ANTI-INFLAMMATORY ACTIVITY OF MEDICINAL LEECH (*Hirudo medicinalis, Hirudinidae*) SALIVA EXTRACT ON CARRAGEENAN-INDUCED EDEMA IN WISTAR RATS (*Rattus norvegicus)*: PRE-FORMULATION OF MEDICINAL GEL

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ABSTRACT

This research study focused on the medicinal leech (*Hirudo medicinalis, Hirudinidae*) saliva extract and its anti-inflammatory activity. Qualitative analysis for proteins, biological assays, product pre-formulation and accelerated stability tests were done. The qualitative analysis for proteins confirmed the presence of proteins in the saliva extract. The first biological assay, using the saliva crude extract, determined the subject's anti-inflammatory activity. Upon product pre-formulation, four gel concentrations were made (25%, 50%, 75%, and 100%). The accelerated stability tests revealed that the most suitable storage condition for the medicinal gel is at 25-30°C. The second biological assay, using the compounded medicinal gel, once again confirmed the medicinal leech saliva extract's anti-inflammatory property. Statistical analysis showed that all the prepared gel concentrations were able to inhibit edema formation, with 75% as the most effective.

Key words: *Hirudo medicinalis, hirudinidae, anti-inflammatory activity, edema, gel pre-formulation, saliva extract*

INTRODUCTION

Inflammation, when it functions properly, is a quick natural response designed to help the body heal. It results from the activation of the immune system in response to a broad range of different stimuli. The immune system is a highly complex and evolutionary optimized defense system with cellular and humoral components. The course of an inflammatory response is influenced by the immune condition of the host, the virulence, and the fine tuning of the local tissue reaction, which may be influenced by individual genetic factors (Co, 2016).

Physicians have been using the medicinal leech (*Hirudo medicinalis*) for more than 2,500 years. It was used for bloodletting in Ancient Egypt. Avicenna, in the canon of medicine, emphasized the use of leeches even for skin diseases. Later in the 12th century, Abdul Latif Baghdadi wrote that leech could also be used for cleaning the tissues after surgical operations. Leeching reached the height of its popularity in the middle 19th century, when it was exposed by the French physician Francois Broussais (1722-1838). Broussais treated diseases such as typhoid fever, syphilis, tuberculosis, and even mental illness by applying leeches to abdomen. In the early 19th century, an American physician from Georgia wrote that bloodletting is the most important treatment, whenever there appears sign of local congestion,

inflammation or that sluggish or torpid action which makes for incapacity in the circulation vessels. He continued to discuss the use of leeches as treatment for myocarditis, peritonitis, pleuritis, hepatitis, gastritis, tonsillitis, nephritis, pneumonia, whooping cough, dysentery, hemorrhoids, acne and pimples. The advantages of leech therapy include: the possibility to avoid side effects and complications of pharmacotherapy; high curative effectiveness which permits to reduce the rates of treatment and prolong the time of remissions; and the limited number of contraindications for the method and complications were the sought out principles after it. With the advent of modern pathology, physiology and microbiology in the late 19th century, bloodletting with leeches fell out of favor. The leeches were brought back into the medical domain in the middle of the 20th century; its use was called hirudotherapy. Hirudotherapy, also known as leech therapy, pertains to a non-invasive treatment involving the application of medicinal leeches on the surface of the body as therapy for certain diseases (Itrat et al., 2013).

Modern medicine has just recently focused on the possible modes of action. Extensive researches had been undertaken to isolate and characterize the active contents from leech saliva especially peptides and proteins. Eglin is an inhibitor of inflammatory factors. Inhibition by eglin causes decreasing levels of free oxygen radicals in neutrophils and prevents tissue inflammation and destruction. According to the study of Han Dong et al, leech therapy's anticoagulant, antithrombin activity, and hypolipidemic effect as well as protective effect against cerebral ischemia-reperfusion injury. These effects could be attributed to its anti-inflammation and detumescence (Rahul et al., 2014; Sig et al., 2017).

In relation to this, the study was conducted as a modern approach to hirudotherapy. The researchers aim to achieve the primary goal of emphasizing the anti-inflammatory property of medicinal leech *(Hirudo medicinalis)* saliva, as well as to compound a topical drug product from the crude saliva extract of the experimental subject.

Research Questions

Generally, this study aimed to investigate and to determine the anti-inflammatory activity of medicinal leech (*Hirudo medicinalis*) by compounding a pharmaceutical gel out of its saliva extract and testing it on carrageenan-induced paw edema in male wistar rats (*Rattus norvegicus)*.

Specifically, it aimed to answer the following questions:

1. What are the phytochemical constituents of Leech saliva extract?
2. What is the degree of paw edema/ inflammation as evidenced by the volume of water displacement of the different treatment groups?
    a. Post-induction of Carrageenan
    b. 1 hour post-treatment

c. 2 hours post treatment
d. 3 hours post treatment
e. 4 hours post treatment
f. 5 hours post treatment

3. Is there a significant difference in degree of inflammation of subjects after induction of inflammation and after 1, 2, 3, 4, and 5 hours post-treatment?
4. Is there a significant difference in the degree of inflammation of the different treatment groups 1, 2, 3, 4, and 5 hours post-treatment?
    a. Positive control (25mg/kg Diclofenac Sodium)
    b. 25% Leech saliva extract
    c. 50% Leech saliva extract
    d. 75% Leech saliva extract
    e. 100% Leech saliva extract
    f. Negative control

Hypotheses

- There is no significant difference between the positive control and medicinal leech saliva extract gel.
- There is no significant difference between the negative control and medicinal leech saliva extract gel.
- There is no significant difference between the positive control and negative control.

