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A Randomized Double-Blind Placebo-Controlled Study on the Efficacy of Oregano (Origanum Vulgare) Leaf Ointment on the Reduction of 24-Hour Colony Forming Units of Staphylococcus Aureus in Infected Open Wounds of Children Aged 8-12 Years Old

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ABSTRACT

The practice of traditional medicine has been a vital part of Philippine medical practice, and, as a consequence, has led to innumerable researches conducted by the Department of Health (DOH) as to their role in the Filipino well-being. Out of the 10 named herbal plants studied by the department, oregano (*Origanum vulgare*) was chosen for its uses on the disorders of the skin, gastrointestinal and respiratory tract in the communities; and was therefore used in this study for the evaluation of its antimicrobial property, formulated as a leaf ointment against *Staphylococcus aureus*.

OBJECTIVES: Specifically, this research aimed to determine a significant difference between the colony forming units (CFUs) on infected wounds treated with the placebo and oregano (*Origanum vulgare*) ointments.

METHODOLOGY: A randomized double-blind placebo-controlled research study was conducted to the 60 infected wounds of the students of Dona Juana Actub Lluch Memorial Central School and Ubaldo Laya Memorial Central School with respondents aging 8-12 years old. Pre-treatment wound swabbing was done for 24-hour microbial culture, from which CFUs were counted. Post-treatment swabbing was performed after 5 days of treatment and subsequent CFUs were obtained. Using 0.05 level of significance, Mann-Whitney Pairwise Comparison Test was used to determine the significant difference in CFU count reduction between the two treatments. Findings showed reduction of CFUs in both ointments with greater reduction of CFUs in wounds treated Oregano leaf ointment as compared to those with the placebo. Statistical analysis of the study revealed that there was a significant difference between Placebo and Oregano (*Origanum vulgare*) on the reduction of CFUs in a 24-hour bacterial culture of infected open wounds.

Keywords: Oregano, Ointment, Wound

INTRODUCTION

It is a remarkable evolution of medicine as a practice to be influenced by religion, superstition and

folklore. In the Philippines, traditional medicine has been accepted as an integral part of well-being as stated in Republic Act 8423, otherwise known as the Traditional and Alternative Medicine Act (TAMA) of 1997.However, as to date, there are only ten (10) herbal plants scientifically studied and endorsed by the Department of Health (DOH). This has become the opportunity of health-affiliated personnel to conduct logical researches in order to prove that other herbs are beneficial to human health – and in this study, the researchers have chosen to focus in the use of Oregano (*Oreganum vulgare L*) leaves as antimicrobial ointment.

Oregano, locally known as *Kalabo*, is a perennial herb that grows abundantly in the Philippines. It is a common species of *Origanum* under the genus of the mint family *Lamiaceae*. Traditionally, by boiling one cup of fresh leaves in 3 cups of water for 10-15 minutes, it can be taken as treatment for common colds. For insect bites, wounds and stings, the oregano leaves are applied directly as poultice on the affected area. Its concentrated juice is also being used to relieve chronic coughs, rheumatism, asthma and dyspepsia (Philippine Herbal Medicine, 2014).

Objectives of the Study

This study was conducted mainly to evaluate the antimicrobial property of Oregano (*O. vulgare* L.) leaf ointment against *Staphylococcus aureus*, as compared to the placebo ointment.

This research specifically aims to,

1. determine the number of colony forming units (CFUs) in a 24-hour bacterial culture of the infected open wound prior to treatment.
2. determine the number of CFUs in a 24-hour bacterial culture of the infected open wound after the 5-day treatment with placebo ointment.
3. determine the number of CFUs in a 24-hour bacterial culture of the infected open wound after the 5-day treatment with Oregano ointment.
4. determine the significant difference between the placebo ointment and oregano ointment in reducing the number of CFUs in the bacterial culture of the infected open wound.

Hypothesis

H_0: There is no significant difference between the placebo ointment and oregano ointment in reducing the number of colony forming units in the bacterial culture of the infected open wound.

H_a: There is a significant difference between the placebo ointment and oregano ointment in reducing the number of colony forming units in the bacterial culture of the infected open wound.

Significance of the Study

The Oregano ointment is a cheaper alternative to the commercial ointment used to treat infected open wounds. Secondarily, the oregano ointment is easy to replicate in the community setting, and can be produced within 15-20 minutes only. Moreover, the ointment can last for 30 days, thus the availability of this herbal medication decreases the careless use of other herbs that do not provide medicinal benefits.

Limitations of the Study

The antimicrobial property of Oregano against *Staphylococcus aureus* was assessed microscopically in terms of the reduction of the number of colony forming units (CFUs) in a 24-hour bacterial culture of the

infected open wound pre- and post- treatment with Oregano leaves ointment. Its antimicrobial property against other strains of bacteria was not included.

Its role in facilitating and accelerating the process of wound healing was not assessed. Thus, a reduction in the wounds' surface area does not have bearing on this study. A phytochemical analysis on oregano extract was also not done.

Operational Definition of Terms

Open Wounds - a disruption in the integrity causing underlying tissues of the skin to be exposed, including any type of wound except incised wound.

Signs of infection - purulent discharge, abnormal smell coming from the wound site, and discoloration of tissues both within and around the wound margins.

Wound Healing - the reduction in the number of colony forming units in the bacterial culture of the infected open wound.

REVIEW OF RELATED LITERATURE

Wound Healing

A. Physiology of Wound Healing

Wound healing is a complex cellular and biochemical cascade that leads to restitution of integrity and function. Wound healing progression comprised systematical processes of events which repair the damaged tissue partially or completely (Kondo, 2007). Normal wound healing follows a predictable pattern that can be divided into overlapping phases defined by characteristic cellular populations and biochemical activities: (a) homeostasis and inflammation, (b) proliferation, and (c) maturation and remodelling. All wounds need to progress through this series of cellular and biochemical events that characterizes the phases of healing in order to successfully re-establish tissue integrity (Schwartz, 2010). It involves the activity of an intricate network of blood cells, cytokines, and growth factors which ultimately leads to the restoration to normal condition of the injured skin or tissue (Clark, 1991). The aim of wound care is to promote wound healing in the shortest time possible, with minimal pain, discomfort, and scarring to the patient and must occur in a physiologic environment conducive to tissue repair and regeneration (Bowler *et al*., 2001).

Wound healing process is promoted efficiently by the use of traditional remedies which are mainly based on plant sources. These remedies have been shown to affect one or more stages of the healing process. In this context, traditional medicines provide a vast source for the discovery of original drug leads (Akkol *et al*., 2011).

B. Wound Infections

The most common cause of healing delays is wound infection. About three-fourths of all wound infections are superficial, involving skin and subcutaneous tissue only. Clinical diagnosis is easy when a wound looks edematous and erythematous and is tender (Sabiston, 2012). Early phase of infection is characterized by a waxy appearance of the skin. The wound appears irregular, raised above the surface, and has a white, pearly discoloration. A bacterial count which exceeds 10^5 organism of gram per tissue or an infection by *Staphylococcus* species which is the most frequent infecting organism, coagulase-negative *Streptococcus*, *Enterococci* and *E. coli*, usually causes a delay in wound healing (Schwartz, 2010).

Oregano (*Origanum vulgare* L.)

A. Description

O. vulgare L. is a medicinal and perennial plant, locally known as Jungali Tulsi or Oregano or Himalayan marjoram. The taxonomic classification of the plant is:

Kingdom: Plantae
Class: Magnoliopsida
Order: Lamiales
Family: Lamiaceae
Genus:*Origanum* L.
Species:*Origanum vulgare* L.

Recently, the World Health Organization estimated that 80% of people worldwide rely on herbal medicines for some aspect of their primary healthcare (Chhetri *et al*., 2010). Oregano has been a valuable source of natural products for maintaining human health for a long period of time, especially in last decade, with more intensive studies for natural therapies (Force *et al*., 2000). Genus *Origanum* is important medicinally as it has antimicrobial, antifungal, antioxidant, antibacterial, antithrombin, antimutagenic, angiogenic, antiparasitic and antihyperglycemic activities (Chishti, 2013). Oregano oil has been used for the first aid treatment of wounds, abscess and burns (Cuttle *et al*., 2009; Steflictsch, 2009). The volatile oil of Oregano has been used traditionally for respiratory disorders, indigestion, dental caries, rheumatoid arthritis, and urinary tract disorders (Chaudhry *et al*., 2007).

