	58
Error Mean Square	0.017728

Number of Means	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Critical Range	.1539	.1619	.1671	.1710	.1739	.1763	.1782	.1798	.1812	.1824	.1835

Means with the same letter are not significantly different.

Duncan Grouping		**Mean**	**N**	**Treatment**
	A	0.50000	6	C100
	A			
	A	0.50000	6	C200
	A			
	A	0.50000	6	R13C300
	A			
	A	0.48833	6	R13C200
	A			
	A	0.47167	6	R14C100
	A			
B	A	0.45000	6	C300
B	A			
B	A	0.42833	6	R12C100
B	A			
B	A	0.41667	6	R14C200
B	A			
B	A	0.37333	6	R13C100
B	A			
B	A	0.37167	6	R12C200
B	A			
B	A	0.33833	6	R14C300

The SAS System

The ANOVA Procedure

Duncan's Multiple Range Test for Inhibition

Means with the same letter are not significantly different.				
Duncan Grouping		**Mean**	**N**	**Treatment**
B				
B		0.28833	6	R12C300

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The SAS System

The ANOVA Procedure

Class Level Information		
Class	**Levels**	**Values**
Treatment	12	C100 C200 C300 R12C100 R12C200 R12C300 R13C100 R13C200 R13C300 R14C100 R14C200 R14C300
rep	3	1 2 3

Number of Observations Read	73
Number of Observations Used	72

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The ANOVA Procedure

Dependent Variable: Inhibition

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	13	0.04788472	0.00368344	2.73	0.0045
Error	58	0.07824722	0.00134909		
Corrected Total	71	0.12613194			

R-Square	Coeff Var	Root MSE	Inhibition Mean
0.379640	7.558037	0.036730	0.485972

Source	DF	Anova SS	Mean Square	F Value	Pr > F
Treatment	11	0.01954861	0.00177715	1.32	0.2385
rep	2	0.02833611	0.01416806	10.50	0.0001

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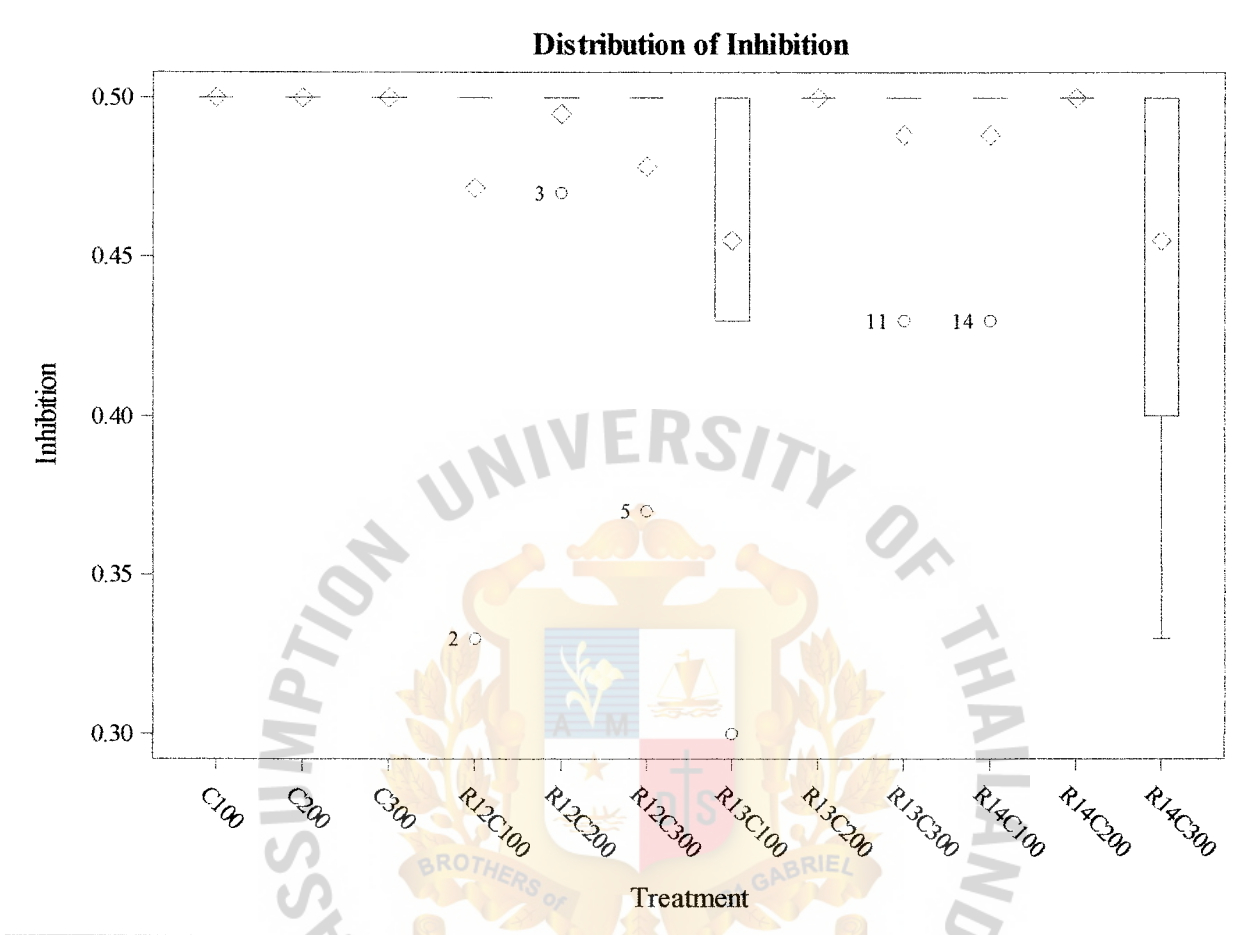
The SAS System