Significance of the Study

This study aims in bringing forth a meaningful development to the following:

To the researchers, for providing a firm grasp on the importance of medicinal leech saliva extract and why it is crucial to be brought forward in to the integrated medical systems of the future; to the community, so they will be able to understand more clearly the value of integrating traditional medicinal leech saliva extract with that of the modern medicinal approach; and how, when practiced properly, can contribute to a higher standard of health care beneficial to patients. And to other researchers, that this may serve as a versatile guide in their forthcoming studies on other systems and properties related to that of medicinal leech saliva extract.

Literature Review

Leeches *(Hirudo medicinalis)*

Leeches are hematophagous animals segmented worm-like creatures. It swims with a vertical motion and moves, when out of water by means of discs or suckers, flattening itself first by one and then by other, alternately stretching out and

contracting its body (Itrat, Zamigar & Haque, 2013). *Leeches have likely more than 650 species worldwide. One of the most extensively studied annelids and the most widely used species in the therapeutic setting is* **Hirudo medicinalis**. *Its scientific name reflects its importance in medicine. The classification of leeches is as follows; Kingdom (Animalia), Phylum (Annelida), Class (Clitellata), and Subclass (Hirudinea)* (RICARIMPEX, 2010).

In centuries, a common European species (*Hirudo medicinalis)* of leeches were a commonly used tool by doctors, who believed that many diseases were the result of "imbalances" in the body that could be stabilized by releasing blood (Gale, 2006). After the recession period of leech therapy, it has resurged after the mid-20th century with new applications in many medical fields, including surgical and reconstitution procedures, vascular diseases, arthritis, and migraine (Abdualkader, Ghawi, Alaama, Awang, & Merzouk, 2013).

In addition to *H. medicinalis* of Europe, the Algerian dragon (*H. troctina*) is also used. *Gnathobdella ferox* is commonly used in Asia. After *H. medicinalis* was introduced into North America, it established itself there as a wild species. Other land leeches that attack humans are primarily of the genus *Haemadipsa* in Asia, the Philippines, the East Indies, and Madagascar (Encyclopædia Britannica, 2011). The taxonomy of leeches depends on the locality where it is commonly found. Some species of medicinal leeches are: *Hirudo manillensis* which is locally found in the Philippines (Lesson, 1842), *Hirudo sanguisorba* in Sri Lanka (Tennent, 1859), together with *Hirudo multistriata* (Schmarda, 1861) *Hirudo luzoniae* in Manila, Philippines (Kinberg, 1866), *Hirudo maculosa,* locally found in Singapore (Grube, 1868), *Hirudo maculata* in the locality of Thailand (Baird, 1869), *Limnatis (Poecilobdella) granulosa* in Java, Indonesia (Blanchard, 1893), and another species- *Hirudo boyntoni* (Wharton, 1913) which is also found in the Philippines (Tangient, 2017).

The effectiveness of leech saliva is the results of specific thrombin inhibitors, hirudin, which was first isolated from *H. medicinalis* and was shown to possess a potent inhibitory effect on both free and clot-bound thrombin. Furthermore, other thrombin inhibitors were identified from different leech species. For instance, bufrudin was isolated from *H. manillensis* with a chemical structure closely similar to hirudin. A tight-binding thrombin inhibitor named haemadin was identified from the whole body extract of the leech species *Haemadipsa sylvestris*. Another antithrombin named granulin-like was isolated from the leech species *H. nipponia*. Finally, a human granulocyte and monocyte protein inhibitor known as theromin was characterized from the head extract of *Theromyzon tessulatum* leech species with an antithrombin activity.

Anti-inflammatory

Leech is a modern therapeutic agent introduced by the Food and Drug Administration (FDA) and it contains different peptides and proteins like histamine, serotonin, steroid hormones and modulators, enzymes, protease inhibitors, and anti-microbial agents (Shakouriet al., 2017). Leech bites allow its immunological medicinal enzymes from its own saliva to penetrate the host. These enzymes are often anti-inflammatory and anticoagulant, crucial in producing various therapeutic effects in humans. To increase efficiency of feeding, leeches have also evolved bioactive saliva that decreases the host's normal humoral and cellular immune responses, including inflammation, pain and swelling. Active investigation of the chemicals in leech saliva is currently under way, and one anticoagulant drug, hirudin, is derived from the tissues of *Hirudo medicinalis* (Gale, 2006). Some bioactive substances can function as analgesics, vasodilators, bacteriostatics, anti-inflammatories, anti-edematous, and anti-coagulants. These bioactive enzymes may improve blood circulation, increase thrombolysis, and enhance anti-inflammatory responses (Cooper & Mologne, 2016).

These bioactive substances are: Hirudin, a heparin-like substance the most potent known natural inhibitor of thrombin; Hyaluronidase, a spreading or diffusing substance that modifies the permeability of connective tissue, therefore increasing the rate of absorption; Calin, which has the ability to suppress collagen-induced platelet aggregation as well as adhesion of platelets to collagen coated micro-carrier beads; Destabilase possesses glycosidase activity; Apyrase a non-specific inhibitor of platelet aggregation; Eglins are small proteins present in *Hirudo medicinalis* that is a potential therapeutic agent for the treatment of diseases associated with inflammation; Bdellins, another bioactive substance could be used as a plasmin inhibitor to control bleeding; and Hirutasin is a potent inhibitor of blood coagulation (Zaidiet al., 2011).