B. Biological Components of Oregano (*Origanum vulgare* L.)

Whole plant extracts have many components. These components work together to produce desired therapeutic effect. The most important components of Oregano are the limonene, gamma-cariofilene, rho-cymenene, canfor, linalool, alpha-pinene, carvacol and thymol (Arcila-Lozano *et al*., 2004). Thymol and carvacrol are the main components of the essential oil of Oregano (Tian and Lai, 2006), which are responsible for its antioxidative, antimicrobial and antifungal effects (Proestos *et al*., 2005).

Origanum oil, mainly rich in carvacrol, is used as a painkiller in rheumatism by rubbing externally on painful limbs (Bakkali *et al*., 2008). The Oregano water, rich in carvacrol, is consumed to check gastrointestinal disorders, reduce blood cholesterol and glucose level and also for tumor suppressive activities (Goze *et al*., 2010); against whooping and convulsive coughs, digestive disorders and menstrual problems. In Northern Peru, leaves and stems, fresh or dried, of oregano are employed as traditional remedies for menstrual cramps, menstruation and lower stomach cramps related to premenstrual stages (Bussmann and Glenn, 2010). In Turkish folk medicine thyme or oregano oils obtained from various "kekik" species (mainly *Origanum* sp., *Thymus* sp., *Thymbra* sp. or *Satureija* sp.) have been used as a remedy against wounds and burns (Dursun *et al*., 2003; Altiok *et al*., 2010).

C. Phytochemical constituents of *O. vulgare* (Oregano) and its mechanism of action on Wound Healing

Flavonoids inhibit a perplexing number and variety of enzymes and have a tremendously wide range of antimicrobial activities. They have been reported to possess many useful properties, including anti-inflammatory activity, estrogenic activity, enzyme inhibition, antimicrobial activity antiallergic activity, antioxidant activity, vascular activity and cytotoxic antitumor activity (Cushnie and Lamb, 2005).

Tannins act by iron deprivation, hydrogen bounding or non specific interactions with vital proteins such as enzymes (Scalbert, 1991).

The main *indoloquinoline alkaloid* causes cell lysis and morphological changes of *S. aureus*, but the antimicrobial effects of the alkaloid may be through another mechanism, since the compound is known to be a DNA intercalator and an inhibitor of DNA synthesis through topoisomerase inhibition (Sawer *et al*., 2005).

Pure *saponin* fraction exhibited remarkable antibacterial activity. The antibacterial activity of extracts was found to be comparatively higher than the standard antibiotics which provide evidence for the antagonistic activity of steroidal saponin against tested bacterial pathogens (Khanna, 2009).

A literature survey revealed significant biological activity of natural *anthraquinones* including astringent, anti- inflammatory, antitumor, purgative, and bactericide effects. The mechanism of action involves an increase in the levels of superoxide anion O_2^- and/or singlet molecular oxygen O_2 (Albesa *et al*., 2011).

D. Traditional Uses of Oregano in the Philippines

Oregano (*O. vulgare* L.), an aromatic plant, is considered of great interest for its flavor and medicinal properties (Falco *et al.*, 2013). In other countries, it is primarily used as culinary herb. Volatile oils in oregano are believed to be responsible for slowing the process of spoilage of food, thus, minimizing the risk of ingesting harmful bacteria, parasites and fungi.

In the Philippines, Oregano is used primarily as herbal medicine for its antibacterial and strong antioxidant properties. In order to maximize its efficacy as herbal medicine, preparations vary according to use. For common colds: 1 cup of fresh leaves is boiled in a 3 cups of water for 10 to 15 minutes, half a cup is taken 3 times a daily. For the relief of chronic coughs, rheumatism, bronchitis, asthma, and dyspepsia: 6 tablespoons of juice of oregano leaves is taken every 6 hours. For insect bites, wounds and stings, leaves are applied as poultice directly on the afflicted area. For sore throat, 2 tablespoonful of dried oregano leaves are boiled in a pint of water, and taken 2 hours before or after meals and for the prevention of degenerative arthritis and general health: drink oregano decoction directly (Philippine Herbal Medicine, 2014).

E. Susceptibility of Staphylococcus organisms to Oregano essential oil, carvacrol and thymol

Increasing antibiotic resistance has prompted a search for new compounds with anti-microbial activity. Several studies have been conducted among traditional herbal plants with components which may be potential for the said purpose. Among these plants is the Oregano plant whose extract was identified as one of the most potent anti-microbial compounds in its essential oil including carvacrol and thymol. Carvacrol is an important component of essential oils in Oregano and recently has attracted much attention as a result of its biological properties, such as a wide spectrum of antimicrobial activity (Blanco *et al*., 2009).

In disk diffusion studies, it has been shown that oregano was bacteriostatic for *Staphylococcus aureus* (*S. aureus*) and methicillin-resistant *S. aureus*, (MRSA) but bactericidal for seven other microorganisms. The same study conveyed results that oregano extracts can be formulated into ointments that showed broad antimicrobial activity including activity against methicillin-resistant *Staphylococcus aureus*. It has shown that 1-10% oregano could inhibit most organisms except for *Proteus mirabilis* and *Proteus vulgaris*, which required 20% and *Pseudomonas* which could not be inhibited even at the highest concentration of 80% (Norman, 2010).

METHODOLOGY

Research Design

The study was a randomized double-blind placebo-controlled research with two variables, the placebo and the treatment. It was conducted last January to February 2015.

Study Population

A total of seventy-two (72) students with infected open wounds were screened from the different primary schools of Iligan City, namely: Dona Juana Actub Lluch Elementary School (DJAL), Brgy. Palao, and Ubaldo D. Laya Memorial Central School (UDLMCS), Brgy. Ubaldo Laya.

Screening Criteria

Inclusion Criteria : Subjects, of any gender, aged 8 to 12 years old, with (a.) at least one open wound (b.) wounds with signs of infection, (c.) wounds with surface area of at least two square centimeter, (d.) wounds not treated with any ointment or poultice, (e.) growth of colonies upon analysis of bacterial culture. Each infected wound was counted as one entry, with a total of seventy-two (72) infected open wounds included in the data analysis.

Exclusion Criteria: Incised wounds were excluded. Subjects who were taking anti-inflammatory and/or antibiotic medications for 7 to 10 days prior treatment were also excluded in this study. Moreover, wounds having no colony growth upon analysis of pre-treatment bacterial culture were excluded for data analysis.

Site of the Study

Preparation of the placebo ointment and oregano ointment was done in Palao, Iligan City. The preparation and analysis of artificial media for bacterial culture was done in MSU-Iligan Institute of Technology, College of Medicine (CSM).

Preparation of Materials

A. Preparation of Placebo Ointment

Three hundred (300) millilitres of coconut oil was heated at 30^{o}C, added with 30 grams of paraffin wax. Then, 20 mL of essential oil and food coloring was mixed to the solution. This placebo ointments were packed in plastic cellophanes measuring 1ml each, allowed to cool down, and was coded appropriately.

B. Collection of *Origanum vulgare* leaves

Fresh leaves of oregano (*O. vulgare*) were obtained. The plant samples were gathered within Iligan City, Lanao del Norte and was selected through purposive sampling design. Young leaves were identified by their yellow green color and rough texture.

The leaves were thoroughly cleaned and washed with distilled water, chopped into smaller pieces.

C. Preparation of Oregano Ointment

Two hundred fifty (250) grams of the leaf pieces were heated in 500 mL of coconut oil at 30^{o}C for 5 minutes. After heating, the oil extract was strained with a cheese cloth and allowed to cool down. Three hundred (300) mL of the oil extract was mixed with 30g of paraffin (ointment base). The solution was heated at 30^{o}C for 5 minutes. Similar in placebo ointment, 20 mL essential oil and food coloring was mixed to the solution. The Oregano ointments were packed in plastic cellophanes measuring 1ml each, allowed to cool down, and was coded appropriately.

Data Collection

A. Preliminary Study

The subjects were selected based on the criteria specified on the previous sections. Each subject was oriented to the nature, purpose, method and duration of the study. All subjects affirmed their voluntary involvement in the study and had secured a letter of consent signed by their parent/guardian. Pre-treatment swabs were obtained prior to ointment application, for the determination of CFUs.

B. Implementation of double-blind method

All 72 wounds were coded and had been randomly assigned by the coder to which ointment (i.e. BDEY1, GHHT2) was used throughout the 5-day application. The master list for the codes is shown in Appendix D and E in the discussion of results.

