# Wound healing activity of Broussonetia luzonica (Blanco) Bur. (himbabao) leaf extract in ovo duck chorionic allantoic membrane (CAM) assay

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***Abstract –*** *Wound healing is a normal process of restoring the tissue and cellular structures. However, this process is usually delayed due to infection and other severe complications. This study makes use of plant extract as an alternative to the invasive and costly effects brought by the current procedures in treating wounds. Broussonetia luzonica is an endemic plant reported for its medicinal and antibacterial activity. Using in ovo duck chorionic allantoic membrane model, the researchers used different concentrations of crude ethanolic extracts of the leaves from 100ug/ml, 300ug/ml and 500ug/ml to evaluate for wound healing potency. Phytochemical screening revealed the presence of various compounds in ethanolic extract including tannin, flavonoids, and alkaloids. In wound assay, measurements were made on alternative days up to day 5 of observation post wounding and results showed significant difference observed on the multiple comparison conducted on the negative control and not significant difference when compared to Diclofenac. This suggests that the B. luzonica plant extract has high wound closure activity, which is comparable with the standard drug, Diclofenac sodium. Another assay assessed the formation of new blood vessels was performed to assess the second stage in the wound healing process. Thus, B luzonica leaf extract has wound healing activity starting at 100 ug/ml with no dose-dependency. Angiogenesis was found to be involved in wound healing activity of B. luzonica leaf extract but was not statistically supported. Therefore, it can be used as an alternative instead of chemically manufactured antibiotics.*

***Keywords –*** *Broussonetia luzonica, chorionic allantoic membrane, duck embryo, wound healing*

## INTRODUCTION

Wound is the continuous destruction of cells due to physical, chemical, thermal, infectious or immunological injury to the skin [1]. Wound healing is a natural physiological process done by the body but still need attention due to risk of infection and other severe complication [2]. It is a highly regulated process that is essential in the repair of the first function of the skin which is to serve as a protective barrier to the underlying tissues [3]. Tissue integrity is being restored as a natural response to injury that replaces damaged cells into living ones [4]. Poor healing occurs when the damaged skin is prone to a number of infections. The process of wound healing has three phases: inflammatory phase, proliferative phase, and remodeling phase [5]. Tissue damage triggers primary hemostasis to constrict the blood vessels and form a platelet plug to prevent further blood loss. When inflammatory cells and macrophages engulfed the bacteria and other foreign debris, inflammatory phase begins. As a result, inflammation occurs to localize the affected area. Proliferation or regenerative phase is characterized by the formation of new blood vessels, new collagen matrix and the development of granulation tissues [7]. New blood vessels that form from existing vasculature are called angiogenesis [8]. It plays a vital role in physiological and pathological developments like wound healing, embryonic development, and development of atherosclerosis Excessive angiogenesis may lead to arthritis, diabetes, psoriasis, tumor growth and metastasis while insufficiency may result to poor wound healing, tissue formation and blood circulation. In remodeling phase, cells that are no longer needed undergo the process of apoptosis and remodeling occurs [9]. Skin wound epithelialization depends on the location, depth, size, microbial contamination, related health conditions and genetics [10]. Acute wounds have short duration and inflammatory phase while wounds that are deep, large or severely infected need more time to heal because of longer hemostasis. Non-healing wounds of all means remains to be an enormous burden in the health care setting worldwide. An estimate of over $25 billion is spent every year on the management of chronic wounds [10].

Nowadays, wound healing studies receive lots of attention not just in the field of medicine but also, in business standpoint. According to a study, wound healing is a huge commercial enterprise, with the market for wound care products exceed US$15billion dollars.

Current methods used to treat chronic wounds include invasive and costly procedures such as debridement, irrigation, antibiotics, tissue grafts and proteolytic enzymes, which have unwanted side effects and major drawbacks to the patient. In spite of new approaches and technology to increase the efficacy of wound healing, wound care has returned to the roots of medicine and is embracing some of the remedies used millennia ago [11]. Alternative to the expensive cost of traditional medicine, researches focus on plants as a natural remedy for a myriad of diseases because broad class of active metabolites is a natural constituent of medicinal plants [12].

These serve as foundation of successful medicine, and will constantly be a significant part of screening com¬pounds. Recent study shows that there are plants that can be used as alternative in the process of wound healing. Increasing demands of phytochemical-based therapies both in developing and developed countries can be used to enhance both acute and chronic wound healing. Plants that are known to exhibit wound healing usually have antioxidant, anti-inflammatory, and anti-microbial properties. Several species of Moraceae family particularly the genus Ficus was prove to have wound healing capacity [13].

Broussonetia luzonica (Blanco) Bur. (himbabao) is an edible plant from the Moraceae family and is endemic in the Philippines [14]. Study shows that this plant is proven to contain phytol, a phytochemical that exhibits antioxidant and antinociceptive effects. Tannins on the other hand, not only heal burns and stop bleeding, but can help stop infection and form a protective layer over the exposed tissue to keep the wound from being infected even more [15].

**Figure 1. *Broussonetia luzonica* leaves**

Studies on angiogenesis, metastasis and tumor cell invasion were performed used on chorioallantoic membrane (CAM) assays [16]. Plant extracts can be utilized to screen drugs through quantitation of vessels in a lot of CAM models [17]. The most effective advantage in the use of CAM is its accessibility, cost effectiveness and rapid growth [18].

To date, there is currently no study of the plant's effectiveness in promoting faster wound healing in vivo as well as in vitro. However, according to previous study conducted using this plant, phytochemical screening suggests that B. luzonica leaf extract has biological activities which support the medicinal application of the plant [19].

