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Evaluation of the Wound Healing Activity of the Seeds of *Dolichos lablab*, *Phaseolus lunatus*, and *Glycine soja*

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Abstract **Original Research Article**

The study aimed to investigate the wound healing activity of the seeds of *Dolichos lablab*, *Phaseolus lunatus*, and *Glycine soja*. In the present study the aqueous extracts of the seeds were orally administered to the Sprague Dawley rats. Evaluation of wound healing activity was done using the incision and excision wound models. In each model, group I served as the control group, group II as the test group treated with *Dolichos lablab* extract, group III as the group treated with *Phaseolus lunatus* extract and group IV with *Glycine soja* extract. Incision wound model rats were treated for 10 days and the wound healing activity was assessed by the tensile strength and histopathological studies of the granulation tissue. Excision wound model rats were treated until the wounds were completely closed. In this model, wound healing activity was assessed by the rate of wound closure and free radical scavenging activity was assessed using 1,1-diphenyl-2-picrylhydrazyl (DPPH) method. In both models, hydroxyproline content in the scab was determined. The results of the study indicated that the extract of *Dolichos lablab* possessed better wound healing and antioxidant activity than *Phaseolus lunatus* and *Glycine soja* extracts.

Keywords: *Dolichos lablab*; *Phaseolus lunatus*; *Glycine soja*; Wound healing; Incision; Excision.

INTRODUCTION

Injury involving a break in the skin is usually experienced as a result of everyday activities. Poor wound healing impedes resolution of minor breaks in the skin, allowing them to enlarge and to become infected. These infections complicate illness and even the less serious wounds are distressing, often irritant and disfiguring, and can lead to death. Popular alternatives nowadays are herbal remedies due to high price of commercially available wound healing preparations.

Philippines have been widely known for its plant diversity which displays an array of both potentially medicinal and therapeutic plants. Without prior researches and explorations, traditionally, these plants have been used as alternatives for medicine and therapy against diseases and ailments by indigenous groups. Medicinal plants have been used since time immemorial for treatment of various ailments of skin and dermatological disorders especially cuts, wounds and burns [1]. Plants and their extracts have immense potential for the management and treatment of wounds.

In an investigation of the antioxidant activity of commonly cultivated legumes in the Philippines, *Dolichos lablab*, *Phaseolus lunatus*, and *Glycine soja* showed remarkable free radical scavenging activity which aids in the healing of wounds [2]. Antioxidants hasten the process of wound healing by inactivating free radicals. Elimination of reactive oxygen species (ROS) is an important strategy in healing of wounds since it results in oxidative stress leading to cell death and delayed wound healing.

Discovering a wound healing agent from a local plant source, which has long been part of the diet with comparable efficacy but relatively cheaper than the ones available in the market, would provide a better alternative. Therefore, this research is conducted to evaluate the wound healing activity and relate it to the antioxidant activity of the seeds.

MATERIALS AND METHODS

Collection and preparation of plant samples

The seeds were removed from their respective fruits and stored separately in polyethylene bags in a place under controlled temperature of 30-40˚C. Sample seeds were brought to the Philippine National Museum for authentication.

Two hundred grams each of the three legume seeds was added to a liter of boiling water and heated for an hour at 50-60ºC. The extracts were filtered and the residue was re-extracted under the same conditions. The combined filtrates were evaporated to dryness under steam bath of 40ºC to obtain the H_2O-soluble constituents.

Evaluation of wound healing activity

Preparation of experimental animals

Healthy Sprague-Dawley albino rats of either sex and of approximately the same age, weighing between 120-150 grams were used for the study. They were individually housed, maintained in clean cages, and fed with commercial rat pellets and water ad libitum.

Twenty-four animals were divided into two groups, the first half were inflicted with incision wound and the other half with excision wound. Each group was further subdivided into four groups namely, control, *Dolichos lablab*, *Phaseolus lunatus*, and *Glycine soja*. The extracts were administered daily via oral gavage. During the study, gum acacia in normal saline was used as a vehicle into which the extracts were dissolved. Two grams of gum acacia was dissolved in 100 ml of normal saline. From this, 10 ml of solution, which contains 200 mg of gum acacia was used for dissolving 1 gram of aqueous extract. Hence, each ml of solution contains 100 mg of extracts.

Wound models

The procedure was carried out using chloral hydrate sedated rats in two the different wound models.