C. Application of Placebo and Oregano Ointment

Each wound was irrigated with distilled water prior to ointment application. The researcher applied 1 ml of the coded ointment per 1 cm^2 of the open wound. Using a cotton applicator, the ointment was spread evenly on the wound surface, and was then covered with sterile gauze.

The ointment application was done twice a day: once in the morning (8:00-10:00am) and once in the afternoon (4:00-6:00pm). Five (5) days of ointment application was completed, for a total of 10 applications per wound for the entire study.

Post-treatment swabs were obtained after the 5-day application, for the determination of CFUs.

Data Analysis

A. Determination of Colony Forming Units (CFUs)

The number of CFUs will be determined using the following equation:

$$\text{Colony Forming Units (CFUs)/ml} = \frac{\text{No. of colonies}}{\text{(Dilution factor) x (Volume of culture plate)}}$$

B. Statistical Test

Two-sample t test will be used to determine the significant difference between post-treatment of placebo ointment and post-treatment of Oregano ointment and between pre-treatment and post-treatment of placebo and oregano ointment on the number of CFUs.

ALGORITHM OF METHODOLOGY

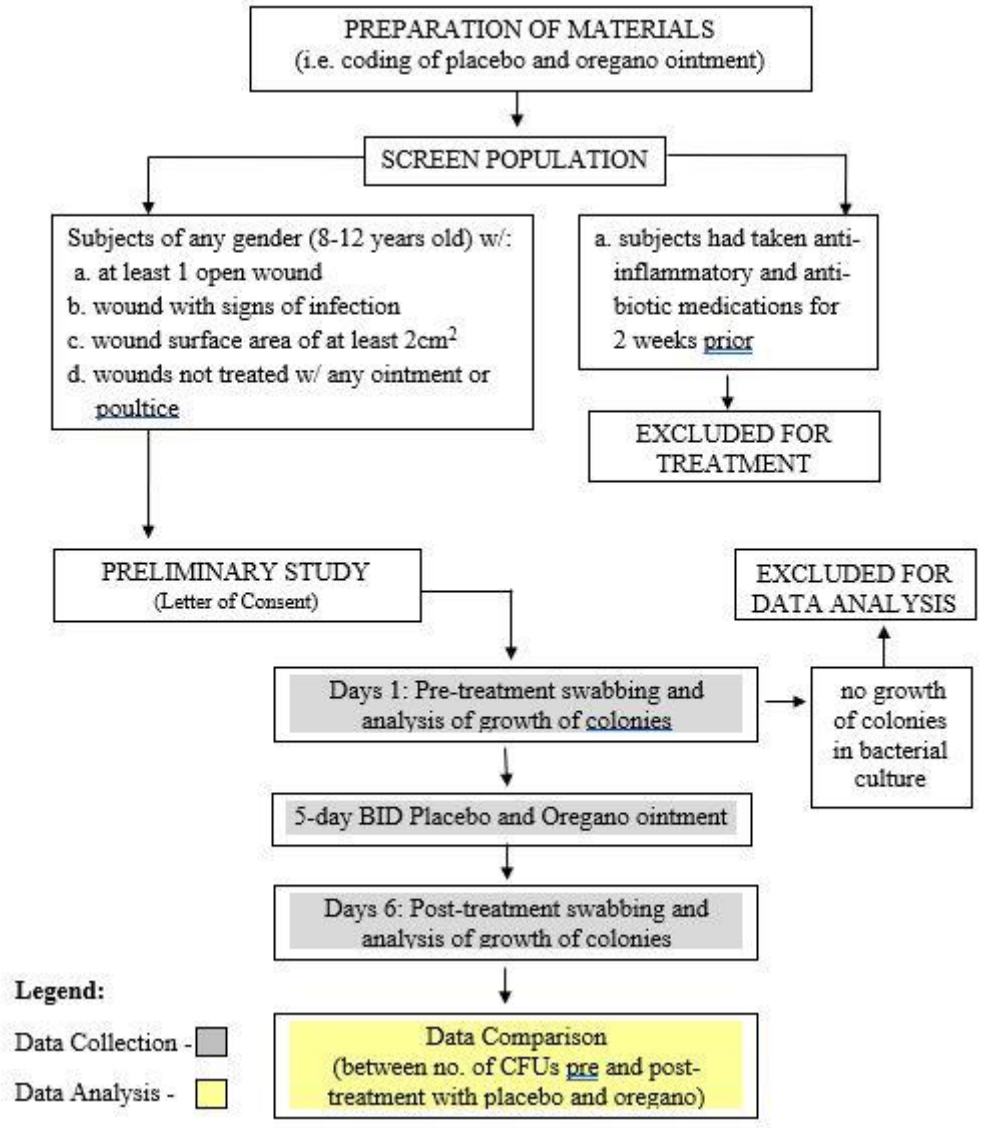

Figure 1. Algorithm of Methodology

RESULTS AND DISCUSSION

A total of 47 respondents participated in this study, with 20 students from DJAL and 27 students from UDLMCS. The distribution of the respondents was as follows: Grade 1 (3 students), Grade 2 (5 students), Grade 3 (7 students), Grade 4 (13 students), Grade 5 (16 students), and Grade 6 (3 students).

With random selection, the population was divided into two groups, A and B which will receive treatment with Placebo and Oregano ointment respectively. For group A (Placebo), the male-female ratio is 1.3:1. The mean age is 10 and the mean wound size is 2.8cm^2. For group B (Oregano), the male-female ratio is 1.5:1. The mean age is 10.1 and the mean wound size is 2.82cm^2.

A total of 72 wounds qualified in the initial screening based on the inclusion criteria. However, 12 were excluded upon conducting the microbiological assay for *S. aureus* microbial growth. The mean pre-treatment CFU count treated with Oregano ointment was 83.1x10^5/ml and 59.87x10^5/ml in Placebo ointment (Figure 1).

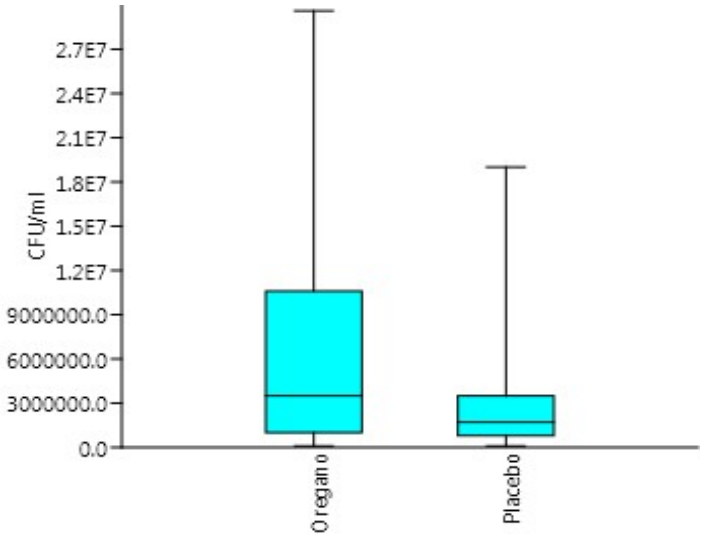

***Figure 2*. Colony Forming Units (CFUs) of 24-hr microbial cultures from wounds prior to treatment with placebo and oregano ointment.**

The antimicrobial effect of the leaf oil extract of *O. vulgare* on the growth of *S. aureus* was determined through counting the number of CFUs in a 24-hour bacterial culture. Using Mannitol salt agar (MSA), *Staphylococcus aureus* colonies were isolated in a 24-hour bacterial culture (Figure 2). It contains 7.5% sodium chloride and thus selects for those bacteria which can tolerate high salt concentrations. MSA also distinguishes bacteria based on the ability to ferment the sugar mannitol, the only carbohydrate in the medium (Shields and Tsang, 2013). With a pH indicator in the medium, phenol red turns into yellow which marks the presence of acid by-products of mannitol fermentation (Figure 3). Although both *Staphylococcus epidermidis* and *Staphylococcus aureus* can tolerate the high salt content of MSA, only *S. aureus* can ferment mannitol,