Many protein compounds exist in Leech saliva which reduce inflammation in the joint (Koeppen et al.) reported that pain was relieved in patients a day after, which seemed to be the result of saliva containing anti-inflammatory ingredients (Shakouri et al., 2017).

Eglins are small protein inhibitors isolated from the leech *Hirudo medicinalis* (Seemueller et al., 1977). A specific inhibitory activity makes eglin a valuable tool as an anti-inflammatory agent by inhibition of achymotrypsin chymase, substilisin, elastase and cathepsin G (Rink et al., 1984). Eglin C is an inhibitor of human neutrophil elastase and cathepsin. These two enzymes are immune serine proteases in chymotrypsin family that are stocked in azurophil granules of polymorphonuclear neutrophils and released as part of the inflammatory response. Inhibition by eglin C can decrease the levels of free oxygen radicals in neutrophils and prevent tissue inflammation and destruction (Sig et al., 2017).

Traditional and Alternative Medicine Act of 1997

The utilization of herbal plants has long been practiced since the ancient times. Even today, it holds a household name for a lot of communities, especially in the rural areas. Ancestries upon ancestries of varying communities have utilized different parts of these so-called “halamong gamot” to be of use in the prevention, alleviation and management of bacterial infections, and other common endemic diseases.

A study of alternative medicine in the Philippines is, inevitably, a study of the origins of its people and the amalgam of cultures and influences: centuries of Spanish colonial rule and the indelible consequences of its religion, hundreds of years of trade with China and assimilation of its healing arts, and tribal and provincial diversities with its profusion of folklore and mythologies, all redounding into the Filipino's easy disposition for superstitions and the allure for the esoteric, mystical, and fringe (Stuart, 2003). Just like Hirudotherapy, one of the most popular and successful alternative medicines sought after by the public**.** It offers a simple and inexpensive alternative, especially for those who prefer to go the ‘natural’ way.

In connection to these, Republic Act 8423 (RA 8423) known as Traditional and Alternative Medicine Act (TAMA) of 1997 aims on improving the quality and delivery of health care services to the Filipino people through the development of traditional and alternative health care and its integration into the national health care delivery system. The creation of Philippine Institute of Traditional and Alternative Health Care (PITHC) promulgates a nationwide campaign to boost support for the realization of the objectives of this Act. The said institute also formulates and implements research programs on the indigenous Philippine traditional health care practices performed by “traditional healers” using scientific research methodologies (TAMA Act of 1997). In this way, indigenous people will be able to share to the wider community their profound knowledge about the utilization of herbal and alternative medicines.

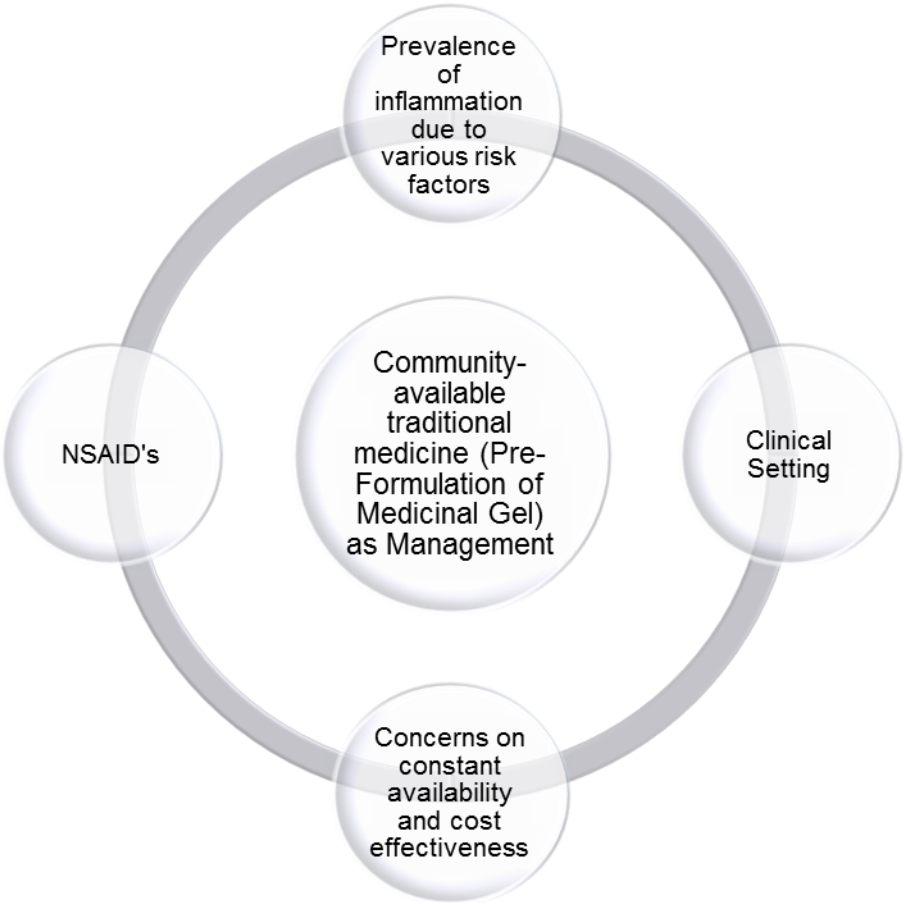

Figure 1. *Research Simulacrum*

The figure above was adapted from the research simulacrum of Determination of the Antibacterial Activity of the Hand Sanitizer with the Crude Leaf Extract of Alibangbang (*Bauhinia malabarica*) against *Escherichia coli* and *Staphylococcus aureus* (March, 2015).