The study aims to determine the wound healing effect of B. luzonica leaf extract using CAM assay as an alternative to in vivo models. Specifically, it aims to determine the phytochemical substances present in B. luzonica leaf extract, determine the most effective concentration capable of wound healing and compare the effect of varying concentrations if there is dose-dependency.

Also it aims to detect the formation of new blood vessels or angiogenesis that usually happened in the second stage of wound healing. It also aims to check if there is dose dependency of B. luzonica leaf extract necessary in wound healing. If proven to be effective, these findings would contribute to the list of potential plants-based pharmaceuticals being directed towards the development of new medication used in treating wounds. Being an endemic plant, B. luzonica will be of low cost and readily available alternative remedy to chemically manufactured drugs.

## MATERIALS AND METHODS

### Plant material

Fresh leaves of *B. luzonica* were collected from Matabungkay, Lian, Batangas. The plant was identified and authenticated by a taxonomist at the University of the Philippines Herbarium, Los Baños, Laguna.

### Plant extraction

The leaves were dried in an open air and ground using a blender. Fifty grams of the ground leaves were extracted with ethanol and filtered through eight layers of muslin cloth. The procedure was repeated twice with 250 ml of ethanol in each extraction. The pooled *B. luzonica* leaf extract was concentrated by evaporation inside the laminar flow hood to prevent contamination. The residue was stored in stock vials for further use.

The prepared *B. luzonica* leaf extract was qualitatively assessed for phytochemicals by standard [20].

**Phytochemical screening**

**Flavonoids**

To confirm for the presence of flavonoids, 2mL of *B. luzonica* leaf extract was added to a test tube containing 1mL of 2N NaOH. Yellow color indicates the presence of flavonoids [21].

**Alkaloids**

For the purpose of detecting alkaloids, 0.2g of *B. luzonica* leaf extract was added in each test tube and 3mL of hexane was mixed in it, shaken, and filtered. Then 5mL of 2% HCl were poured in a test tube having the plant extract and hexane. Formation of yellow color indicates the presence of alkaloids [22].

**Tannins**

To detect for the presence of tannins, 1mL of *B. luzonica* leaf extract was added to 2mL of 5% ferric chloride. Dark blue or greenish black indicates positive result [23].

**Saponins**

The presence of saponin was detected by shaking 0.5g of *B. luzonica* leaf extract in 2mL of water. If foam persists for ten minutes it indicates the presence of saponins [24].

**Embryo collection**

Fertilized white duck eggs were purchased in a farm at Victoria, Laguna. The outer surface of the embryos was cleansed with 75% ethanol and incubated at 37°C throughout the study. Eggs with prominent heartbeat were chosen. Five groups containing five eggs per group were used for each assay [25].

**Preparation of saturated filter disk for wound assay**

Whatman No. 1 filter paper was purchased from Puljed trading. Small disks were prepared using a standard 5mm hole puncher, sterilized by autoclaving and stored for use. The pre-sterilized filter disks were saturated with different concentrations of *B. luzonica* leaf extract, from 100, 300 and 500 µg/mL with distilled water as negative control (Chris, David, & Dwayne, 2005) and Diclofenac sodium (50µg/mL) in 4% ethanol as positive control. The said drug was proven to accelerate healing of wounds because it is an anti-inflammatory and analgesic which relieve pain and inflammation [26].

**Wound assay**

The fertilized eggs were incubated inside an incubator at 37°C for 11 days with an automatic rotator to allow slow but constant movement of the egg. The humidity of the incubator was maintained at 62% by putting trays of distilled water under the egg tray and replacing the water every day to prevent contamination and to allow good maturation of the chorioallantoic membrane [27]. On the 12$^{th}$ day of incubation, the eggs were placed in the laminar flowhood). Ethanol was used to sterilize the outer surface [28]. To visualize the developing embryo, egg candler was used [29]. Under aseptic conditions a tiny hole was made carefully in the eggshell with a surgical scalpel and a small window of the shell was opened exposing the opaque inner shell membrane. About 0.5-1 mL sterile saline was added for the inner shell membrane to be translucent. This layer was peeled to visualize the CAM layer. Using sterile forceps and small dissecting scissors, the layer was pulled gently and excised wound of approximately 3 mm diameter. The drug saturated disc labeled with the corresponding concentrations and controls were placed on the CAM at the junction of two blood vessels. Parafilm was used to cover the window on the eggshell and the eggs were returned to the incubator.

Measurements the internal diameter of wounds was made on alternative days up to day 5 of observation post wounding. The wound closure was measured as wound contraction percentage (WC %) using the formula [30].

$$WC\ \% = \frac{\text{Initial wound size} - \text{Specific day wound size} \times 100}{\text{Initial wound size}}$$

**Angiogenesis assay**

The vascular network forms well in the developing embryo by 7 days of incubation. On the 8$^{th}$ day, the outer shell was wiped with 75% ethanol to sterilize the surface. Under aseptic conditions, a tiny hole was made carefully in the egg shell with a needle and a small window of the shell was cracked open to expose the CAM layer. The drug saturated disc labeled with the corresponding concentrations and controls were placed on the CAM at the junction of two blood vessels. The window on the eggshell was covered with parafilm and the eggs were returned to the incubator. After 3 days of incubation, results were observed and photographed. Macroscopically, photographs were printed and evaluated using an acetate paper. Blood vessel branch points or the points of intersection were counted in 6 random areas of each Chorionic Allantoic Membrane (CAM) in a clockwise direction [27].