The ANOVA Procedure



Level of Treatment	N	Inhibition Mean	Inhibition Std Dev
C100	6	0.50000000	0.00000000
C200	6	0.50000000	0.00000000
C300	6	0.50000000	0.00000000
R12C100	6	0.47166667	0.06940221
R12C200	6	0.49500000	0.01224745
R12C300	6	0.47833333	0.05307228
R13C100	6	0.45500000	0.08093207
R13C200	6	0.50000000	0.00000000
R13C300	6	0.48833333	0.02857738
R14C100	6	0.48833333	0.02857738
R14C200	6	0.50000000	0.00000000
R14C300	6	0.45500000	0.07314369

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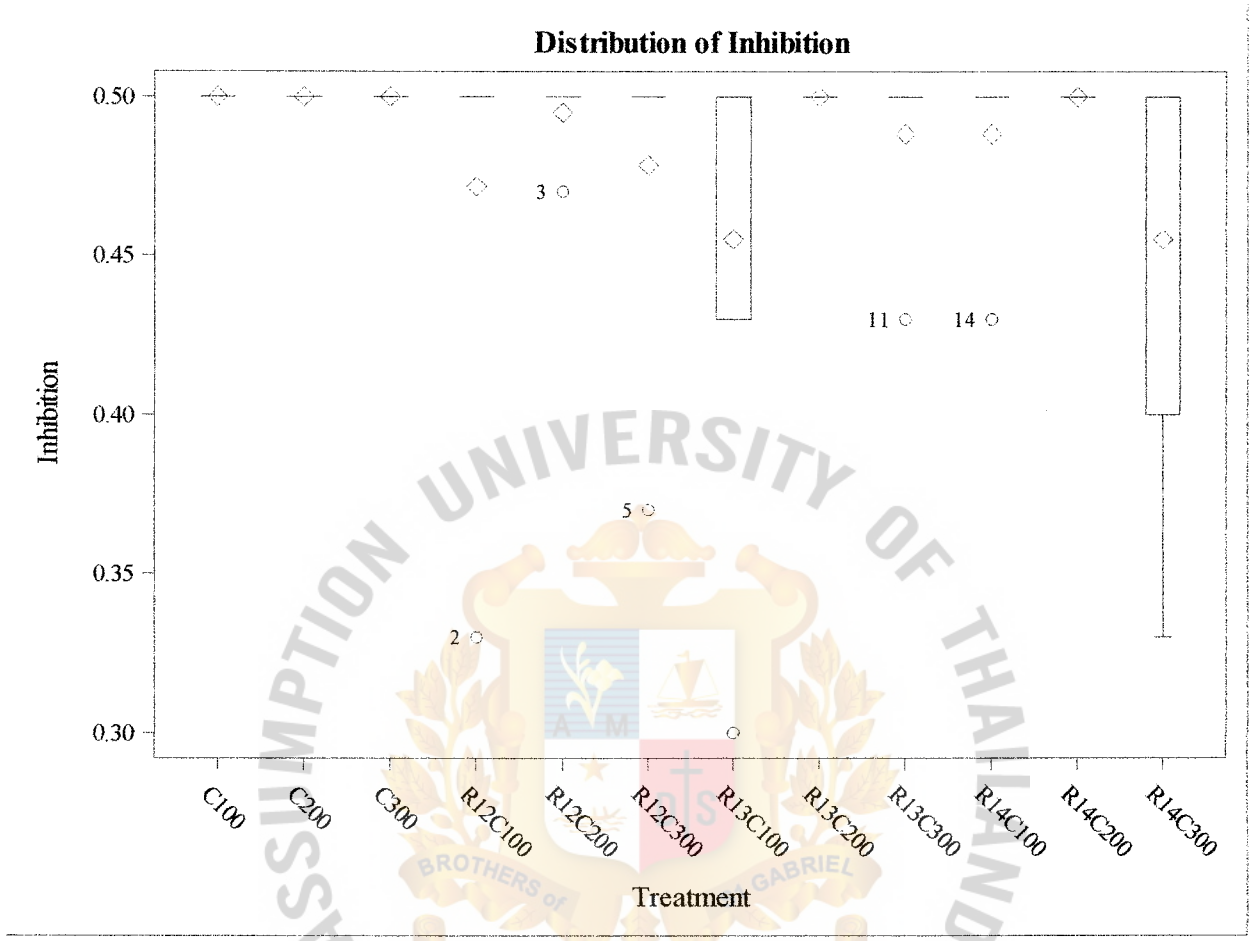
The SAS System

The ANOVA Procedure



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The SAS System

The ANOVA Procedure

Duncan's Multiple Range Test for Inhibition

Note: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	58
Error Mean Square	0.001349

Number of Means	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Critical Range	.04245	.04465	.04611	.04716	.04798	.04863	.04916	.04961	.04999	.05032	.05061

Means with the same letter are not significantly different.			
Duncan Grouping	**Mean**	**N**	**Treatment**
A	0.50000	6	C100
A			
A	0.50000	6	C200
A			
A	0.50000	6	C300
A			
A	0.50000	6	R13C200
A			