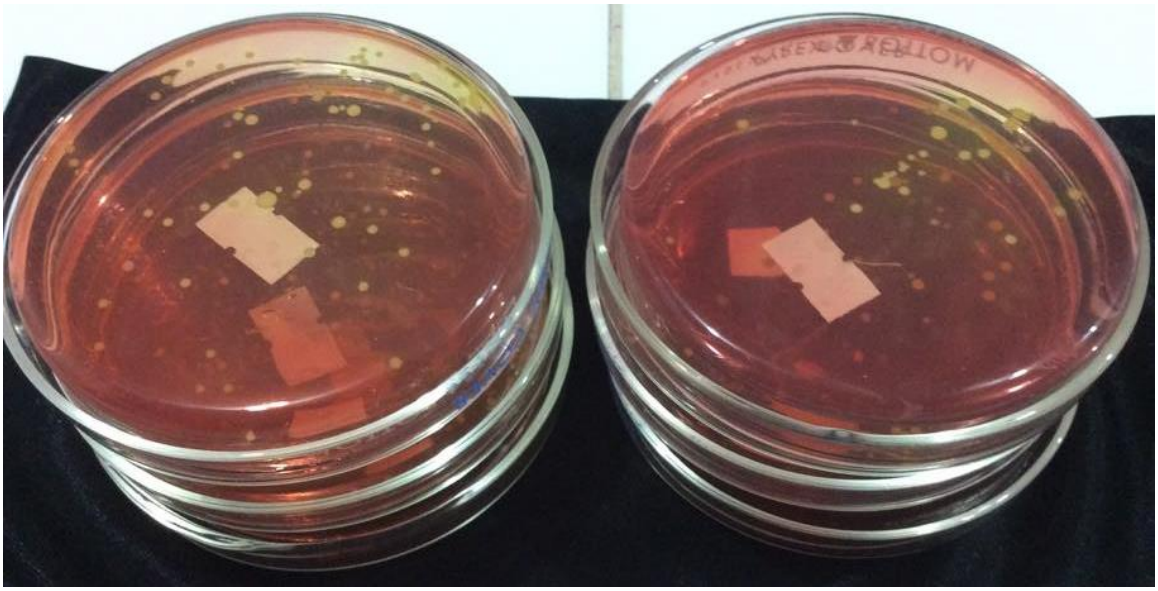

Figure 3. Twenty-four-hour bacterial culture of *Staphylococcus aureus*.

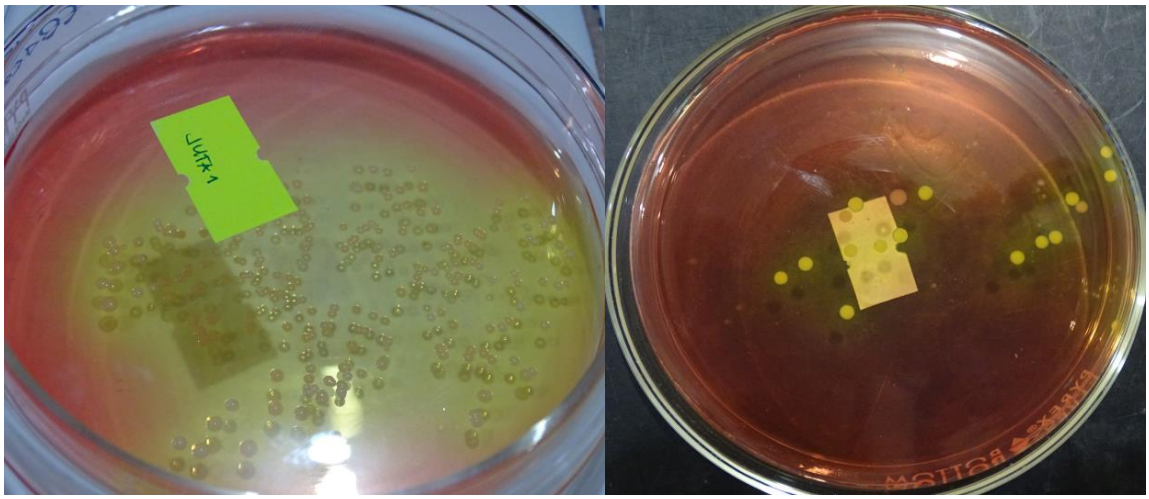

Figure 4. Fermentation of mannitol by *S. aureus*, showing a color change from red to yellow which indicates the presence of acid by-products.

After 24 hours of incubation of microbial culture in the pre-treatment phase, morphological characteristics of the colonies grown were described. The colonies observed were light yellow, smooth, varying in sizes, circular, some were flat and others were elevated. Phenotypic characterization of colonies was also done through Gram staining and viewed under a microscope. Cells were stained bluish purple which indicates thick peptidoglycan layer and thus, gram positive (Figure 5). Slide catalase test was also performed by adding a drop of hydrogen peroxide to an inoculum of bacteria in a glass slide. Upon mixing, bubbles were observed which indicates catalase positive. The ability to ferment mannitol, catalase positive, gram-positive, spherical cells which are arranged in irregular clusters, gray to golden yellow and round colonies strongly suggests *Staphylococcus aureus* (Jawetz, 2010).

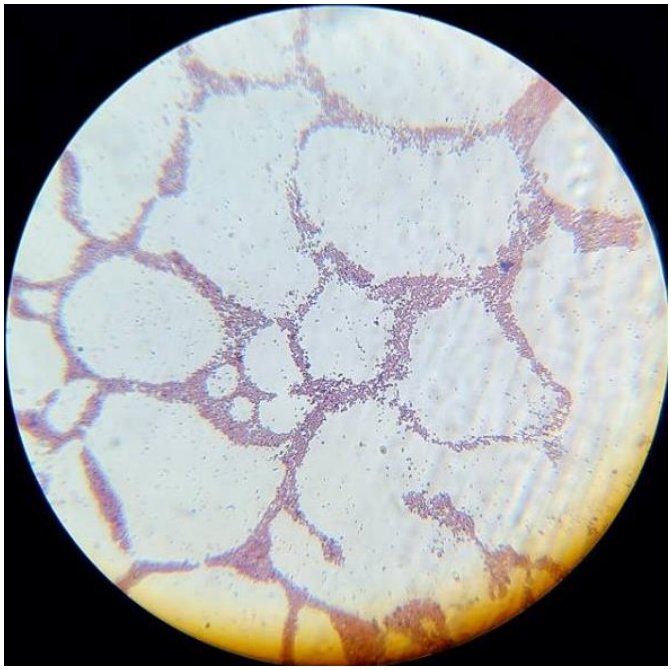

Figure 5. Gram stain of *Staphylococcus aureus* showing gram-positive cocci in irregular clusters. Magnification 400x.

After the post-treatment phase, description of the morphologic characteristics of the bacterial colonies, gram staining and catalase test were performed. Similar findings were noted in both treatment phase. Infected open wounds treated with Oregano (*Origanum vulgare*) leaf ointment showed a reduction in the mean number of colony forming units from $83.1x10^5$/mL to $8.9x10^5$/mL. The mean difference between pre-treatment and post-treatment number of CFUs of infected open wounds treated with Oregano ointment was $-74.2x10^5$/mL. CFU counts of Oregano (*Origanum vulgare*) treated wounds can be observed in Figure 6 and Table 1, as follows.

Table 1. Difference between pre-treatment and post-treatment number of colony forming units (CFUs) of infected open wounds treated with Oregano (*Origanum vulgare*) leaf ointment.

CODE	Pre-treatment CFUs (10^5/mL)	Post-treatment CFUs (10^5/mL)	Difference (10^5/mL)
CYBU	296	0	296
DAAI	22	12	10
JEMA	35	0	35
JODI 1	14	1	13
JODI 2	27	1	26
JODI 3	8	1	70
JODI 4	63	17	46
JODI 5	7	20	-13
ALSA	91	1	90
JAPA	280	18	262
SCTE 1	205	1	204
SCTE 2	106	15	91
SCTE 3	1	5	-40
HAGA 1	1	20	-19
HAGA 2	163	0	163
HAGA 3	2	40	-38
JMMA 1	290	2	288
JMMA 2	25	10	15
ROAM	37	0	37
CMMA	17	8	90
LEAR	24	1	23
JUTA	12	1	11
JOGA	68	20	48
EJCA	10	12	-20
AMSU	94	2	92
JRSE	285	20	265
ACCA	200	2	198
DEEN	100	19	81
OJBO	5	10	-50
WAMA	5	8	-30
TOTAL:	**2493**	**267**	**2226**

MEAN:	**83.1**	**8.9**	**74.2**

Infected open wounds treated with Placebo ointment showed a slight reduction in the mean number of CFUs from 33.23x10^5/mL to 30.93x10^5/mL. The mean difference between pre-treatment and post-treatment CFU count of infected wounds treated with Placebo ointment was -2.3x10^5/mL as presented in Table 1. CFU counts of Placebo treated wounds can be observed in Figure 7 and Table 2, as shown below:

Table 2. Difference between pre-treatment and post-treatment number of colony forming units (CFUs) of infected open wounds treated with Placebo ointment.