Incision Wound

Two paravertebral incisions (3 cm long) were made through the full thickness of the skin on either side of the vertebral column. Wounds were closed with interrupted sutures 1 cm apart using a surgical thread (No. 000) and curved needle (No. 11). The wounds were left undressed. The sutures were removed on the 7th day and tensile strength was measured on the 10th post-wounding day using an improvised tensiometer apparatus.

Excision Wound

A circular piece of full thickness skin (~300 mm^2) was cut off from a predetermined area on the back of the rat. Wounds were traced on a 1 mm^2 graphing paper on the day of wounding and subsequently on the alternate days, until healing was complete. Changes in wound area were calculated giving an indication of the rate of wound contraction. Scars were traced on complete epithelization to assess wound contraction by noting scar size and shape. Round or oval, large scars indicated poor contraction while stellate-shaped or linear scars indicated enhanced wound contraction.

Histopathological studies

For histopathological studies, granulation tissues from the incised rats were preserved in 10% formaldehyde solution and sent for histopathological examination at the UP College of Medicine.

Determination of hydroxyproline content

Six-point hydroxyproline standards (0-25 µg/ml) in 5 µg/ml increments were prepared. Triplicate samples of each legume treated group and control group were done. Samples then received in order: 1 ml each of 0.01 M copper sulfate solution, 2.5 N sodium hydroxide, and 6 percent hydrogen peroxide. The solutions were mixed and shaken occasionally during a period of 5 minutes, and were then placed in a water bath at 80ºC for 5 minutes with frequent vigorous shaking. The tubes were chilled in an ice and water bath and 4 ml of 3.0 N sulfuric acid were added with agitation, followed by 2 ml of p-dimethylaminobenzaldehyde solution were then added with thorough mixing.

The tubes were placed in a water bath at 70ºC for 16 minutes and then cooled in tap water. Samples were read at 513 nm with a Genesys™ 10 UV/Vis Spectrophotometer.

Evaluation of antioxidant activity

Fractionation

The dry residues from water extract of each legume were suspended (100 mg/mL) in water-acetone (2:1) and extracted with chloroform followed by ethyl acetate in a separatory funnel. Each of the fractions were dried and weighed.

1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay

The potential antioxidant activity of plant extracts was assessed on the basis of the scavenging activity of the stable DPPH free radical by the spectrophotometric method. The changes in color from deep violet to light yellow when DPPH radical reacts with an antioxidant were measured at 517 nm on a UV-visible light Genesys™ 10 UV/Vis Spectrophotometer.

The dried residue from each of the fractions was suspended in methanol to prepare a 200 µg /mL solution. Ascorbic acid which acted as control was also prepared at similar concentrations. Different volumes (2, 1, 0.5, 0.2, 0.1 and 0.05 mL) of each solution were mixed with 2 mL methanolic solution of DPPH (100 µg/mL) in a 10 mL volumetric flask and diluted to volume with methanol. The absorbances were measured with a spectrophotometer at 520 nm. The percent increase in absorbance was determined: as [($Abs_{initial}$-Abs_{final}/$Abs_{initial}$)] x 100. The concentration of the test solution required to give a 50% decrease in absorbance compared to that of a blank solution (EC_{50}) was determined by linear regression. Two trials of each sample were performed.

Statistical analysis

The data were statistically analyzed using one-way Analysis of Variance (ANOVA). Aqueous extracts of the legumes' seeds were compared with the control group. ANOVA was used to identify differences between groups. Data were considered significant at ρ-values of 0.05 or less.

RESULTS

Wound models and extract administration

Dolichos lablab, *Phaseolus lunatus*, and *Glycine soja* seeds were extracted with boiling water in accordance to the previous study where the aqueous extract exhibited remarkable antioxidant activity in induced liver toxicity. The amount of H_2O-soluble constituents was recovered through evaporation of the solvent via steam bath maintaining the temperature to 400ºC. The amounts produced for *Dolichos lablab*, *Phaseolus lunatus*, and *Glycine soja* were 17.4573 g (8.73%), 40.9765 g (20.49%), and 60.1231 g (30.06%), respectively. The residues were stored in amber bottles under controlled temperature of 30-400ºC. This is done to prevent triggering of oxidation and destruction of desired constituents.

To evaluate the wound healing activity of the legume seeds, incision and excision wound models were used. Twenty-four previously weighed rats were divided into each wound model group and three rats were used for each extract and standard. According to Food National Research Institute, 7.5-10.0 g per 50 kg individual or 150-200 mg/kg is the normal daily intake of legumes. Therefore, the dose used for the treatment of wound was 150 mg/kg. Toxicity studies were not done since it appears that the samples are usual human food commodities.