This representation shows that inflammation occurs due to various risk factors such as rheumatoid arthritis, arthritis, osteoarthritis, obesity, autoimmune diseases and many more. A body's inflammatory response is a sign of defense mechanism, but the unpleasant feeling that comes with it cannot easily be neglected, that's why the public resorts to the most commonly sought after medication—the Non-Steroidal Anti-Inflammatory Drugs (NSAIDs) to relieve the said condition.

Due to continuous demand for the management and relief of inflammation using NSAIDs, great numbers of concern have been and are continuously being raised for its constant availability and cost effectiveness. Given that a vast number of anti-inflammatory medicines can only be purchased with the presence of prescriptions, there is another need for professional consultation with expensive fees, which is essential for acquiring the said prescriptions and costly medications. Because of these factors, the community may consider a traditional-based anti-inflammatory pharmaceutical product in the management of inflammation.

METHODS

Research Design

Experimental method was applied in this research endeavor, which included product formulation, product variation, and pre-clinical stage of product testing. It was conducted in the Pharmacy and Chemistry Laboratories of University of Saint Louis, Tuguegarao City, Cagayan and in the Philippine Institute of Traditional and Alternative Health Care (PITAHC), Carig Sur, Tuguegarao City, Cagayan.

Subjects of the Study

The sanguivorous medicinal leeches (*Hirudo medicinalis*) were accumulated from farmlands in Ilagan, Isabela and were brought to Tuguegarao City, Cagayan for tending. On the other hand, the male wistar rats (*Rattus norvegicus*) on the other hand, were bought from a registered and duly licensed breeder in Malvar, Santiago City, Isabela and were housed in the Philippine Institute of Traditional and Alternative Health Care (PITAHC) at Carig Sur, Tuguegarao City, Cagayan.

Sampling Technique

The identification and confirmatory test of the medicinal leeches was conducted in the Fisheries Laboratory of the Bureau of Fisheries and Aquatic Resources (BFAR)-RFO 02, at Carig Sur, Tuguegarao City, Cagayan.

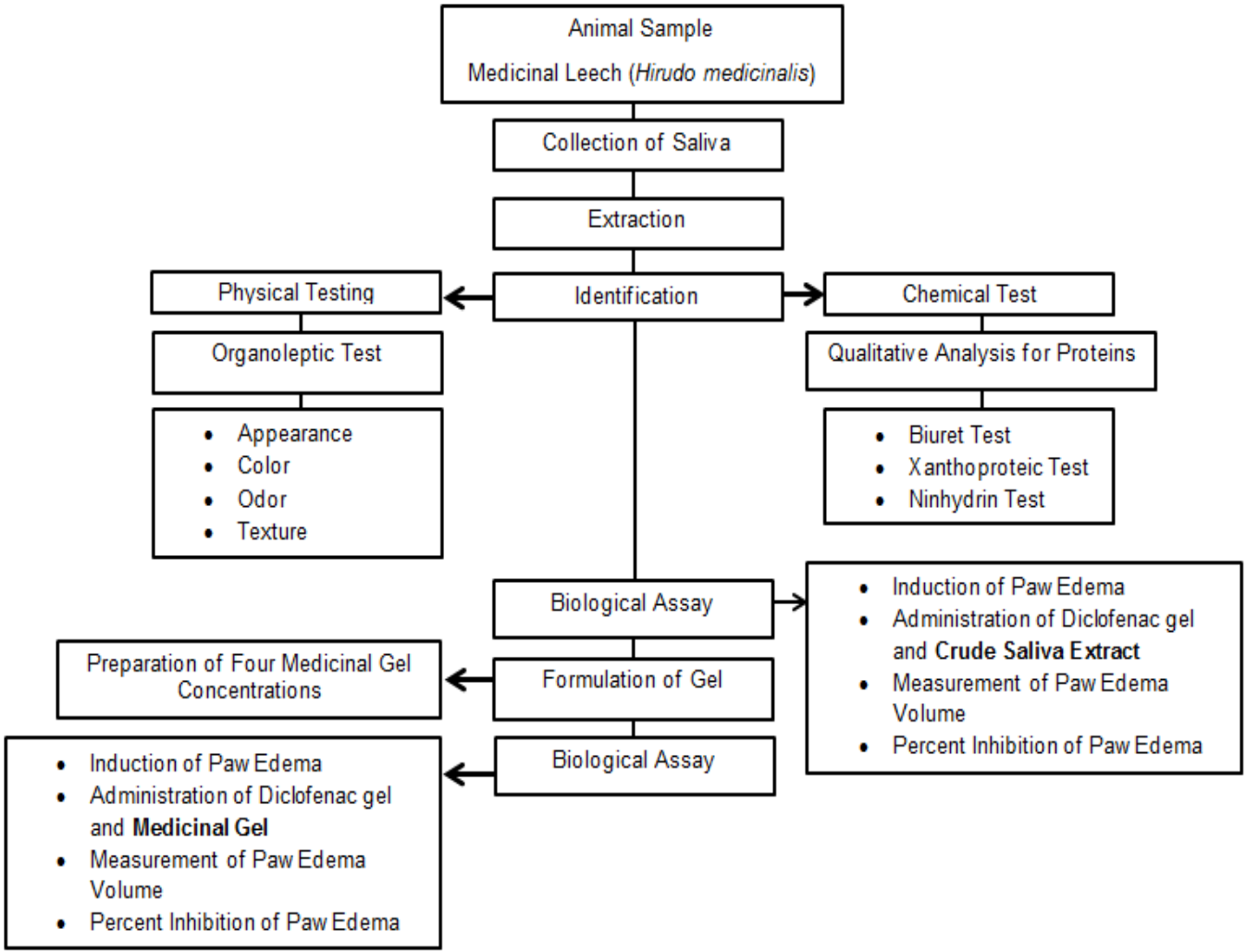

Figure 2: Methodological flowchart

Procedures of Data Gathering

1. Collection and Preparation of Animal Control

1.1. Collection of Animal Samples

The researchers collected medicinal leeches (*Hirudo medicinalis*) from farmlands in Ilagan, Isabela. They were kept in clear glass containers with perforated lid to allow sufficient air circulation. Rain water was used as the main source for non-chlorinated type of water and was changed every seven days.