CODE	CFU count in Pre-treatment phase (10^5/mL)	CFU count in Post-treatment phase (10^5/mL)	Difference (10^5/mL)
ERCA 1	31	27	4
ERCA 2	17	2	-3
JAMA 1	9	7	2
JAMA 2	22	18	4
JAMA 3	7	6	1
BEGA	59	53	6
JAAL 1	14	11	3
JAAL 2	184	163	21
JAAL 3	73	14	5.9
MATA	202	190	12
ANPA	67	61	6
RABA	52	1	51
DABE 1	100	54	50
DABE 2	8	10	-2
ROSA 1	35	31	4
ROSA 2	19	15	4
DIAB 1	116	12	104
DIAB 2	28	19	9
DIAB 3	11	8	3
SHMA	68	59	9
MADA	17	16	1
EMFA	190	201	-11
AIMA 1	16	15	1
AIMA 2	8	9	-1
MAAN	74	69	5
NOSA	95	4	91
FRSI	203	103	100
JODE 1	10	16	-6
JODE 2	39	36	3
MIGA	22	20	2
TOTAL:	**1796**	**1250**	**478.9**
MEAN:	**59.87**	**42.27**	**17.73**

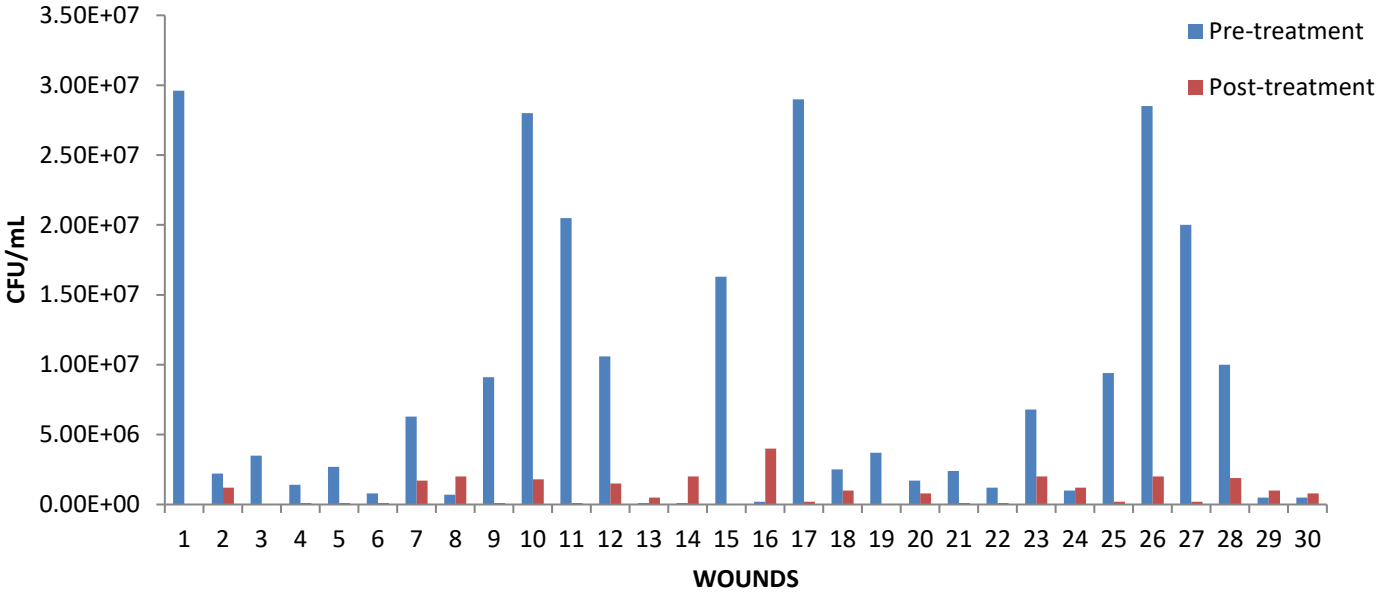

***Figure 6*. Comparison of Colony forming unit count (CFU/mL) in a 24-hour bacterial culture pre- and post-treatment with Oregano (*Origanum vulgare*) leaf ointment.**

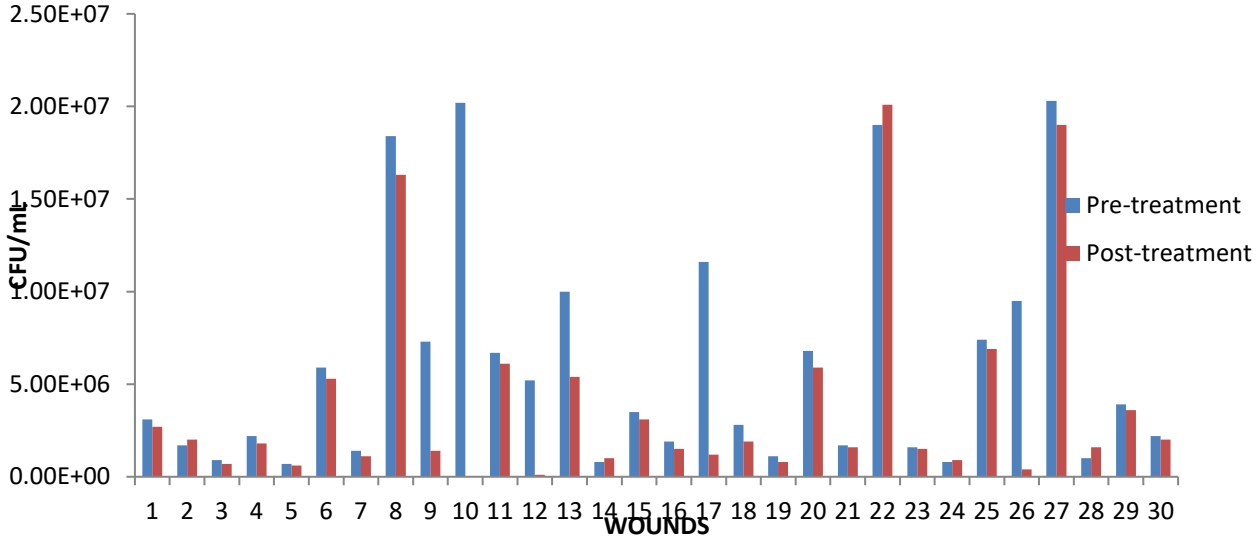

***Figure 7*. Comparison of Colony forming unit count (CFU/mL) a 24-hour bacterial culture of infected wounds pre- and post-treatment with Placebo ointment.**

Using 0.05 level of significance, Two-sample t test was used to determine if there is significant difference between Placebo and Oregano (*Origanum vulgare*) leaf ointment treated wounds in terms of CFU count. Table 3 suggests that there was no significant difference in the pre-treatment CFU count between placebo and Oregano ointment (p=0.2807). As can be noticed, difference in post-treatment CFU count between Placebo and Oregano (*Origanum vulgare*) treated wounds was significant (p=0.0058) which rejects the null hypothesis of the study and approves the alternative hypothesis. Moreover, mean differences in pre-treatment and post-treatment CFU count were also calculated between Placebo ($17.73x10^5$/mL) and

Oregano ($74.2x10^5$/mL) treated wounds which both mark a reduction in CFU count. However, reduction of CFU count in Oregano treatment was greater compared in Placebo treatment which indicates that their mean CFU differences were significant (p=0.0049).

Based on the results and comparative analysis, we can infer that Oregano (*Origanum vulgare*) leaf ointment is efficient in reducing the number of colony forming units in a 24-hour bacterial culture of infected open wounds. Thereupon, null hypothesis is rejected and alternative hypothesis is accepted.

Table 3. Differences in colony forming unit counts between infected open wounds treated with placebo and Oregano ointment.

	Difference between means	*t value*	p value
Placebo vs. Oregano			
Pre-treatment mean CFU count	$23.23x10^5$	1.089	**0.2807**
Post-treatment mean CFU count	$28.37x10^5$	-2.8677	***0.0058**
Mean difference	$56.47x10^5$	-2.9287	***0.0049**
Oregano Pre-treatment vs. Post-treatment CFU count	$74.2x10^5$	4.0761	***0.0001**
Placebo Pre-treatment vs. Post-treatment CFU count	$17.6x10^5$	1.1775	**0.2438**

Table 4. Data Analysis of pre-treatment, post-treatment and mean difference of colony forming unit (CFU) count in infected open wounds treated with Placebo and Oregano ointment using Two-sample t test.