Tensile strength

On the 10th post-wounding day of incision rats, healed skin was taken and its tensile strength was tested using an improvised tensiometer. Table 1 shows that the average tensile strengths were 90.95, 87.5, 113.89 and 76.74 grams for *Dolichos lablab*, *Phaseolus lunatus*, *Glycine soja* and control groups respectively. This shows that *Glycine soja* had the greatest tensile strength, followed by *Dolichos lablab* then *Phaseolus lunatus*, and finally the control group. However, statistical analyses (Table 2) of the results showed there was no statistical difference between the tensile strength of the control compared to each of the three samples.

Table-1: Tensile Strength of Incised Wound

Sample	Trial 1	Trial 2	Trial 3	Average	$\bar{x}$
Control 1	85.4	58.5	114.9	86.27	76.74
Control 2	70.2	49.4	82.0	67.2	
Control 3	54.3	82.8	93.15	76.75	
D. lablab 1	96.5	83.1	107.0	95.53	90.95
D. lablab 2	43.8	105.5	118.7	89.3	
D. lablab 3	103	82.5	78.6	88.03	
P. lunatus 1	29.1	63.1	133.0	75.07	87.5
P. lunatus 2	94.3	115.8	81.0	97.03	
P. lunatus 3	112.4	53.7	105.1	90.4	
G. soja 1	206.7	144.2	139.6	163.5	113.89
G. soja 2	65.9	34.0	144.3	81.4	
G. soja 3	87.5	94.4	108.4	96.77	

Table-2: Statistical Result of Incision Wound for the Samples

Source of Variation	Sum of Squares	Degree of Freedom	Mean Squares	Computed f
Between Column	6,597.38	3	2,199.13	2.77
Within Column	25,411.77	32	794.12	

f .05= 2.90

Rate of wound contraction

Table 3 shows the excision wound contraction rate of the different groups. It can be seen that *Dolichos lablab* has the fastest closure rate, followed by *Glycine soja* then *Phaseolus lunatus*. Scars formed from the *Dolichos lablab* were stellate and smaller compared to control while *Phaseolus lunatus* and *Glycine soja* scars were oblongate and linear. This indicates that the scar formed from the *Dolichos lablab* showed better wound healing activity since there are more collagen deposition and greater wound contraction. However, statistical analyses (Table 4) of the results showed there was no statistical difference between the rate of wound contraction of the control group compared to each of the three samples.

Table-3: Wound Contraction Rate of Excised Wound

Group	Number of Days	Area	% Wound Closure
Control 1	1	394	0.00
	4	270	31.47
	8	126	68.02
	12	30	92.39
	16	12	96.95
	20	0	Complete healing
Control 2	1	244	0.00
	4	215	11.89
	8	92	62.30
	12	66	72.95
	16	13	94.67
	20	3	98.77
	24	0	Complete healing
Control 3	1	356	0.00
	4	282	20.79
	8	117	50.28
	12	21	94.10
	16	5	98.60
	20	0	Complete healing
D. lablab 1	1	335	0.00
	4	198	40.90
	8	59	82.39
	12	6	98.21
	16	5	98.51
	20	0	Complete healing
D. lablab 2	1	421	0.00
	4	302	28.27
	8	142	66.27
	12	50	88.12
	16	9	97.86
	20	8	98.10
	22	5	98.81
D. lablab 3	1	356	0
	4	184	48.31
	8	70	80.34
	12	5	98.60
	16	2	99.00
	20	0	Complete healing
P. lunatus 1	1	270	0.00
	4	221	18.15
	8	121	55.19
	12	18	93.33
	16	7	97.41
	20	4	98.15
	22	5	Complete Healing
P. lunatus 2	1	405	0.00
	4	271	33.09
	8	204	49.63
	12	109	73.09
	16	35	91.36
	20	2	99.51
P. lunatus 3	1	357	0
	4	210	41.18
	8	112	68.63
	12	50	86.00
	16	8	97.76

	20	7	Complete healing
G. soja 1	1	348	0.00
	4	213	38.79
	8	84	75.86
	12	19	94.54
	16	5	98.56
	20	2	99.43
G. soja 2	1	437	0.00
	4	280	35.93
	8	258	40.96
	12	123	71.85
	16	62	85.81
	20	25	94.28
	22	11	97.48
G. soja 3	1	325	0.00
	4	270	16.92
	8	144	55.69
	12	42	87.08
	16	8	97.54
	20	3	99.08
	20	3	99.08