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**Statistical analysis**

Collected data were analyzed using Analysis of Variance (ANOVA) and multiple comparisons was made thru the use of Tukey's Multiple Comparison post hoc analysis test and Dunnett's Multiple Comparison Test. Both tests were used to compare the level of significance between the experimental treatments and control groups. The values are presented as mean difference. In the above statistical tool, the probability value $p<0.05$ is considered as significant level.

## RESULTS AND DISCUSSION

**Plant Extract**

Crude ethanolic extract was obtained from dark-brown colored dried leaves of B. luzonica. Sticky, dark green extract as described by Casuga et al. [31] weighing 16.64 grams was obtained as seen in Figure 2B. Total percent yield was 33.28%. The results are different with the study of Choa et al. [32] in which the amount of extract using methanol was 273.663 grams and it yielded 21.048%. This was probably due to difference in the solvents used.

**Phytochemical Screening**


**Figure 2. *B. luzonica* (A) Dried leaves (B) Crude extract**

Screening of metabolites revealed the presence of various compounds of B. luzonica leaves in ethanolic extract including tannins, flavonoids and alkaloids.

In our findings, the plant is positive for the presence of alkaloids. The positive result of this metabolite in *B. luzonica* is in parallel with the findings of Choa et al., [32]. Anti-inflammatory, antiviral, antibacterial, antioxidant activity of the plant, aiding wounds to heal faster may be attributed to the presence of alkaloids [33]. According to a study conducted by Manjunatha et al. [34] flavonoids have been known to increase the vascularity of vessels, resulting to more viable collagen fiber and can reduce lipid peroxidation.

**Table 1. Phytochemical Result**

| Tests | Result |
|---|---|
| Alkaloids | + |
| Flavonoids | + |
| Saponin | - |
| Tannin | + |

*Legend: (+) presence, (-) absence*

Tannin promotes the rate of epithelization and is also responsible for wound contraction. This phenolic compound also possesses an anti-inflammatory property. It also has antibacterial property and aids in the formation of new blood vessels which is beneficial to cutaneous wound healing. Therefore, these studies may support that the presence of alkaloids, flavonoids and tannins in the plant extract are probably responsible for wounds to heal faster [35].

**Wound Assay**

Wound closure (WC) is defined as a measurement of how much of the wound injury was healed (Espiritu et al., 2016). It was measured on Wound Contraction Percentage (WC%).

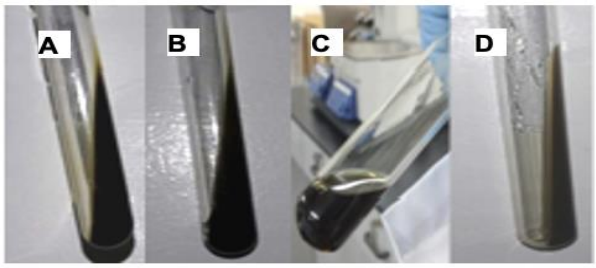


**Figure 3. (A) Alkaloid (yellow ppt), (B) Flavonoid (Yellow color), (C) Saponin (negative), (D) Tanin (greenish black)**

**Table 2. Wound Closure Percentage (%) from Day 3 & Day 5**

| *B. luzonica* leaf extract Concentrations & Controls | Wound Closure Day 3 | Wound Closure Day 5 |
|---|---|---|
| Positive | 56.73 | 75.67 |
| Negative | 23.33 | 50.07 |
| 100 ug/mL | 53.87 | 72.13 |
| 300 ug/mL | 55.80 | 72.00 |
| 500 ug/mL | 54.00 | 73.40 |

As shown in Table 2 and Figure 4, WC% of the positive control were 56.73% on Day 3 and 75.67% on day 5 while the negative exhibited 23.33% on day 3 and 50.07% on Day 5. On day 3, *B. luzonica* leaf extract concentrations of 100 ug/mL, 300 ug/ml, 500 ug/ml has 53.87%, 55.80% and 54.00% wound closure percentage, respectively. On day 5, at similar concentrations, the WC% results were 72.13%, 72.00% and 73.40%, respectively. WC% of the group containing positive control exhibited the highest wound closure activity. This was followed by the 300 ug/mL concentration having 55.8% on day 3 while 500 ug/mL

has wound closure of 73.4% on the 5th day respectively. The wound contraction percentage of the plant is probably attributed to the presence of tannins. As supported in the study conducted by Bin and Wang [35], tannin promotes the rate of epithelization and is also responsible for wound contraction. The negative control on the other hand, has the lowest wound closure percentage of 23.33% on day 3 and 50.07% on day 5. This is because wound healing is natural physiologic mechanism of the body. Therefore, without doing anything, healing of wounds is an automatic process. But despite the fact that it is an expected process, accelerating wound healing requires attention due to risk of infection and other severe complication [36].

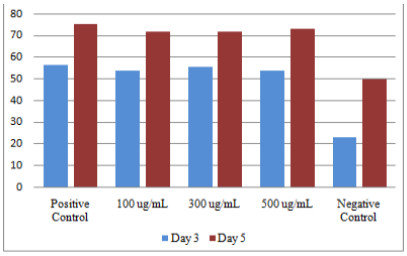


**Figure 4. Wound Closure Percentage**

Figure 4 shows the wound closure percentage of the different concentrations of *B. luzonica* leaf extract as well as the controls used.

Increase percentage of wound closure is seen in the positive control as well as the different concentration of *B. luzonica* leaf extract. This indicates faster wound healing activity. The effect done by the positive control has nearly the same effect done by the varying concentrations of the *B. luzonica* leaf extract. However, the effect done by the negative control (distilled water) has only minimal result in the wound closure as compared with the other groups.