Treatment	Pre-treatment Mean CFU count	Post-treatment mean CFU count	Mean difference	Remarks
Placebo (*n*=30) Vs.	59.87 $x10^5$/mL	$42.27x10^5$/mL	$17.73x10^5$/mL	Decrease in CFU count
Oregano (*n*=30)	$83.1x10^5$/mL	$8.9x10^5$/mL	$74.2x10^5$/mL	Decrease in CFU count

Note: *p<0.05 = significant using Two-sample t test.

In accord to the results presented, there is a significant difference between the Placebo ointment and Oregano (*O. Vulgare*) leaf ointment in reducing the number of CFUs in a 24-hour bacterial culture of open infected wounds. Mean difference of CFU count in Oregano treated wounds is greater compared in placebo treated wounds. This can be attributed to the presence of essential oils of Oregano such as limonene, gamma-cariofilene, rho-cymenene, canfor, linalool, α-pinene, carvacol and thymol (Arcila-Lozano *et al.*, 2004). Proestos et al. (2005) claimed that thymol and carvacol are the main components of Oregano which are responsible for its antimicrobial effect. The two phenolic compnonents mentioned earlier were found to exhibit bactericidal activity (Friedman *et al*., 2002). A study by Nostro et al. (2006) claims that Oregano is effective against *S. aureus* and *S. epidermidis* mainly due to the presence of thymol and carvacol. The same findings were attained by Ozkalp et al. (2010) where the essential oils extracted from the leaf of *Origanum vulgare* was more effective as suppressor of microbial growth than ampicillin against gram-positive bacteria such as *S. aureus*. The same study concluded that *Micrococcus luteus* and *Bacillus cereus* were the most susceptible to oregano oil.

SUMMARY, CONCLUSIONS, AND RECOMMENDATIONS

This randomized double-blind placebo-controlled research was performed with the primary intention of determining the efficacy of Oregano (*Origanum vulgare*) leaf ointment on the reduction of the number of colony forming units (CFUs) in a 24-hour bacterial culture of open infected wounds. This study which was conducted from mid-January to mid-February 2015, was done to determine the significant difference in the reduction of CFUs between control (placebo ointment) and treatment (Oregano ointment) of a 24-hour microbial culture.

The respondents who were included in this research were based on several criteria: gender of 8-12 years with at least one open wound; wounds with signs of infection such as purulent discharge, abnormal smell coming from the wound site and discoloration of tissues both within and around the wound margins; wounds of at least $2cm^2$; wounds untreated with any ointment or poultice; age of wound at least 2 days; and growth of colonies upon microbial culture.

Ocular survey and swabbing of wounds prior to the application of ointment was done in the first and last day of treatment. Ointment application was done twice for seven days. Post-treatment swabbing was performed a day after the last day of application.

The quantification of bacterial colonies was done after 24 hours of incubation in an agar plate and comparison between the pre-treatment and post-treatment of the two ointments was made. Results showed a decrease in number of colony forming units after the treatment period for both placebo and Oregano ointments. However, the decrease in CFU count from pre-treatment to post-treatment culture was greater in Oregano ointment compared to placebo ointment. Using One-way ANOVA test at 0.05 level of significance, it was revealed that there is a significant difference between the two comparative variables (placebo and Oregano ointment) in terms of CFU mean difference and CFU post-treatment count. Therefore, the null hypothesis is rejected and the alternative hypothesis is accepted.

Since Oregano ointment is effective in inhibiting microbial growth of *S. aureus* which are predominantly present in open infected wounds, it is recommended to be used as a topical antibacterial agent. For further studies, it is recommended that the antimicrobial effect of Oregano (*Origanum vulgare*) leaf ointment must be compared to other commercial products commonly used in the community as antiseptic agents such as Bactroban cream, Betadine cream, Mupirocin-topical cream, Neosporin and etc. It is also

recommended that the antimicrobial activity of Oregano must be further evaluated using Kirby-Bauer Disc Diffusion Antibiotic Sensitivity Testing to assess whether it is bacteriostatic or bactericidal.

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APPENDIX A
LETTER OF CONSENT TO THE SCHOOL PRINCIPAL

MINDANAO STATE UNIVERSITY
COLLEGE OF MEDICINE
Research Team, Class 2017

To whom it may concern:

Good day!

The Mindanao State University – College of Medicine (MSU COM) is working under a community-based curriculum. Part of this is the duty of a medical student to formulate a pharmacologic substance that is of therapeutic value.

After careful research, our team had produced an ointment that contains phytochemicals which may enhance wound healing, with the research title, "A RANDOMIZED DOUBLE-BLIND PLACEBO-CONTROLLED STUDY ON THE EFFECT OF OREGANO (*Origanum vulgare* L.) LEAF OINTMENT IN REDUCING THE NUMBER OF 24-HOUR COLONY FORMING UNITS OF *Staphylococcus aureus* IN INFECTED OPEN WOUNDS OF CHILDREN AGES 8-12 YEARS OLD FROM ILIGAN CITY." In line with that, we are asking permission from your good office to conduct this follow-up study to children ages 8-12 years old with infected and/or old wounds who are members of your school. They will be given the treatment twice a day for five (5) days only.

We are hoping for your positive response. Thank you and God bless!

Requested for Approval:

Mr. Eldimson Bermudo
Research Team Representative
MSU-College of Medicine Class 2017
1st Dona Juana, Palao, Iligan City
Noted by:

Dr. Leah Manapat
Research Adviser
MSU – College of Medicine

Received by:

Prof. Enerio Ebisa
Principal, DJAL MCS

APPENDIX B

LETTER OF CONSENT TO RESPONDENTS

MINDANAO STATE UNIVERSITY
COLLEGE OF MEDICINE
Research Team, Class 2017

PARENT'S CONSENT

To whom it may concern:

Ako si Mr/Ms. _____, amahan/inahan/guardian ni _____ nga estudyante sa _____ _____, nagapamatuod nga gitugtan ko siya sa pag-apil sa research study sa Mindanao State University – College of Medicine (MSU COM) nga gipanganlang "A RANDOMIZED DOUBLE-BLIND PLACEBO-CONTROLLED STUDY ON THE EFFECT OF OREGANO (*Origanum vulgare* L.) LEAF OINTMENT IN REDUCING THE NUMBER OF 24-HOUR COLONY FORMING UNITS OF *Staphylococcus aureus* IN INFECTED OPEN WOUNDS OF CHILDREN AGES 8-12 YEARS OLD FROM ILIGAN CITY."

Nahibalo usab ako na sa sulod sa lima ka adlaw, walay laing ointment ang pwede ipahid sa iyang mga samad kung dili ang herbal ointment lamang na gihimo sa research team.

_____

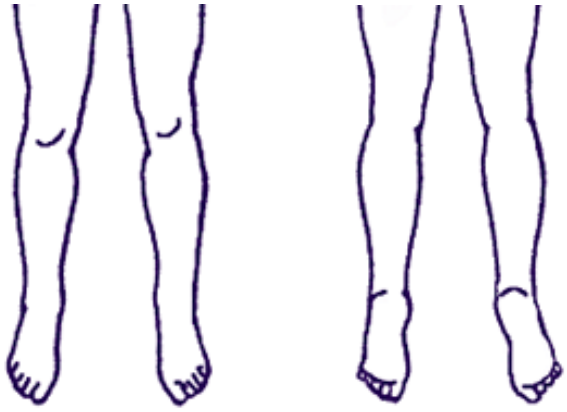
Guardian's Signature over printed name

APPENDIX C
INFORMATION SHEET FOR SCREENING PHASE

SCREENING PHASE
Subject Code: | | | | |
Name:
_____ Grade - Section -
Age: □8 y/o □9 y/o □10 y/o □11 y/o □12 y/o Gender: □ M □ F
School: □ Dona Juana Actub Lluch Elem School □ Brgy. Ubaldo Laya Elem School
Was parent's consent given? □ Yes □ No
If yes, when was the consent given? _____
Total no. of wounds in the lower extremities: □ 1 □ 2 □ 3 □ 4 □ more than 4
Screening of the wound (please fill in the necessary information in the table below):

Wound Code	Wound No.	Size of wound (cm^2)	Onset of infection (days)	Meds taken? (Yes/No)	Pre-test swab: (# of CFUs)	Description of colonies
	1					
	2					
	3					
	Anterior View				Posterior View	