Healthy and pure strain male wistar rats (*Rattus norvegicus*), weighing 100g were bought from a registered and duly licensed breeder in Malvar, Santiago City, Isabela and were nurtured in the Philippine Institute of Traditional and Alternative Health Care (PITAHC) at Carig Sur, Tuguegarao City, Cagayan until they reached the ideal weight of 150-200g for the experiment proper. Commercial pellets and soy beans, together with distilled water were fed to the rats ad libitum for the duration of the observation. The experiments were carried out according to Animal Care and Use Protocol Review Form.

1.2. Collection of Saliva Samples

The aforementioned medicinal leeches were kept in a dimly-lit room and starved for one week. The amount of saliva collected and the presence of proteins were determined.

During the starvation period of the medicinal leeches, the resulting turbid rain water was collected, changed and contained in a clean transparent bottle. The accumulated liquids were strained through a filter paper and centrifuged for 10 minutes at 3000rpm (Abdualkader et al., 2011). The resulting crude saliva extract was then subjected for testing.

2. Qualitative Analysis for Proteins

According to a study conducted in 2011 by Zaidi et al., a leech has several medicinally useful bioactive substances present in its saliva, which are of protein classification—most especially Eglins, rendering its anti-inflammatory property. Various tests for this specific macromolecule were then conducted to determine the presence of proteins in the accumulated leech saliva extract, to support this claim. The procedures were conducted according to the standard experimental procedures for proteins.

2.1. Biuret Test

Take a small quantity of the crude saliva extract in a test tube and add 2mL of NaOH solution into it. Then add 4-5 drops of 1% $CuSO_4$ solution and warm the mixture for about 5 minutes.

2.2. Xanthoproteic Test

Take about 2mL of the sample in a test tube and add a few drops of concentrated HNO_3 into it and heat the test tube.

2.3. Ninhydrin Test

Take 2mL of the sample in a test tube and add 3-4 drops of Ninhydrin solution and boil the contents.

3. Biological Assay

Before compounding a medicinal gel out of the crude saliva extract, the said extract underwent the mandatory biological assay, to confirm the presence of its anti-inflammatory property.

3.1. Preparation of 1% Carrageenan suspension

A freshly prepared solution of 1% carrageenan solution was used to induce edema in the male wistar rats. This was prepared by mixing 100mg of laboratory grade Carrageenan powder with 100mL of Plain Normal Saline Solution (0.9% Na Cl).

3.2. Induction of Paw Edema

Male wistar rats weighing 150-200g were divided into six groups: one positive control group, one negative control group, and four experiment control groups. All rats were contained in an observation chamber for ten minutes to minimize stress related behavioral changes (S. Koliyote, et.al, 2013) prior to the start of the experiment proper. The standard drug, diclofenac gel (1% Voltaren Emulgel®) and experimental drug medicinal leech crude saliva extract with varying concentrations of 25%, 50%, 75%, and 100% (A.Shakouri, et.al., 2017), were then gently rubbed on the subplantar surface of the right hind paw fifty (50) times using the index finger, thirty minutes prior to the subcutaneous injection of the freshly prepared 1% Carrrageenan suspension in all six groups (S. Amdekar, et. al, 2012). They were all given a fair volume of distilled water for the duration of the experiment.

3.3. Measurement of Paw Edema

After the induction, the paw edema volume was measured by using the volume displacement technique to determine edema formation size at 0, 1, 2, 3, 4 and 5 hours.

3.4. Calculation of Percent Inhibition of Edema

The percentage of inhibition of edema was calculated using the following formula (S. Koliyote, et.al, 2013):

$$\frac{Paw\ volume\ post-carrageenan\ injection\ (mL) - Paw\ volume\ after\ particular\ time\ (mL)}{Paw\ volume\ post-carrageenan\ injection\ (mL)} \times 100$$

3.5. Gel Formulation

After the initial testing of the anti-inflammatory activity of the medicinal leech crude saliva extract, a finished product consisting of a topical medicinal gel was prepared by cold method.

The weighed amount of carbopol together with a previously diluted methyl paraben and propyl paraben was placed in a mortar. Triethanolamine was added drop-wise with constant trituration. After a gel was formed, the weighed amount of medicinal leech saliva extract which has been previously mixed with ethanol was then incorporated into the carbopol mixture. Eucalyptus was added drop-wise for aesthetic purposes. The finished product was then stored in a gel tube at controlled room temperature.

3.6. Dilution of Gel Concentrations

During the compounding of the medicinal gel, the accumulated saliva extracts were diluted with ethanol to concentrations of 25%, 50%, 75%, and 100% (A.Shakouri, et.al., 2017), in connection to this, the specific volumes of the extract were needed. The dilution formula was lifted from Jenkin's Quantitative Pharmaceutical Chemistry, 1977:

$$\%\ Concentration = \frac{weight}{volume} \times 100$$

4. Statistical Analysis

Comparison of the various paw edema measurements per hour of the positive control (Diclofenac 1% topical gel), negative control and experiment control (Medicinal leech saliva extract gel) was analyzed by ONE-WAY ANOVA. The difference between the positive control and negative control was also determined using t-test (two-tailed). The Tukey HSD test was utilized to find out which means were significantly different. Level of significance was set at $P<0.05$. All statistical manipulations were carried out using IBM-SPSS, Version 20.