**Table 3. Comparison on the wound assay (Day 3 and 5) against negative control**

| Varying concentrations of *B. luzonica* leaf extract | Mean Diff. Day 3 | Mean Diff. Day 5 | P-value | Interpretation |
|---|---|---|---|---|
| positive control | 1.002 | 0.7680 | *** | Highly Significant |
| 100 ug/ml | 0.9160 | 0.6620 | *** | Highly Significant |
| 300 ug/ml | 0.9740 | 0.6460 | *** | Highly Significant |
| 500 ug/ml | 0.9200 | 0.7000 | *** | Highly Significant |

Table 3 shows comparison of the data gathered during day 3 and day 5 of post treatment. Positive control has a mean difference of 1.002 in day 3 and 0.7680 in day 5 and a p-value of <0.001. Thus there is a highly significant difference observed as compared to the wound healing activity done by the negative control. Diclofenac sodium is a non-steroidal drug proven to have anti-inflammatory property and can increase wound healing activity [37].

Moreover, varying concentrations of *B. luzonica* leaf extract from 100, 300 and 500 ug/ml produced mean differences of 0.9160, 0.9740 and 0.9200 in day 3, respectively, and 0.6620, 0.6460 and 0.7000 Increase percentage of wound closure is seen in the positive control as well as the different concentration of B. luzonica leaf extract. This indicates faster wound healing activity. The effect done by the positive control has nearly the same effect done by the varying concentrations of the B. luzonica leaf extract. However, the effect done by the negative control (distilled water) has only minimal result in the wound closure as compared with the other groups.

Table 3 shows comparison of the data gathered during day 3 and day 5 of post treatment. Positive control has a mean difference of 1.002 in day 3 and 0.7680 in day 5 and a p-value of <0.001. Thus there is a highly significant difference observed as compared to the wound healing activity done by the negative control. Diclofenac sodium is a non-steroidal drug proven to have anti-inflammatory property and can increase wound healing activity [37]. Moreover, varying concentrations of B. luzonica leaf extract from 100, 300 and 500 ug/ml produced mean differences of 0.9160, 0.9740 and 0.9200 in day 3, respectively, and 0.6620, 0.6460 and 0.7000.

**Table 4. Comparison on the wound assay (Day 3 and 5) against positive control**

| Varying concentrations of *B. luzonica* leaf extract | Mean Diff. Day 3 | Mean Diff. Day 5 | P-value | Interpretation |
|---|---|---|---|---|
| negative control | -1.002 | -0.7680 | *** | Highly Significant |
| 100 ug/ml | -0.08600 | -0.1060 | ns | Not Significant |
| 300 ug/ml | -0.0280 | -0.1220 | ns | Not Significant |
| 500 ug/ml | -0.08200 | -0.06800 | ns | Not Significant |

Table 4 shows that the negative control has a mean difference of -1.002 in day 3 and -0.7680 in day 5 against the positive control and a p-value 0f <0.0001. Thus, there is a highly significant difference observed when negative control is compared with the positive control. This clearly shows that distilled water has no wound healing activity. According to a study conducted by Fernandez and Griffiths [38], there is no strong evidence that cleansing wounds using distilled water per se increases healing or reduces infection. Varying concentrations of B. luzonica leaf extract from 100, 300 and 500 ug/ml have mean differences of -0.08600, -0.0280and -0.08200 in day 3, respectively, and -0.1060, -0.1220 and -0.06800 in day 5, respectively. When compared with the positive control, there is no significant difference observed because the wound healing activity exhibited by varying concentrations of B. luzonica leaf


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extract was found to be similar with the positive control, Diclofenac sodium. Phytochemicals present in B. luzonica were probably responsible for its wound healing activity. Tannins, present in B. luzonica leaf extract was known to promote the rate of epithelization and was also responsible for wound contraction [35]. However, as a standard drug, Diclofenac sodium showed better wound healing activity than B. luzonica leaf extract.

Table 5 shows the multiple comparison of the wound healing activity of varying concentrations of *B. luzonica* leaf extracts and controls in wound assay (Day 3 & 5). For negative control, when compared with the positive control, the resulting p-value was <0.0001; therefore, there was a highly significant difference observed in their wound healing activity.

When compared with 100, 300 and 500 ug/ml concentrations, the result was highly significant because the p-value was <0.0001. Therefore, there was a large difference in terms of their effectiveness. When positive control was compared with the negative control, the resulting p value was <0.0001; thus, there is highly significant difference in their wound healing activity confirming the results in Table 3 and Table 4.

However, there is no significant difference when positive control was compared with 100, 300 and 500 ug/ml concentrations, respectively, because the p-value is greater than 0.05. This only means that the positive control and the varying concentrations of *B. luzonica* leaf extract has almost the same activity. For 100 ug/ml, when compared with the positive control, the result has no significant difference because the p-value was greater than 0.05. When compared with the negative control, there is highly significant difference because the p-value is <0.0001. When compared with 300 and 500 ug/ml concentration, the results showed no significant difference because the p-value was >0.05. For 300 ug/ml, when compared with the positive result showed no significant difference because the p-value is >0.05. When compared with the negative control, the result showed highly significant difference because the p-value is <0.0001. When compared with 100 ug/ml and 500 ug/ml concentrations, the result showed no significant difference because the p-value is >0.05. For 500 ug/ml, when compared with the positive control the result was of no significant difference because the p-value is greater than 0.05. When compared with the negative control, the result showed highly significant difference because. When compared with the 100 and 300 ug/ml concentrations, the resulting p-value is > 0.05 showing no significant difference in the effect done by the varying concentrations of *B. luzonica* leaf extract.