Location of the wound (please indicate the wound number in the illustration below):



Characteristics of an infected wound (please check the signs evident for each wound indicated):

Wound No	Erythema/ redness	Waxy Appearance	Presence / or drainage of pus	Discoloration of tissues within	Discoloration of tissues at the	Tenderness at wound margins	Appears swollen /inflamed	Warm to touch

				the margins	wound margins			
1								
2								
3								

APPENDIX D

Wound codes of respondents from Doña Juana Actub Lluch Memorial Central School

Wound Code	Age	Grade	Sex	Section
CYBU	10	5	M	3
DAAI	12	5	M	4
JEMA	11	4	F	7
JODI 1	10	4	M	5
JODI 2	10	4	M	5
JODI 3	10	4	M	5
JODI 4	10	4	M	5
JODI 5	10	4	M	5
ALSA	9	2	M	2
JAPA	10	4	F	7
SCTE 1	10	5	F	1
SCTE 2	10	5	F	1
SCTE 3	10	5	F	1
HAGA 1	12	5	M	4
HAGA 2	12	5	M	4
HAGA 3	12	5	M	4
ERCA 1	11	5	M	3
ERCA 2	11	5	M	3
JAMA 1	9	4	M	6
JAMA 2	9	4	M	6
JAMA 3	9	4	M	6
BEGA	8	3	M	2
JAAL 1	11	5	M	3
JAAL 2	11	5	M	3
JAAL 3	11	5	M	3
MATA	10	5	F	2
ANPA	12	6	F	5
RABA	9	4	M	3
DABE 1	9	3	M	3
DABE 2	9	3	M	3
CDDA 1*	10	4	M	2
RSPO 1*	8	2	M	3

RSPO 2*	8	2	M	3
RSPO 3*	8	2	M	3
ABRE 1*	9	3	M	2
DSDA 1*	8	2	F	1

APPENDIX E

Wound codes of respondents from Ubaldo D. Laya Memorial Central School

Wound Code	Age	Grade	Sex	Section
JMMA 1	10	5	F	Sunflower
JMMA 2	10	5	F	Sunflower
ROAM	11	5	F	Sunflower
CMMA	10	5	F	Sunflower
LEAR	10	5	F	Sunflower
JUTA	11	5	M	Yellowbells
JOGA	12	5	M	Yellowbells
EJCA	8	1	M	Gumamela
AMSU	8	3	F	Champaca
JRSE	8	2	M	Carnation
ACCA	8	2	M	Rose
DEEN	8	1	M	Gardenia
OJBO	8	1	M	Gladiola
ROSA 1	9	3	M	Champaca
ROSA 2	9	3	M	Champaca
DIAB 1	10	4	F	Daffodil
DIAB 2	10	4	F	Daffodil
DIAB 3	10	4	F	Daffodil
SHMA	11	4	M	Daffodil
MADA	12	6	F	Carnation
EMFA	10	5	F	Daisy
AIMA 1	9	4	F	Daffodil
AIMA 2	9	4	F	Daffodil
MAAN	8	5	F	Champaca
NOSA	8	4	F	Daffodil
FRSI	11	6	F	Carnation
JODE 1	9	5	M	Daisy
JODE 2	9	5	M	Daisy
MIGA 1	10	4	F	Tulip
WAMA 1	11	4	F	Tulip
SMCA 1*	10	3	F	Champaca
ETAM 1*	9	4	F	Tulip
ETAM 2*	9	4	F	Tulip
MCMI 1*	11	5	M	Daisy
MCMI 2*	11	5	M	Daisy

MCMI 3*	11	5	M	Daisy

APPENDIX F

Pretreatment wound condition of the subjects from Doña Juana Actub Lluch Memorial Central School

Wound Code	E	W	P	D_1	D_2	T	S	H
CYBU	+	+	+	+	+	+	+	+
DAAI	+		+	+	+			
JEMA				+	+	+		+
JODI 1			+	+	+		+	
JODI 2			+	+	+			
JODI 3			+	+	+			
JODI 4			+	+	+	+	+	
JODI 5			+	+	+		+	
ALSA	+	+		+	+		+	
JAPA	+	+	+	+	+	+		
SCTE 1	+		+	+	+	+	+	+
SCTE 2	+		+	+	+	+	+	+
SCTE 3	+		+	+	+	+	+	
HAGA 1	+	+	+	+	+	+		
HAGA 2		+	+	+	+		+	+
HAGA 3	+	+	+	+	+	+	+	+
ERCA 1	+	+	+	+	+	+		
ERCA 2		+		+	+		+	+
JAMA 1			+	+	+	+	+	+
JAMA 2	+	+	+	+	+	+	+	+
JAMA 3	+	+	+	+				
BEGA			+		+	+	+	+
JAAL 1	+	+	+	+		+		
JAAL 2	+	+		+		+		
JAAL 3	+	+	+				+	
MATA		+	+	+	+		+	+
ANPA		+		+	+		+	
RABA	+		+	+		+	+	
DABE 1	+	+	+		+			
DABE 2		+		+		+	+	
CDDA 1*	+		+	+		+	+	+
RSPO 1*	+		+	+		+		+
RSPO 2*		+	+		+	+		
RSPO 3*	+	+		+				+
ABRE 1*	+		+		+	+		+
DSDA 1*	+		+	+		+		+

Legend: **E**-erythema; **W**-waxy appearance; **P**-presence of pus; **D_1**-discoloration of tissues within the margins; **D_2**-discoloration of tissues at the wound margins; **T**-tenderness; **S**-swelling; **H**-warm to touch.

APPENDIX G

Pretreatment wound condition of the subjects from Ubaldo D. Laya Memorial Central School

Wound Code	E	W	P	D_1	D_2	T	S	H
JMMA 1	+	+	+	+	+	+	+	+
JMMA 2	+	+	+	+	+	+	+	+
ROAM	+	+	+	+	+	+	+	+
CMMA	+	+	+	+	+	+	+	
LEAR	+	+	+	+	+	+	+	+
JUTA	+	+	+	+	+	+	+	+
JOGA	+	+		+	+			
EJCA	+	+	+	+	+	+	+	+
AMSU	+		+	+	+		+	
JRSE	+	+	+	+	+		+	
ACCA	+	+	+	+	+		+	+
DEEN	+	+	+	+	+	+	+	+
OJBO	+	+	+	+	+	+	+	+
ROSA 1	+	+	+	+	+	+	+	
ROSA 2		+	+	+	+	+	+	+
DIAB 1	+	+	+	+	+	+		
DIAB 2		+	+	+	+	+	+	
DIAB 3	+	+	+	+	+	+	+	+
SHMA		+		+	+	+	+	+
MADA	+	+	+	+	+	+	+	+
EMFA		+	+	+	+	+	+	
AIMA 1	+	+	+	+	+	+	+	
AIMA 2		+	+	+	+	+	+	+
MAAN	+	+	+	+	+	+		+
NOSA		+	+	+	+	+	+	
FRSI	+	+	+	+	+	+	+	
JODE 1		+	+	+	+	+	+	+
JODE 2	+	+	+	+	+	+		
MIGA 1			+	+	+	+	+	
WAMA 1		+	+	+	+	+	+	
SMCA 1*	+	+	+	+	+			
ETAM 1*	+	+	+	+	+	+	+	+
ETAM 2*		+	+	+	+	+	+	
MCMI 1*	+	+	+	+	+	+	+	
MCMI 2*		+	+	+	+	+	+	+
MCMI 3*		+	+	+	+	+	+	+

Legend: **E**-erythema; **W**-waxy appearance; **P**-presence of pus; **D_1**-discoloration of tissues within the margins; **D_2**-discoloration of tissues at the wound margins; **T**-tenderness; **S**-swelling; **H**-warm to touch.

APPENDIX H
LETTER OF CONSENT TO MSU-IIT FOR DATA ANALYSIS

MINDANAO STATE UNIVERSITY
COLLEGE OF MEDICINE
Pala-o, Ilign City

To: DR. SUKARNO D TANGGOL, DPA
Chancellor, MSU-IIT

Thru: DR. JINKY B. BORNALES
Vice Chancellor for Research and Extension
DR. CESAR G. DEMAYO
Chair, Department of Biological Sciences
College of Science and Mathematics

Dear Sir or Madam:

The Mindanao State University – College of Medicine (MSU-COM) is under a community-based curriculum, part of which involves pharmacology research. We, the members of Class 2017 re currently working on our study entitled "A RANDOMIZED DOUBLE-BLIND PLACEBO-CONTROLLED STUDY ON THE EFFECT OF OREGANO (*Origanum vulgare* L.) LEAF OINTMENT IN REDUCING THE NUMBER OF 24-HOUR COLONY FORMING UNITS OF *Staphylococcus aureus* IN INFECTED OPEN WOUNDS OF CHILDREN AGES 8-12 YEARS OLD FROM ILIGAN CITY."