A	0.50000	6	R14C200
A			
A	0.49500	6	R12C200
A			
A	0.48833	6	R14C100
A			
A	0.48833	6	R13C300
A			
A	0.47833	6	R12C300
A			
A	0.47167	6	R12C100
A			
A	0.45500	6	R13C100

The SAS System

The ANOVA Procedure

Duncan's Multiple Range Test for Inhibition

Means with the same letter are not significantly different.			
Duncan Grouping	**Mean**	**N**	**Treatment**
A			
A	0.45500	6	R14C300

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The ANOVA Procedure

Class Level Information		
Class	**Levels**	**Values**
Treatment	12	C100 C200 C300 R12C100 R12C200 R12C300 R13C100 R13C200 R13C300 R14C100 R14C200 R14C300
rep	3	1 2 3

Number of Observations Read	73
Number of Observations Used	72

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The ANOVA Procedure

Dependent Variable: Inhibition

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	13	0.66118472	0.05086036	2.48	0.0092
Error	58	1.18774722	0.02047840		
Corrected Total	71	1.84893194			

R-Square	Coeff Var	Root MSE	Inhibition Mean
0.357604	33.16189	0.143103	0.431528

Source	DF	Anova SS	Mean Square	F Value	Pr > F
Treatment	11	0.20254861	0.01841351	0.90	0.5467
rep	2	0.45863611	0.22931806	11.20	<.0001