**Table 5. Multiple Comparison on the effect of varying concentrations of B. *luzonica* leaf extracts and controls in wound assay (Day 3 & 5)**

| Different concentrations of *B. luzonica* leaf extract | | Mean Diff. Day 3 | Mean Diff. Day 5 | P-value | Interpretation |
|---|---|---|---|---|---|
| Negative Control | positive control | 33.40 | 25.60 | *** | Highly Significant |
| | 100 ug/ml | -30.53 | -22.06 | ns | Not Significant |
| | 300 ug/ml | -32.47 | -21.53 | ns | Not Significant |
| | 500 ug/ml | -30.67 | -23.33 | ns | Not Significant |
| Positive Control | negative control | 33.40 | 25.60 | *** | Highly Significant |
| | 100 ug/ml | 2.864 | 3.534 | *** | Highly Significant |
| | 300 ug/ml | 0.9300 | 4.066 | *** | Highly Significant |
| | 500 ug/ml | 2.728 | 2.26 | *** | Highly Significant |
| 100 ug/ml | positive control | -2.864 | -3.534 | ns | Not Significant |
| | negative control | 30.53 | 22.06 | *** | Highly Significant |
| | 300 ug/ml | -1.934 | -0.01600 | ns | Not Significant |
| | 500 ug/ml | -0.1360 | -1.268 | ns | Not Significant |
| 300 ug/ml | positive control | -0.9300 | -4.066 | ns | Not Significant |
| | negative control | 32.47 | 21.53 | *** | Highly Significant |
| | 100 ug/ml | 1.934 | 0.01600 | ns | Not Significant |
| | 500 ug/ml | 1.798 | -1.800 | ns | Not Significant |
| 500 ug/ml | positive control | -2.728 | -2.266 | ns | Not Significant |
| | negative control | -30.67 | -23.33 | *** | Highly Significant |
| | 100 ug/ml | 0.1360 | 1.268 | ns | Not Significant |
| | 300 ug/ml | -1.798 | -1.800 | ns | Not Significant |

Based on the multiple comparison, it was found out that during the second reading, there was a significant difference observed on the multiple comparison conducted on the negative control and 100ug/ml, 300ug/ml and 500ug/ml concentrations since the computed p-values were < 0.001 whereas, in comparison with the positive control, there is no significant difference since the results of the different concentrations of *B. luzonica* is similar with the effect done by the Diclofenac sodium. It is evident that there is no dose-dependency in the rate of wound closure because the result was still the same regardless of the concentration used. Therefore, the most effective concentration was 100 ug/ml because it is the lowest concentration capable of exhibiting wound closure. Phytochemicals present in *B. luzonica* were probably responsible for its wound healing activity. Tannins, present in *B. luzonica* leaf extract was known to promote the rate of epithelization and was also responsible for wound contraction [35]. Another study specified that the anti-inflammatory, antiviral, antibacterial, antioxidant activity of the plant, aiding wounds to heal faster may be attributed to the presence of alkaloids [33]. Therefore, these studies may support that the presence of alkaloids, and tannins present in the *B. luzonica* plant extract are probably responsible for wounds to heal faster [14]. However, being the standard drug used in treating wounds, Diclofenac sodium still exhibited the most effective result in accelerating the healing of wounds.

**Table 6. Average of Total, Main and Capillary Branch Vessels (Day 3)**

| *B. luzonica* leaf extract Concentrations & Controls | Total Branch Vessels | Main Branch Vessels | Capillary Branch Vessels |
|---|---|---|---|
| Negative | 29 | 17.4 | 11.6 |
| Positive | 36.8 | 21 | 15.8 |
| 100 ug/mL | 42.6 | 24 | 18.6 |
| 300 ug/mL | 43.8 | 24 | 19.6 |
| 500 ug/mL | 33.8 | 18.2 | 15.6 |

## Angiogenesis Assay

New blood vessels that are formed from pre-existing ones play an important role in physiological developments like healing of wounds and embryonic development [11]. Table 6 shows the average, total and capillary branch vessels during day 3. The negative control which is composed of distilled water. It was found out that there were 29 total branch vessels, 17.4 main branch vessels and 11.6 capillary branch vessels counted. Though new blood vessels were observed, this can be attributed to the natural wound healing process and not on the capacity of distilled water to induced wound healing. Distilled water can be used as a cleansing agent for wounds but there is no strong evidence that cleansing wounds using distilled water per se increases healing or reduces infection [38]. Production of new blood vessels occur because wound healing is a natural process done by the body to restore tissue integrity [39].

On the other hand, an average number of 36.8 total branch vessels, 21 main branch vessels and 15.8 capillary branch vessels were counted in the positive control that is composed of 50ug Diclofenac sodium in 4% ethanol. Diclofenac sodium is a non-steroidal drug proven to have anti-inflammatory property [37]. According to Kant et al. [40] anti-inflammatory properties of certain drugs and extracts caused faster and better wound healing because it decreases the persistence of inflammatory state via decreased expression of TNF-alpha, IL-1beta and MMP-9 and increased levels of IL- 10 at the wound site.

Varying concentrations *of B. luzonica* leaf extract from 100, 300 and 500 ug/ml concentrations showed an average number of 42.6, 43.8 and 33.8 total branch vessels, 24, 24, and 18.2 main branch vessels and 18.6, 19.6 and 15.6 capillary branch vessels, respectively. At 300 ug/ml concentration, the highest total branch vessels with a mean of 43.8 was seen, followed by the 100 ug/ml with a mean of 42.6 mean difference. This is probably because of the presence of alkaloids and flavonoids. Alkaloids present in the plant are proven to have antibacterial property and aids in the formation of new blood vessels which is beneficial for cutaneous wound healing [29]. Furthermore, flavonoids have been known to increase the vascularity of blood vessels [34]. However, at 500ug/ml, the total branched vessels were decreased to 33.8 probably because the extract has already too much flavonoid content.