Due to a limited microbiology laboratory equipment and materials, we are humbly asking for your permission to allow us to use the resources in the Department of Biological Sciences – College of Science and Mathematics. The specified materials and equipment are enlisted in the attached document.

We are planning to conduct these procedures on January 28, 2014 (Wednesday) at 4:00-7:00pm and on February 2, 2014 (Monday) at 4:00-7:00pm only.

Your approval is essential for the success of our study. Thank you and God bless.

Yours truly,
MR. ELDIMSON BERMUDO
Research Team Representative

APPENDIX I
LIST OF MATERIALS USED

ITEMS	QUANTITY
Petri Plates	120 pcs
Test tubes (10 ml)	600 pcs
Autoclave	1
Clean bench	1
Alcohol lamp	3 pcs

L-rods	3 pcs
Stirring rods	3 pcs
Beakers (500 ml)	3 pcs
Graduated cylinder (15 ml)	3 pcs
Graduated cylinder (500 ml)	1 pc
Erlenmeyer flask (500 ml)	3 pcs
Micropipettor (200-microliters)	1 pc
Micropipette tips	20 pcs

***Nothing follows.

APPENDIX J
DOCUMENTATION (Part 1 of 3)

Figure 8. Preparation of Materials (Washing of Petri plates, test tubes and rack)

APPENDIX J
DOCUMENTATION (Part 2 of 3)

Figure 9. Preparation of Materials for Culture and Swabbing

Figure 10. Preparation of Placebo and Oregano Ointments

APPENDIX J
DOCUMENTATION (Part 3 of 3)

Figure 11. Data collection (Pre-treatment and Post-treatment swabbing)

APPENDIX K
INFORMATION SHEET FOR TREATMENT PHASE

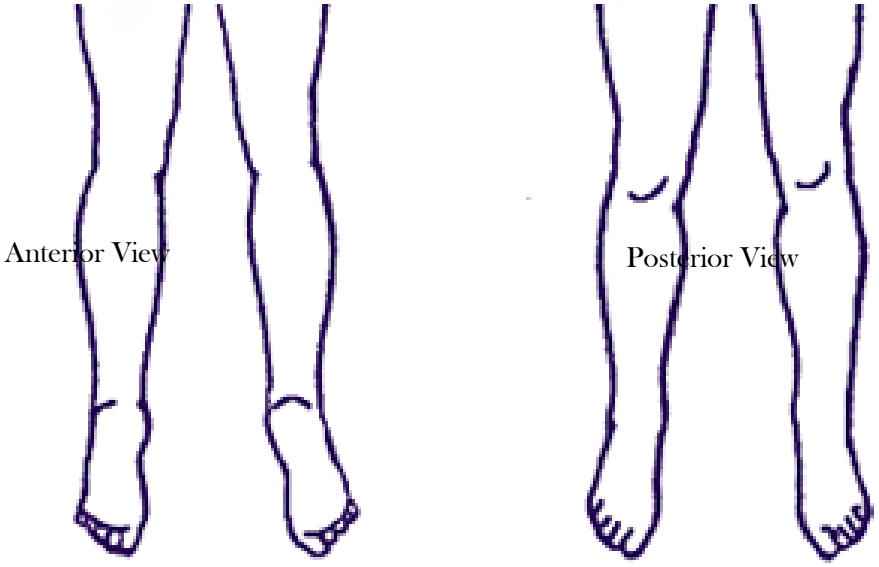
TREATMENT PHASE
Subject Code:

Name: _____ Grade - Section -

Age: □8 y/o □9 y/o □10 y/o □11 y/o □12 y/o Gender: □ M □ F
School: □ Dona Juana Actub Lluch Elem School □ Brgy. Ubaldo Laya Elem School
Total no. of wounds in the lower extremities: □ 1 □ 2 □ 3 □ 4 □ more than 4
Treatment checklist (please indicate check marks on Day 1- Day 5 to table below):

Treatment Code	Wound No	Day 1		Day 2		Day 3		Day 4		Day 5		Post-test swab (# of CFUs)	Description of colonies
		AM	PM	AM	PM	AM	PM	AM	PM	AM	PM		

Location of the wound (please indicate the wound number in the illustration below):



Researcher assigned: _____

Information Sheet collected by:_____

Date: _____

APPENDIX L
SAMPLE 24-HR BACTERIAL CULTURES

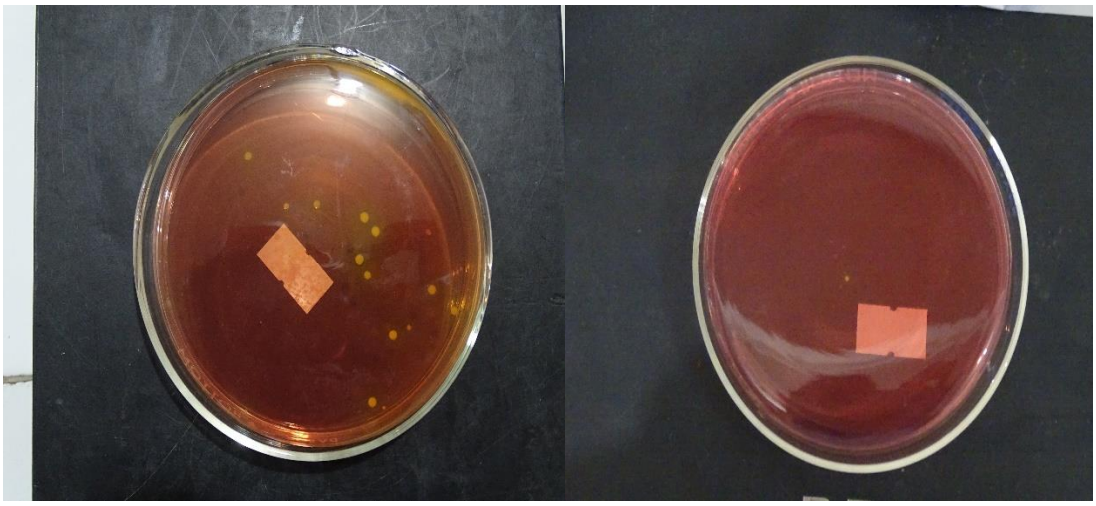


Figure 12. Pre-treatment (Left) and Post-treatment (Right) bacterial cultures of Oregano (*Origanum vulgare*) ointment

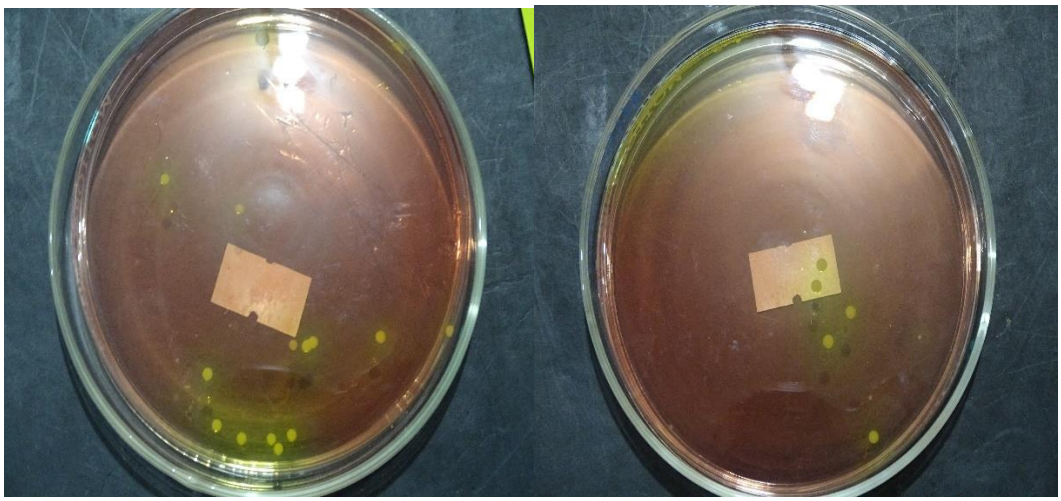


Figure 13. Pre-treatment (Left) and Post-treatment (Right) bacterial cultures of Placebo ointment