4.1. Accelerated Stability Testing

After the medicinal leech saliva extract gels were tested for their anti-inflammatory property, the compounded products underwent accelerated stability testing to measure their capability to maintain their physical attributes through time.

4.2. Temperature at 2-8°C

The prepared medicinal leech saliva extract in its appropriate container was stored for one month in a refrigerator. At the pull out period, the sample was subjected for physical testing.

4.3. Temperature at 25-30°C

The prepared medicinal leech saliva extract in its appropriate container was stored for one month in the laboratory with controlled room temperature. At the pull out period, the sample was subjected for physical testing.

4.4. Temperature at 40°C

The prepared medicinal leech saliva extract in its appropriate container was stored for one month in a hot air oven at 40°C. At the pull out period, the sample was subjected for physical testing.

Ethical Consideration

This research study underwent the University Research Ethics Board (UREB)'s review of protocols prior to its conduction of experiment.

All wistar rats used in the experiment were surrendered in the Philippine Institute of Traditional and Alternative Health Care (PITAHC) for their proper segregation and post-experimental care. The leeches, on the other hand, were nurtured and cultivated back to the farmlands in Ilagan, Isabela.

RESULTS

Table 1. *Qualitative Analysis for Proteins of Medicinal Leech Saliva Extract*

Chemical Test	(+) Result	Saliva Extract Reaction
Biuret Test	Purple/Mauve Color	+
Xanthoproteic Test	Deep Orange-Yellow Color	+
Ninhydrin Test	Yellow Color	+

The table shows the presence of proteins in the medicinal leech saliva extract. A research conducted by S.M. Abbas Zaidi et.al, in 2011, stated that Eglin, which is the major bioactive substance responsible for the anti-inflammatory property of medicinal leech saliva, is of protein classification.

Table 2. *Measurement of Paw Edema among the different Treatment Groups*

Treatment Group	After Carrageenan Induction	Post-Treatment				
		1 hour	2 hours	3 hours	4 hours	5 hours
Positive Control (Diclofenac Gel)	1.200	1.280	.920	.760	.620	.480
Medicinal Leech Saliva Extract, 25%	1.240	1.100	.840	.800	.560	.440
Medicinal Leech Saliva Extract, 50%	1.120	.960	.656	.640	.520	.440
Medicinal Leech Saliva Extract, 75%	1.120	.880	.720	.640	.440	.420
Medicinal Leech Saliva Extract, 100%	1.320	1.160	.940	.780	.600	.440
Negative Control (Distilled Water)	1.480	1.420	1.320	1.260	1.320	1.420

The table above presents the measurement of paw edema which was used as measure for assessing degree of inflammation manifested by the subjects. It can be further observed that a decreasing trend in the degree of inflammation can be observed in all treatments except in the negative control where there is a decrease in paw edema 2 and 3 hours after treatment followed by an increase in paw edema 4 to 5 hours after treatment.

Table 3.1. *Test of Significant Difference of the Degree of Inflammation of Subjects under the Positive Control Treatment after Induction of Inflammation and Post-treatment*

Pairs	t-value	p-value	Decision
Post-Carrageenan Administration- 1 hour Post Treatment	-1.000	.374	Accept Ho
Post-Carrageenan Administration- 2 hours Post Treatment	5.715	.005	Reject Ho
Post-Carrageenan Administration- 3 hour Post-Treatment	11.000	.000	Reject Ho
Post-Carrageenan Administration-4 hours Post Treatment	29.000	.000	Reject Ho
Post-Carrageenan Administration-5 hours Post Treatment	14.697	.000	Reject Ho

It can be gleaned from the table that one hour post treatment of Diclofenac, no significant decrease in paw edema was observed. However, after 2, 3, 4 and 5 hours of treatment significant decrease in paw edema was observed.

Table 3.2. *Test of Significant Difference of the Degree of Inflammation of Subjects under the 25% Leech Saliva Treatment after Induction of Inflammation and Post-treatment*

Pairs	t-value	p-value	Decision
Post-Carrageenan Administration- 1 hour Post Treatment	3.500	.025	Reject Ho
Post-Carrageenan Administration- 2 hours Post Treatment	6.325	.003	Reject Ho
Post-Carrageenan Administration- 3 hour Post-Treatment	5.880	.004	Reject Ho
Post-Carrageenan	8.500	.001	Reject Ho

Administration-4 hours Post Treatment			
Post-Carrageenan Administration-5 hours Post Treatment	12.649	.000	Reject Ho

It can be gleaned from the table that a significant decrease in paw edema was observed from the subjects 1, 2, 3, 4 and 5 hours after treatment of 25% Leech saliva extract.

Table 3.3. *Test of Significant Difference of the Degree of Inflammation of Subjects under the 50% Leech Saliva Treatment after Induction of Inflammation and Post-treatment*

Pairs	t-value	p-value	Decision
Post-Carrageenan Administration- 1 hour Post Treatment	4.000	.016	Reject Ho
Post-Carrageenan Administration- 2 hours Post Treatment	2.730	.052	Reject Ho
Post-Carrageenan Administration- 3 hour Post-Treatment	9.798	.001	Reject Ho
Post-Carrageenan Administration-4 hours Post Treatment	9.487	.001	Reject Ho
Post-Carrageenan Administration-5 hours Post Treatment	8.500	.001	Reject Ho

It can be gleaned from the table that a significant decrease in paw edema was observed from the subjects 1, 2, 3, 4 and 5 hours after treatment of 50% Leech saliva extract.