Flavonoids also contain antioxidant potential which prevents angiogenesis. [7]. Because of its presence in the *B. luzonica* leaf extract, *B. luzonica* has pro and anti-angiogenic activity which may induce the formation of new blood vessels but at the same time, may suppress vessel growth when too much flavonoids is present. This probably explains that at 100 and 300ug/ml, blood vessel formation is abundant because of the presence of alkaloids and flavonoids. However, at 500ug/ml, the blood vessel formation is partially inhibited due to increase flavonoid content that may suppress the growth of blood vessels.

Several histologic measurements showed that significant elevation of liver enzymes and alkaline phosphatase which can damage the liver in prolonged intake may be attributed to Diclofenac toxicity (Amer, 2010). On the other hand, *B. luzonica* is an endemic plant here in the Philippines. Because of its availability in the country, it can be used as an alternative remedy to the standard drug Diclofenac sodium in attaining faster wound closure as well as preventing the adverse effects brought by the drug.

On gross morphology as seen in Figure 5, blood vessels were developed around the embryo without any


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noticeable deformation indicating that eggs were alive during observation of results.

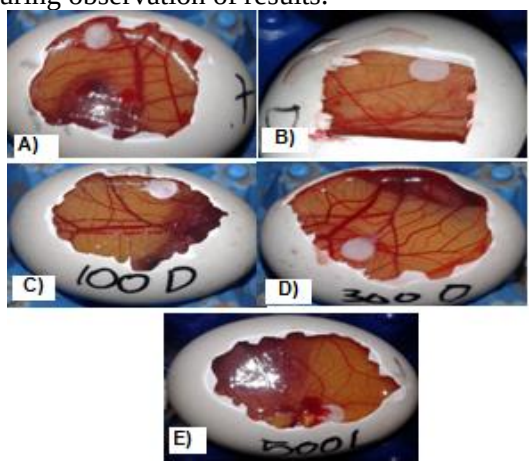


**Figure 5. Gross morphology of the embryo (A) Positive control, (B) Negative control, (C) 100ug/ml, (D) 300ug/ml, (E) 500ug/ml**

Statistical comparison on the angiogenesis assay (capillary branched vessels) of the varying concentrations of *B. luzonica* leaf extract is shown in Table 7 and Table 8. Table 7 shows that when compared to the positive control, the negative control having a mean difference of 0.0140 has no significant difference in terms of the formation of new blood vessels. All other concentrations of *B. luzonica* leaf extract from 100, 300 and 500 ug/ml has a mean difference of -0.03600, -0.05000 and 0.008000, also has no significant difference.

**Table 7**
**Comparison on the Angiogenesis Assay (CBV) Against Positive Control**

| Varying concentrations of *B. luzonica* leaf extract | Mean Diff. | Interpretation |
|---|---|---|
| negative control | 0.0140 | Not significant |
| 100 ug/ml | -0.03600 | Not significant |
| 300 ug/ml | -0.05000 | Not significant |
| 500 ug/ml | 0.008000 | Not significant |

*Legend: * p < 0.50; ** p < 0.01; *** p < 0.001;**** p < 0.0001*

Table 8 shows that when compared to the negative control, positive control has a mean difference of -0.0140 and the varying concentrations of *B. luzonica* leaf extract from 100, 300, and 500 ug/ml have mean differences of -0.05000, -0.06400 and -0.006000, respectively; thus, all groups differ insignificantly with the other groups. This only means that the effects were the same when compared to each group. This is possibly because the comparison considered many variances that have caused the varying and unparallel findings. It is inevitable in any statistical analysis that all results will agree with another [41]. In contrast to the study conducted by Shrmanker et al. [42] wherein improved angiogenesis would be contributing significantly to wound healing activity, the researchers found out that angiogenesis is not a sole indicator of wound healing activity because of pro and anti- angiogenic properties exhibited by the *B. luzonica* leaf extract.

**Table 8**
**Comparison on the Angiogenesis Assay (CBV) Against Negative Control**

| Varying concentrations of *B. luzonica* leaf extract | Mean Diff. | Interpretation |
|---|---|---|
| Positive control | -0.0140 | Not significant |
| 100 ug/mL | -0.05000 | Not significant |
| 300 ug/mL | -0.06400 | Not significant |
| 500 ug/mL | -0.006000 | Not significant |

*Legend: * p < 0.50; ** p < 0.01; *** p < 0.001;**** p < 0.0001*

To further verify the results of macroscopical observation of newly formed capillaries, it is visualized representative sample of each group under the dissecting microscope as shown in the Figure 6. There is abundance of blood vessel formation in the Figures A, C, D, and E.

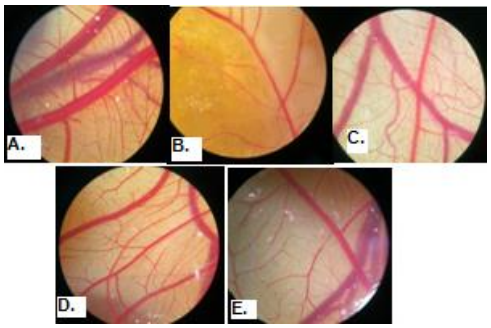


**Figure 6. Microscopical Observation (A) Positive control, (B) Negative control, (C) 500ug/ml, (D) 300ug/ml, (E) 100ug/ml**

However, as seen microscopically, the positive control still exhibited the most number of blood vessels because it is a standard drug proven to increase healing of wounds. On the other hand, Figure B or the negative control exhibited only minimal blood vessel formation because naturally, the body has the ability to repair damaged tissue and angiogenesis is involved in the second stage of wound healing. This demonstrates a large difference in terms of healing of wounds. Varying concentrations from 100, 300 and 500 ug/ml *of B.*

*luzonica* leaf extract on the other hand, exhibits large numbers of newly formed capillaries as compared to the negative control.