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The SAS System

The ANOVA Procedure

Class Level Information		
Class	**Levels**	**Values**
Treatment	12	C100 C200 C300 R12C100 R12C200 R12C300 R13C100 R13C200 R13C300 R14C100 R14C200 R14C300
rep	3	1 2 3

Number of Observations Read	73
Number of Observations Used	72

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The SAS System

The ANOVA Procedure

Dependent Variable: Inhibition

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	13	0.61680139	0.04744626	1.42	0.1800
Error	58	1.94326389	0.03350455		
Corrected Total	71	2.56006528			

R-Square	Coeff Var	Root MSE	Inhibition Mean
0.240932	52.40182	0.183042	0.349306

Source	DF	Anova SS	Mean Square	F Value	Pr > F
Treatment	11	0.55691528	0.05062866	1.51	0.1524
rep	2	0.05988611	0.02994306	0.89	0.4147

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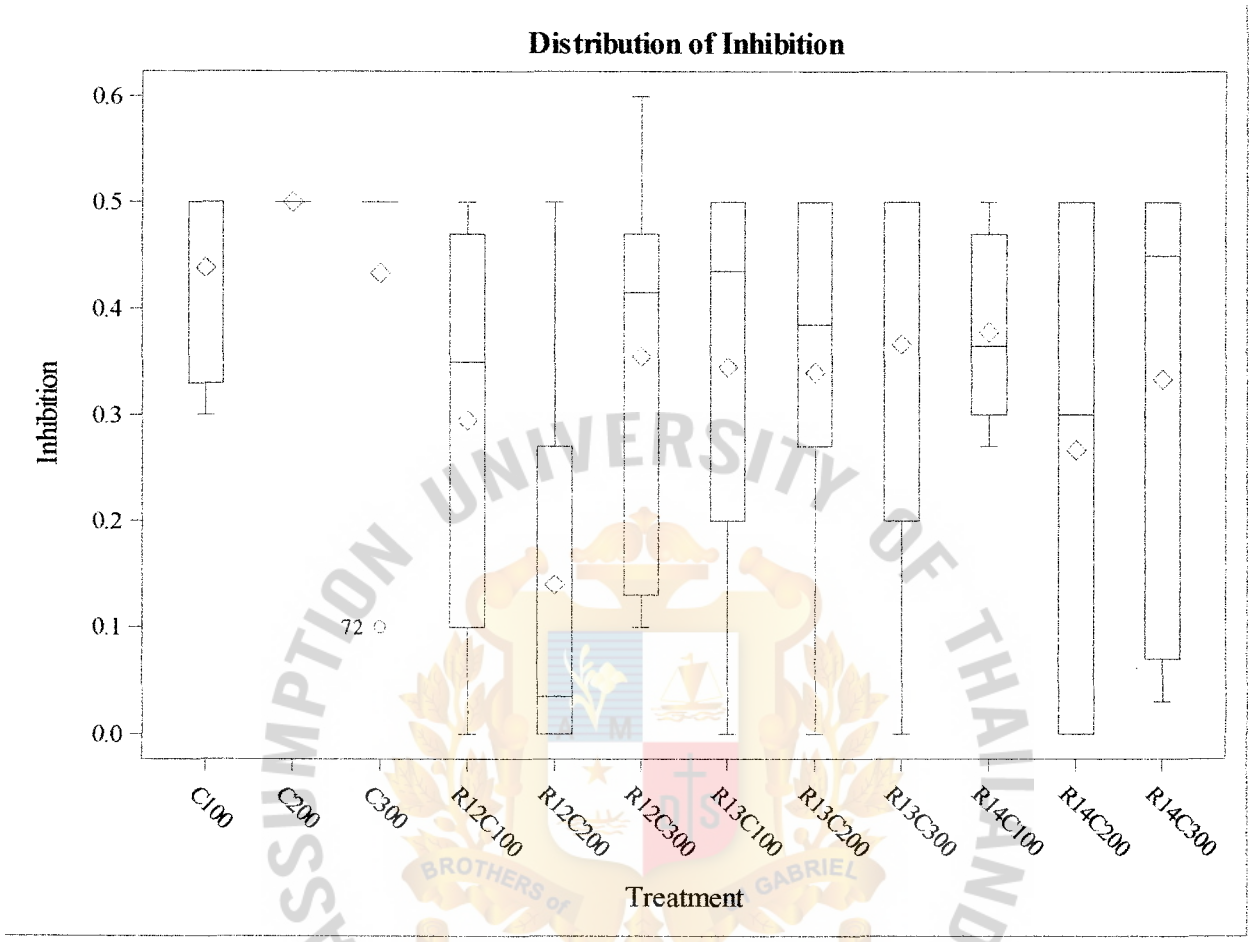
The ANOVA Procedure