Table-4: Statistical Result of the Excision Wound from the Samples

Source of Variation	Sum of Squares	Degree of Freedom	Mean Squares	Computed f
Between Column	779.11	3	259.70	0.34
Within Column	45564.99	60	759.40	

f .05= 2.76

Hydroxyproline assay

The incision and excision wound models were measured for hydroxyproline content. Measurement of hydroxyproline is used as an index for collagen turnover. The standard absorbance curve was determined using increasing amounts of standard and the absorbance obtained from each standard. Linear regression was used to determine the unknown concentration of sample with given amount of absorbance. In the incision model, average amount of hydroxyproline for each group were 2741.33, 4262.33, 3768.00 3955.50 µg/ml for the control, *Dolichos lablab*, *Phaseolus lunatus*, and *Glycine soja* groups respectively. The data in Tables 5 shows that the hydroxyproline content of the granulation was greatest in *Dolichos lablab*, *Phaseolus lunatus* and *Glycine soja* being next, and the control group had the lowest amount.

Table-5: Hydroxyproline concentration in Incision wound

Sample	Absorbance (A)	Concentration (µg/mL)	Concentration (µg/mL) x Dilution Factor (100)	Average
Control 1	1.834	50.17	5017	2741.33
Control 2	1.064	28.94	2894	
Control 3	1.449	39.56	3956	
D. lablab 1	2.011	55.05	5505	4262.33
D. lablab 2	1.520	41.51	4151	
D. lablab 3	1.150	31.31	3131	
P. lunatus 1	1.533	41.87	4187	3768.00
P. lunatus 2	0.589	15.84	1584	
P. lunatus 3	0.904	24.53	2453	
G. soja 1	2.000	54.75	5475	3955.50
G. soja 2	1.004	27.28	2728	
G. soja 3	1.139	31.01	3101	

Granulation tissue from excision model had 2983.50, 4956.33, 3792.67, 4217.33 µg/ml average amount of hydroxyproline for the control, *Dolichos lablab*, *Phaseolus lunatus*, and *Glycine soja* group respectively. It is shown in Table 7 that *Dolichos lablab* had the most amount of hydroxyproline followed by *Glycine soja*, *Phaseolus lunatus*, and control. Therefore, the *Dolichos lablab* group had the most amount of

hydroxyproline in both models and thus had the most collagen turnover.

Table-7: Hydroxyproline concentration in Excision wound

Sample	Absorbance (A)	Concentration	Concentration (µg/mL) x Dilution Factor (100)	Average
Control 1	1.128	30.51	3051	2983.50
Control 2	1.072	29.16	2916	
Control 3	1.096	29.83	2983	
D. lablab 1	2.060	56.40	5640	4956.33
D. lablab 2	1.504	41.07	4107	
D. lablab 3	1.872	51.22	5122	
P. lunatus 1	1.032	28.06	2806	3792.67
P. lunatus 2	2.014	55.13	5513	
P. lunatus 3	1.124	30.59	3059	
G. soja 1	1.978	54.14	5414	4217.33
G. soja 2	1.643	44.90	4490	
G. soja 3	1.011	27.48	2748	

Histopathological studies

The histopathological studies of the granulation tissue of the control group of animals showed absence of active collagen and macrophage. Histological studies of the tissue obtained from the *Dolichos lablab* treated groups showed significant increase in collagen deposition and few macrophages. In the case of *Phaseolus lunatus* treated groups equal amounts of collagen and macrophages were observed. In the case of *Glycine soja* treated groups, moderate collagen deposition, and increase in macrophages were observed. Increased collagen formation may enhance healing activity. Therefore, *Dolichos lablab* treated group with highest increase in collagen deposition had the highest wound-healing activity.

Antioxidant activity

In the analysis of antioxidant activity, DPPH solution was colored violet and was decolorized upon addition of the samples. Maximum absorbance was scanned at 520 nm. Conversion of DPPH to a non-radical form causes a reduction in absorbance. Ascorbic acid reduced DPPH absorption stronger than the legume extracts.

Chloroform, ethyl acetate and water acetone fractions were prepared to evaluate the antioxidant activity of the samples. In Trial 1 (Table 8), the chloroformic and ethyl-acetate fractions of *Dolichos lablab* showed the highest activities which surpassed those of the other two legume seed extracts. However, the water-acetone fractions were not as effective as *Phaseolus lunatus* and *Glycine soja*. *Glycine soja* showed the lowest activity in the chloroformic and water-acetone fractions. In Trial 2 (Table 9), the water-acetone and chloroformic fractions of *Phaseolus lunatus* showed the highest antioxidant activity. The ethyl-acetate of *Dolichos lablab* was greater than that of *Phaseolus lunatus*. The data showed that the antioxidant activity was found greatest in the chloroformic extract. This would indicate that the free radical scavenging activity may likely come from terpenoids and phenolic compounds since this compound have higher affinity to chloroform.