## CONCLUSION AND RECOMMENDATION

Through this study, the researchers have proven the capacity of the Broussonetia luzonica leaf extract in promoting wound healing when compared to the positive and negative controls. 100ug/mL concentration of B. luzonica leaf extract initiated the wound healing effect and succeeding concentrations produced the same results therefore, there is no dose dependency exhibited by the Broussonetia luzonica leaf extract. Thus, B luzonica leaf extract has wound healing activity starting at 100 ug/ml with no dose-dependency. Angiogenesis was found to be involved in wound healing activity of B. luzonica leaf extract but was not statistically supported. Therefore, it can be used as an alternative instead of chemically manufactured antibiotics.

Based on the findings, the researchers recommend the use of other parts of the plant for the observation of wound healing property aside from the leaves. Furthermore, purification of phytochemicals can be done, microscopic means or softwares can be used to quantify the existence of new vessels and lastly, the use of other in vivo assays to determine the wound healing property.

## REFERENCES


[1] Portou, Abraham, Baker, Tsui. (2015). The innate immune system, toll-like receptors and dermal wound healing:Areview. *Vascul,Pharmacol. 2 71*, 31-36.

[2] ShrimankerMitali, Patel N. M. (2013). Preparation and *In-Vitro* Evaluation of Polyherbal Formulation for Wound Healing.*American Journal of PharmTechResearch 3*(6) ISSN: 2249-3387

[3] Han G., & Ceilley R. (2017). Chronic wound healing: A review of current management and treatments, *AdvTher. 34*:599–610, 10.1007/s12325-017-0478-y

[4] Olczyk P., Mencner A., Vassev K. (2014). The role of the extracellular matrix components in cutaneous wound healing.*BioMed Research International* 747584, 8

[5] Quebral E., & Medina P. (2015). Crude anthocyanin extract (CAE) from Ballatinao black rice hastens regeneration in *Dugesiahymanae. J Pharm Biomed Sci. 2; 05*(02):147-153.

*[6]* Brand, H., & Veerman, E. (2013). Saliva and wound healing.*The Journal of Dental Research* 16(1)

[7] Mumtaz, A., Fousia L., Hira A., Laiba A., Muhammad I., Nabeela N., & Nazia T. (2015). Evaluation of pharmacological effect of *Morusnigra* extract on angiogenesis using chorioallantoic membrane(CAM)assay.*CanadianJournal of Applied Sciences; 05*(02): 26-40

*[8]* Brand, H., & Veerman, E. (2013). Saliva and wound healing.*The Journal of Dental Research Volume 16 Number 1*

[9] Sorg H., Hager S., Hauser J., Mirastschijski U., & Tilkorn D. (2017). Skin Wound Healing: An Update on the Current Knowledge and Concepts. *EurSurg Res 58*:81–94.

[10] Sen, C. K., Gordillo, G. M., Roy, S., Kirsner, R., Lambert, L., Hunt, T. K., ... & Longaker, M. T. (2009). Human skin wounds: a major and snowballing threat to public health and the economy. *Wound repair and regeneration*, *17*(6), 763-771.

[11] MajewskaI.,& Darmach E. (2011).Proangiogenic activity of plant extracts in accelerating wound healing — a new face of old phytomedicines endothelial cadherin; VEGF, vascular endothelial growth *58*, No 4/2011449–460

[12] Espiritu A., GuerreroJ., & Lao S. (2016). Burn wound healing potential of *Bixaorellana Linn [Bixaceae]* leaf extracts on albino mice.*Journalof Medicinal Plants Studies 4*(1): 84-87

[13] Alam G., Singh M. & Singh A. (2011). Wound healing potential of some medicinal plants. *International Journal of Pharmaceutical Sciences Review and Research Vol. 9* Issue 1, Article-26.

[14] Ruma O. (2015). Antimicrobial activity and phytochemical screening of selected indigenous food plants from Isabela, Philippines.*Upland Farm Journal 23*, 1.

*[15]* Ashok & Upadhyaya. (2012). Tannins are astringent,*Journal of Pharmacognosy and Phytochemistry ISSN 2278*- 4136 ZDB-Number: 2668735-5 IC Journal No: 8192

[16] Lookman N., Elder A., Ricciardelli C., &Oechler M. (2012). Chick chorioallantoic membrane (CAM) assay as an in vivo model to study the effect of newly identified molecules on ovarian cancer invasion and metastasis.*International Journal ofMolecular Sciences. 13*:9959-9970.

[17] Gamallo, Espere, Carillo, Blanes, Abuda, Labarda, Madelo, & Jumawan (2016) Evaluation of antiangiogenic property of Ocimum basilica ethanolic leaf extract by using duck embryo chorioallantoic membrane (cam) assay and its morphometric analysis. *International Journal of Herbal Medicine 2016; 4*(4): 22-26

[18] Ribatti D. (2012). Chicken chorionic allantoic membrane model.*Methods Mol. Biol;843*:47-57

[19] Casuga F., Castillo A., & Corpuz M. (2016). Bioactive compounds and cytotoxicity of Ethyl Acetate extract from *Broussonetia luzonica* (Moraceae) Blanco leaves against Hepatocellular




ISSN 2704-3568

carcinoma (Hepg2*) Cell Lines. Pharmacogn. J*. 8(5):497-501.