Level of Treatment	N	Inhibition Mean	Inhibition Std Dev
C100	6	0.43833333	0.09600347
C200	6	0.50000000	0.00000000
C300	6	0.43333333	0.16329932
R12C100	6	0.29500000	0.21566177
R12C200	6	0.14000000	0.20503658
R12C300	6	0.35500000	0.19826750
R13C100	6	0.34500000	0.20627651
R13C200	6	0.34000000	0.19503846
R13C300	6	0.36666667	0.21602469
R14C100	6	0.37833333	0.09368387
R14C200	6	0.26666667	0.22589083
R14C300	6	0.33333333	0.22321888

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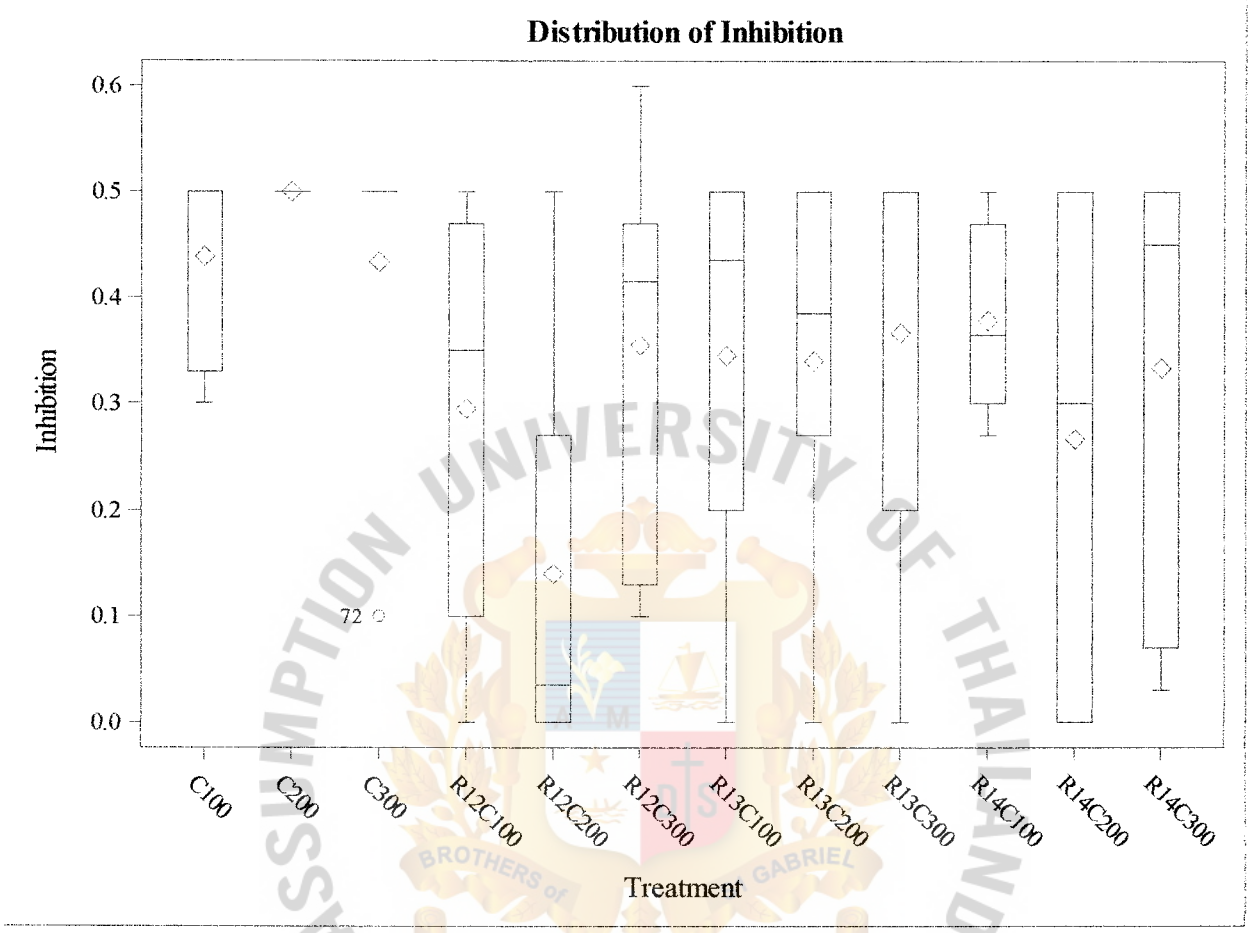
The ANOVA Procedure