Table 3.4. *Test of Significant Difference of the Degree of Inflammation of Subjects under the 75% Leech Saliva Treatment after Induction of Inflammation and Post-treatment*

Pairs	t-value	p-value	Decision
Post-Carrageenan Administration- 1 hour Post Treatment	6.000	.004	Reject Ho
Post-Carrageenan Administration- 2 hours Post Treatment	6.325	.003	Reject Ho

Post-Carrageenan Administration- 3 hour Post-Treatment	9.798	.001	Reject Ho
Post-Carrageenan Administration-4 hours Post Treatment	13.880	.000	Reject Ho
Post-Carrageenan Administration-5 hours Post Treatment	15.652	.000	Reject Ho

It can be gleaned from the table that a significant decrease in paw edema was observed from the subjects 1, 2, 3, 4 and 5 hours after treatment of 75% Leech saliva extract.

Table 3.5. *Test of Significant Difference of the Degree of Inflammation of Subjects under the 100% Leech Saliva Treatment after Induction of Inflammation and Post-treatment*

Pairs	t-value	p-value	Decision
Post-Carrageenan Administration- 1 hour Post Treatment	4.000	.016	Reject Ho
Post-Carrageenan Administration- 2 hours Post Treatment	4.750	.009	Reject Ho
Post-Carrageenan Administration- 3 hour Post-Treatment	4.630	.010	Reject Ho
Post-Carrageenan Administration-4 hours Post Treatment	5.308	.006	Reject Ho
Post-Carrageenan Administration-5 hours Post Treatment	6.016	.004	Reject Ho

It can be gleaned from the table that a significant decrease in paw edema was observed from the subjects 1, 2, 3, 4 and 5 hours after treatment of 100% Leech saliva extract.

Table 3.6. *Test of Significant Difference of the Degree of Inflammation of Subjects under the Negative Control Group after Induction of Inflammation and Post-treatment*

Pairs	t-value	p-value	Decision
Post-Carrageenan Administration- 1 hour Post Treatment	2.449	.070	Reject Ho

Post-Carrageenan Administration- 2 hours Post Treatment	6.532	.003	Reject Ho
Post-Carrageenan Administration- 3 hour Post-Treatment	5.880	.004	Reject Ho
Post-Carrageenan Administration-4 hours Post Treatment	3.138	.035	Reject Ho
Post-Carrageenan Administration-5 hours Post Treatment	1.500	.208	Reject Ho

The table shows that no significant decrease in paw edema was observed after one hour of treatment with distilled water. However, a significant decrease in paw edema was observed 2, 3, and 4 hours after treatment. This was then followed by an increase in paw edema of the subjects which is significantly similar to the level of paw edema present before administration of distilled water.

Table 4. *Test of Significant Difference in the Degree of Inflammation of the Different Treatment Groups 1, 2, 3, 4 and 5 Hours Post-treatment*

Pairs	F-value	p-value	Decision
1 hour Post-treatment	5.057	.003	Reject Ho
2 hours Post-treatment	5.654	.001	Reject Ho
3 hours Post-treatment	9.952	.000	Reject Ho
4 hours Post-treatment	31.988	.000	Reject Ho
5 hours Post-treatment	73.452	.000	Reject Ho

The table above shows that there is a significant difference in the paw edema of the test subjects from different treatment groups.

Table 5.1. *Post-Hoc Analysis of the Test of Significant Difference in the Degree of Inflammation of the Different Treatment Groups 1 hour Post-treatment*

	Mean	Positive control	Exp. Group 1	Exp. Group 2	Exp. Group 3	Exp. Group 4	Negative Control
Positive Control	1.280						
Exp. Group 1	1.100	.165					
Exp. Group 2	.960	.018*	.276				
Exp. Group 3	.880	.004*	.093	.531			

Exp. Group 4	1.160	.349	.637	.125	.036*		
Negative control	1.420	.276	.018*	.001*	.000*	.050*	

**The mean difference is significant at the 0.05 level*

The table shows that 75% and 100% Leech saliva extract has significantly similar effects as the positive control (Diclofenac) in that they are unable to cause significant decrease in paw edema after one hour of treatment. However, the 25% and 50% Leech saliva extract manifested significantly better effect than Diclofenac, such that this treatment caused decrease in paw edema one hour after treatment.

Table 5.2. *Post-Hoc Analysis of the Test of Significant Difference in the Degree of Inflammation of the Different Treatment Groups 2 hours Post-treatment*

	Mean	**Positive control**	**Exp. Group 1**	**Exp. Group 2**	**Exp. Group 3**	**Exp. Group 4**	**Negative Control**
Positive Control	.920						
Exp. Group 1	.840	.571					
Exp. Group 2	.656	.070	.199				
Exp. Group 3	.720	.164	.397	.650			
Exp. Group 4	.940	.887	.479	.052	.127		
Negative control	1.320	.008*	.002*	.000*	.000*	.012*	

**The mean difference is significant at the 0.05 level*

The table shows that all concentrations of Leech saliva extract has the significantly the same anti-inflammatory effect as the positive control (Diclofenac).