[20] Maneemegalai S., Naveen T. (2010*)*. Evaluation of antibacterial activity of flower extracts of *Cassia auriculata. Ethnobotanical Leaflets, 14*, 8-20.

[21] Rohini M., & Padmini E. (2016). Preliminary phytochemical screening of selected medicinal plants of polyherbalformulation. Journal of Pharmacognosy and Phytochemistry; 5(5):277-282.

[22] Wadood A., Ghufran M., Jamal S., Naeem M., Khan A.,Ghaffar R., & Asnad. (2013) Phytochemical Analysis of Medicinal Plants Occurring in Local Area of Mardan. *Biochem Anal Biochem 2*: 144. doi: 10.4172/2161-1009.1000144

[23] Rohini M., & Padmini E. (2016). Preliminary phytochemical screening of selected medicinal plants of polyherbalformulation. *Journal of Pharmacognosy and Phytochemistry; 5*(5):277-282.

[24] Tiwari P., Kumar B., Kaur M., Kaur G., &Kaur H. (2011). Pytochemical screening and extraction: A review. *International Pharmacutica Sciencia 1(1)*

[25] Vaidyanathan L., Thanikachalam D., & Sivaswamy L. (2014). Evaluation of Wound Healing Potency of Cassia auriculata Flower Extract Using Chick Embryo Wound Model,*Int. J. Pharma Sci. Rev.Res, 27*(2): 222

[26] Mohan, & Sharma (2017). Biochemical Changes in the liver of mice after exposure to different Doses of Diclofenac Sodium. *World Academy of Science, Engineering and Technology International Journal of Animal and Veterinary Sciences 11(5).*

[27] Goze, Cetin & Goze. (2010). Investigation of effects of essential oils of O. minutiflorum O SchuarzPh Davis &Cyclotrichiumniveum on angiogenesis in shell-less chick embryo culture. *African Journal of Biotechnology,* 9(14).

[28] Narendra N., Gaurav P., LokeshD., & Naveen. (2009). Wound healing activity of latex of *Calotropisgigantea, International J. of Pharmacy and Pharmaceutical Sciences*, *1*(1), 34-35.

*[29]* Li, Pathak, Rivera, Friedman, Ghiso, &Sikora, (2015). The Inovo CAM Assay as an efficient Xenograft model of Hepatocellular carcinoma*J Vis Exp. 2015 Oct 9;(104).*

*[30]* Flores-Sánchez, I. J., & Garza-Ortiz, A. (2019). Is there a secondary/specialized metabolism in the genus Cuscuta and which is the role of the host plant?. *Phytochemistry Reviews*, *18*(5), 1299-1335.

[31] Casuga F., Castillo A., &Corpuz M. (2016). Bioactive compounds and cytotoxicity of Ethyl Acetate extract from *Broussonetia luzonica*(Moraceae) Blanco leaves against Hepatocellular carcinoma (Hepg2) *Cell Lines. Pharmacogn. J*. 8(5):497-501.

[32] Choa, Lu, Nombrado ,Rayos, Invento & Castañeda (2016). Phytochemical screening of *Broussonetia luzonicus* (Moraceae) leaves.*Journal of Chemical and PharmaceuticalResearch, 8*(2):335-338

[33] Amic, D., Davidovic-Amic, D., Beslo, D., Rastija, V., Lucic, B., & Trinajstic, N. (2007). SAR and QSAR of the antioxidant activity of flavonoids. *Current medicinal chemistry*, *14*(7), 827-845.

[34] Manjunatha BK., Vidya SM., Rashmi KV., Mankani KL., Shilpa HJ., Jagadeesh Singh SD. (2005) Evaluation of wound-healing potency of Vernoniaarborea. Hk. *Indian Journal of Pharmacology*; 37(4);223-226.

[35] Bin L., Wang J. HC. (2011). Fibroblasts and myofibroblasts in wound healing:force generation and measurement.*J Tissue Viab 20*(4): 108-20

[36] Shrimanker Mitali, Patel N. M. (2013). Preparation and *In-Vitro* Evaluation of Polyherbal Formulation for Wound Healing.*American Journal of PharmTechResearch 3*(6) ISSN: 2249-3387

[37] Ulubay, Yurt, Kaplan & Atilla (2017). The use of diclofenac sodium in urological practice: Structural and neurochemical based review. *Journal of Chemical Neuroanatomy* 87, 32-36.

[38] Fernandez R, & Griffiths R. (2012). Water for wound cleansing. *Cochrane Database Syst Rev*. 2012 Feb 15;(2):. doi:10.1002/14651858.CD003861.pub3.

[39] Olczyk P., Mencner A., Vassev K. (2014). The role of the extracellular matrix components in cutaneous wound healing .*BioMed Res International* 747584, 8

[40] Kant, Gopal, Pathak, Kumar, Tandan & Kumar (2014). Antioxidant and anti-inflammatory potential of curcumin accelerated the cutaneous wound healing in streptozotocin-induced diabetic rats. *International Immunopharmacology*. 20 (2). 322-330.

[41] Belouafa, Habti, Benhar, Belafkih, Souad, & Souad (2016). Statistical tools and approaches to validate analytical methods: methodology and practical examples*Int. J. Metrol. Qual. Eng.* 8

[42] Shrimanker Mitali, Patel N. M. (2013). Preparation and *In-Vitro* Evaluation of Polyherbal Formulation for Wound Healing.*American Journal of PharmTechResearch 3*(6) ISSN: 2249-3387