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The SAS System

The ANOVA Procedure

Duncan's Multiple Range Test for Inhibition

Note: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	58
Error Mean Square	0.033505

Number of Means	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Critical Range	.2115	.2225	.2298	.2350	.2391	.2423	.2450	.2472	.2491	.2508	.2522

Means with the same letter are not significantly different.

Duncan Grouping		**Mean**	**N**	**Treatment**
	A	0.5000	6	C200
	A			
	A	0.4383	6	C100
	A			
	A	0.4333	6	C300
	A			
B	A	0.3783	6	R14C100
B	A			
B	A	0.3667	6	R13C300
B	A			
B	A	0.3550	6	R12C300
B	A			
B	A	0.3450	6	R13C100
B	A			
B	A	0.3400	6	R13C200
B	A			
B	A	0.3333	6	R14C300
B	A			
B	A	0.2950	6	R12C100
B	A			
B	A	0.2667	6	R14C200

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The ANOVA Procedure

Duncan's Multiple Range Test for Inhibition

Means with the same letter are not significantly different.				
Duncan Grouping		**Mean**	**N**	**Treatment**
B				
B		0.1400	6	R12C200

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The ANOVA Procedure

Class Level Information		
Class	**Levels**	**Values**
Treatment	12	C100 C200 C300 R12C100 R12C200 R12C300 R13C100 R13C200 R13C300 R14C100 R14C200 R14C300
rep	3	1 2 3

Number of Observations Read	73
Number of Observations Used	72

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The SAS System

The ANOVA Procedure

Dependent Variable: Inhibition

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	13	0.64464167	0.04958782	1.27	0.2547
Error	58	2.25695833	0.03891307		
Corrected Total	71	2.90160000			

R-Square	Coeff Var	Root MSE	Inhibition Mean
0.222168	60.38693	0.197264	0.326667

Source	DF	Anova SS	Mean Square	F Value	Pr > F
Treatment	11	0.26553333	0.02413939	0.62	0.8040
rep	2	0.37910833	0.18955417	4.87	0.0111