Table 5.3. *Post-Hoc Analysis of the Test of Significant Difference in the Degree of Inflammation of the Different Treatment Groups 3 hours Post-treatment*

	Mean	**Positive control**	**Exp. Group 1**	**Exp. Group 2**	**Exp. Group 3**	**Exp. Group 4**	**Negative Control**
Positive Control	.760						
Exp. Group 1	.800	.701					
Exp. Group 2	.640	.255	.133				

Exp. Group 3	.640	.255	.133	1.000			
Exp. Group 4	.780	.848	.848	.187	.187		
Negative control	1.260	.000*	.000*	.000*	.000*	.000*	

**The mean difference is significant at the 0.05 level*

The table shows that all concentrations of Leech saliva extract has the significantly the same anti-inflammatory effect as the positive control (Diclofenac).

Table 5.4. *Post-Hoc Analysis of the Test of Significant Difference in the Degree of Inflammation of the Different Treatment Groups 4 hours Post-treatment*

	Mean	**Positive control**	**Exp. Group 1**	**Exp. Group 2**	**Exp. Group 3**	**Exp. Group 4**	**Negative Control**
Positive Control	.620						
Exp. Group 1	.560	.463					
Exp. Group 2	.520	.226	.623				
Exp. Group 3	.440	.035*	.149	.330			
Exp. Group 4	.600	.806	.623	.330	.058		
Negative control	1.320	.000*	.000*	.000*	.000*	.000*	

**The mean difference is significant at the 0.05 level*

The table shows that all concentrations of Leech saliva extract have the significantly the same anti-inflammatory effect as the positive control (Diclofenac).

Table 5.5. *Post-Hoc Analysis of the Test of Significant Difference in the Degree of Inflammation of the Different Treatment Groups 5 hours Post-treatment*

	Mean	**Positive control**	**Exp. Group 1**	**Exp. Group 2**	**Exp. Group 3**	**Exp. Group 4**	**Negative Control**
Positive Control	.480						
Exp. Group 1	.440	.549					
Exp. Group 2	.440	.549	1.000				

Exp. Group 3	.420	.371	.764	.764			
Exp. Group 4	.440	.549	1.000	1.000	.764		
Negative control	1.420	.000*	.000*	.000*	.000*	.000*	

**The mean difference is significant at the 0.05 level*

The table shows that all concentrations of Leech saliva extract have the significantly the same anti-inflammatory effect as the positive control (Diclofenac).

DISCUSSION

This study aimed to achieve the primary goal of emphasizing the anti-inflammatory property of medicinal leech *(Hirudo medicinalis)* saliva, as well as to compound a topical drug product from the crude saliva extract of the experimental subject. Qualitative analysis of proteins and induction of edema were conducted by the researchers to attain the objectives of this study.

Based on the results of the collected data, the Qualitative Analysis of Proteins- which was done by performing Biuret, Xanthoproteic and Ninhydrin Tests confirmed the presence of proteins in the medicinal leech saliva extract. Previous studies have shown that medicinal leech saliva extract contains bioactive substances such as hirudin, hyaluronidase, destabilase, apyrase, calins, bdellins, decorsin, hirustasin, guamerin, piguamerin, gelin, gamma-Glutamyl Transpeptidase, and eglins, which is primarily responsible for the anti-inflammatory property of medicinal leech saliva. Almost all of a medicinal leech's bioactive substances are of protein classifications, especially eglins (Zaidi et al., 2011).

This study used Diclofenac Gel for the positive control, Medicinal Leech Saliva Extract Gel for the Experimental Control and Distilled Water for the Negative control. The results obtained showed that Medicinal Leech Saliva Extract Gel is more effective than the positive control. Descriptive statistics showed that the most effective concentration based on their percent inhibition is obtained with Medicinal Leech Saliva Extract Gel, 75% (mean= 44.5%), followed by Medicinal Leech Saliva Extract Gel, 25% (mean=39.8%), Medicinal Leech Saliva Extract Gel, 50% (mean=39.7%), and Medicinal Leech Saliva Extract Gel, 100% (mean=38.8%).

As for the stability test done, the most suitable temperature for storage of the medicinal gel is at controlled room temperature or at 25-30°C, for it showed a stable retainment of its physical properties. The medicinal gel stored at 2-8°C also showed a fine texture, but with moist on its surface, which is an early sign for bacterial growth. Storing the medicinal gel at 40°C also revealed a liquid consistency which is not an aesthetically pleasing characteristic of the finished product.

CONCLUSION

Based on the results obtained by the researchers from the qualitative analysis and biological assay, the following conclusions were made for the Anti-inflammatory activity of Medicinal Leech (*Hirudo medicinalis, hirudinidae*) Saliva Extract on Carrageenan-Induced Edema in Wistar Rats (*rattus norvegicus)*: Pre-formulation of Medicinal Gel.

The Qualitative Analysis confirmed the presence of proteins from Medicinal Leech (*Hirudo medicinalis)* Saliva Extract, which firmly supports its anti-inflammatory property.

The biological assays showed that all prepared concentrations (25%, 50%, 75%, 100%) of the medicinal gel were effective, with 75% as the most effective among the four concentrations.

RECOMMENDATION

Based on the previous findings of this study, the following points were recommended for its future progress and enhancement:

1. Isolate Eglins to further prove the leech saliva's anti-inflammatory property.
2. Test other biological macromolecules present in the medicinal leech saliva.
3. Determine medicinal leech saliva's protein concentrations.
4. Try other species of leech, especially the *Hirudo manillensis*, which is locally found in the Philippines.
5. Perform longer duration of stability test with relative humidity.
6. Pre-formulate other dosage forms like ointment which is an oil based topical medication.
7. Use plethysmometer device to measure the microliter changes in the paw edema volume.
8. Conduct skin sensitization test for the finished gel product.
9. Test the treatment properties of medicinal leech saliva.
10. Test other properties of medicinal leech saliva.

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