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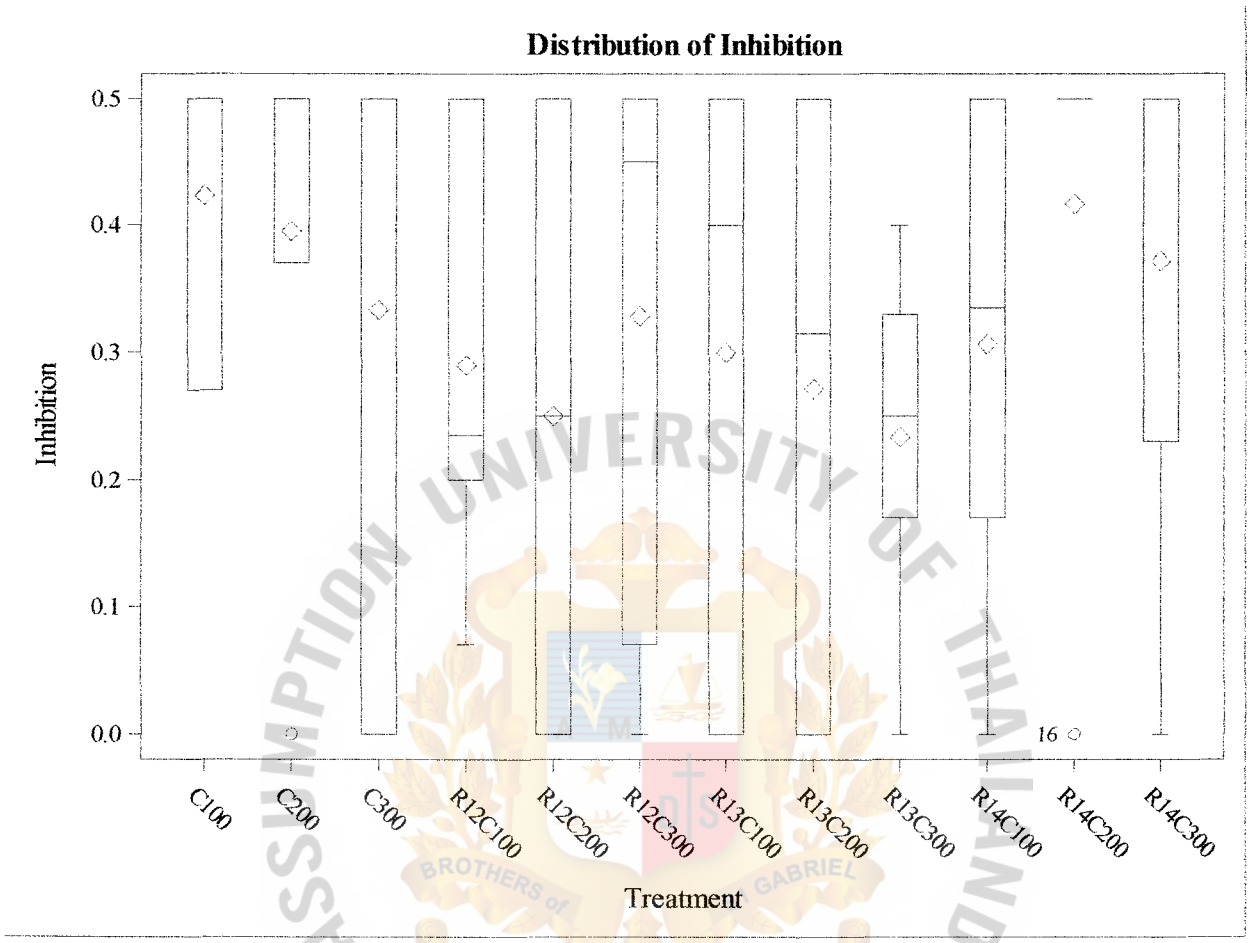
The ANOVA Procedure



Level of Treatment	N	Inhibition Mean	Inhibition Std Dev
C100	6	0.42333333	0.11877149
C200	6	0.39500000	0.20037465
C300	6	0.33333333	0.25819889
R12C100	6	0.29000000	0.17504285
R12C200	6	0.25000000	0.22583180
R12C300	6	0.32833333	0.23155273
R13C100	6	0.30000000	0.24494897
R13C200	6	0.27166667	0.25459118
R13C300	6	0.23333333	0.14222049
R14C100	6	0.30666667	0.19876284
R14C200	6	0.41666667	0.20412415
R14C300	6	0.37166667	0.21169947

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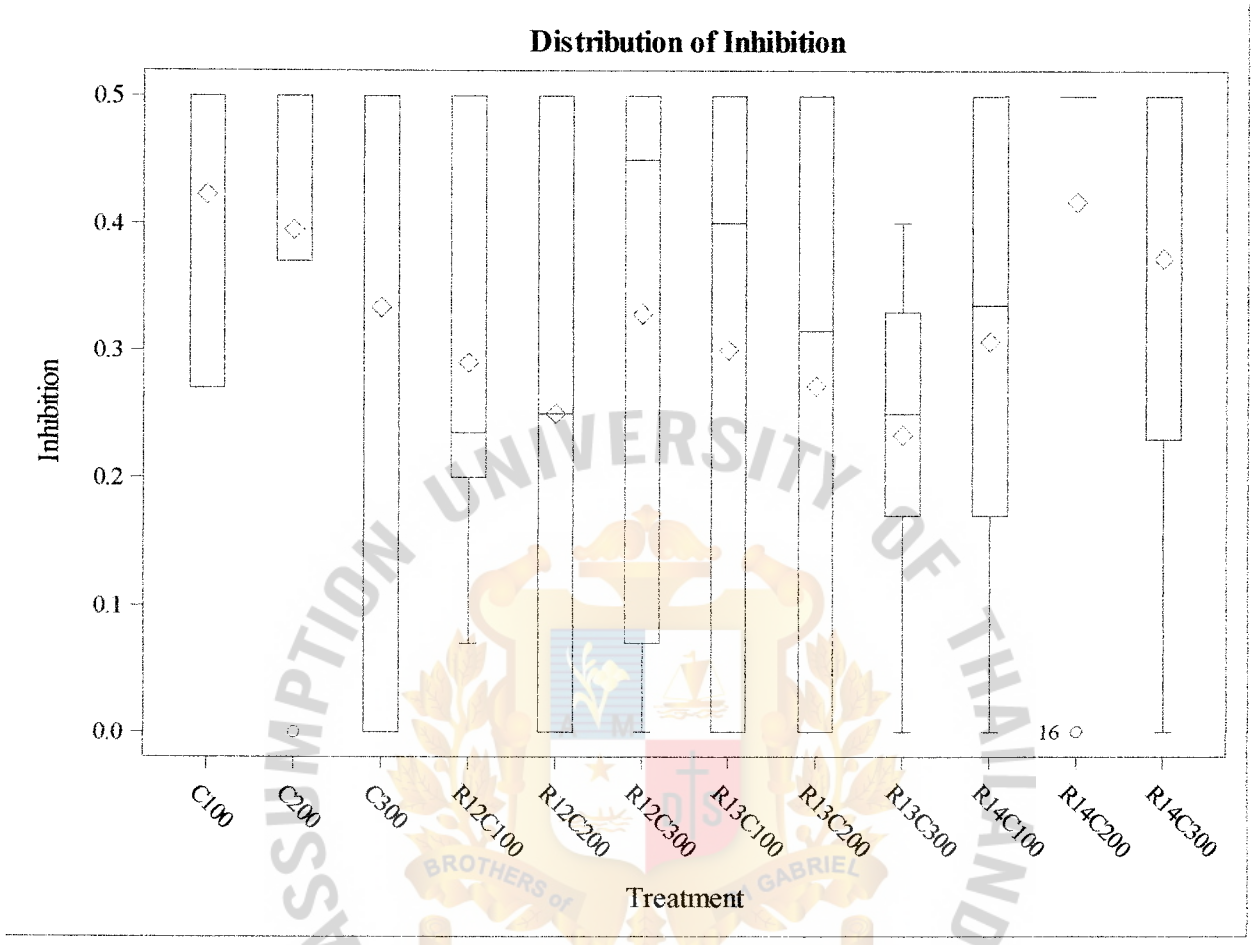
The ANOVA Procedure



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The ANOVA Procedure

Duncan's Multiple Range Test for Inhibition

Note: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	58
Error Mean Square	0.038913

Number of Means	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Critical Range	.2280	.2398	.2476	.2533	.2577	.2612	.2640	.2664	.2685	.2703	.2718

Means with the same letter are not significantly different.

Duncan Grouping	**Mean**	**N**	**Treatment**
A	0.4233	6	C100
A			
A	0.4167	6	R14C200
A			
A	0.3950	6	C200
A			
A	0.3717	6	R14C300
A			
A	0.3333	6	C300
A			
A	0.3283	6	R12C300
A			
A	0.3067	6	R14C100
A			
A	0.3000	6	R13C100
A			
A	0.2900	6	R12C100
A			
A	0.2717	6	R13C200
A			
A	0.2500	6	R12C200

The SAS System

The ANOVA Procedure

Duncan's Multiple Range Test for Inhibition

Means with the same letter are not significantly different.			
Duncan Grouping	**Mean**	**N**	**Treatment**
A			
A	0.2333	6	R13C300

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