The scavenger activities of the samples are expressed as EC_{50}. The activity of the fractions of all samples confirms that these contain powerful antioxidant. *Dolichos lablab* showed the strongest activity relative to the control, followed closely by *Phaseolus lunatus*.

Table-8: Percent Reduction in Absorbance of DPPH and EC_{50} (Trial 1)

		Dolichos lablab			***Glycine soja***			***Phaseolus lunatus***		
µg/mL	Ascorbic	H_2O-Acetone	$CHCl_3$	EtOAc	H_2O-Acetone	$CHCl_3$	EtOAc	H_2O-Acetone	$CHCl_3$	EtOAc
40	96.35	87.65	95.03	96.30	91.65	80.37	65.29	90.45	83.13	82.09
20	96.74	58.43	78.12	73.24	65.62	44.75	51.82	70.65	53.97	47.87
10	96.30	49.98	72.64	64.71	43.93	39.13	48.73	54.43	38.24	28.82
4	72.79	26.65	30.46	28.45	28.45	24.43	22.82	32.27	30.87	24.43
EC_{50}	1.19	17.60	12.28	13.61	16.74	21.73	23.53	15.02	19.61	21.91

Table-9: Percent Reduction in Absorbance of DPPH and EC_{50} (Trial 2)

		Dolichos lablab			*Glycine soja*			*Phaseolus lunatus*		
µg/mL	Ascorbic	H_2O-Acetone	$CHCl_3$	EtOAc	H_2O-Acetone	$CHCl_3$	EtOAc	H_2O-Acetone	$CHCl_3$	EtOAc
40	98.04	90.25	94.73	90.66	86.38	89.10	78.74	95.54	94.32	76.54
20	97.68	69.76	67.27	67.20	61.17	54.48	53.86	65.94	76.76	50.23
10	96.48	56.86	49.78	59.46	33.03	41.56	42.26	62.23	43.24	34.13
4	73.32	32.32	40.65	38.93	20.12	21.01	29.48	35.55	41.12	27.19
EC_{50}	1.12	14.88	14.56	14.14	19.55	19.79	20.15	13.89	14.28	22.10

DISCUSSION

Collagen deposition gives tensile strength in wounds. *Phaseolus lunatus* showed the greatest tensile strength upon subjecting incision wounds to tensiometer. The tensile strength exhibited was arranged as follows: *Glycine soja* > *Dolichos lablab* > *Phaseolus lunatus* > control. This indicates that *Glycine soja* had greatest increase in collagen deposition thus having a tighter closure of the wound.

To have an accurate description of the collagen deposition, histopathological studies were done in incision wounds. The arrangements of collagen deposition in histological studies were as follows: *Dolichos lablab* > *Glycine soja* > *Phaseolus lunatus* > control. *Dolichos lablab* had more active collagen deposition histopathologically than *Glycine soja* thus indicates that there is more collagen. Enhanced healing is attributed to increase collagen formation.

To further assess the wound healing activity, wound closure rate was done to the excision wounds. The orders of wound closure rate were as follows: *Dolichos lablab* > *Glycine soja* > *Phaseolus lunatus* > control. Stellate and smaller scars were formed in *Dolichos lablab* groups indicate better wound healing activity.

Dolichos lablab group had the highest amount of hydroxyproline in both incision and excision wound models. The amount of hydroxyproline was arranged as follows: *Dolichos lablab* > *Glycine soja* > *Phaseolus lunatus* > control for both incision wounds and excision wounds. The measurement of hydroxyproline content was an indicator of collagen synthesis, thus an increase in hydroxyproline content indicates increase in collagen synthesis in wounds. This supports the wound contraction rate results in which there were increase deposition on the wounded site making faster wound healing.

The researchers determined the amount of antioxidant activity of each aqueous extract of the leguminous seeds. These were subjected to fractions of chloroform, ethyl acetate and water acetone. Each extract showed decrease in the original violet color solution and absorbance of samples, thus all extracts showed antioxidant activity. *Dolichos lablab* showed the highest antioxidant activity followed by *Glycine soja* and *Phaseolus lunatus*. Based on the three fractions, chloroformic extracts exhibited highest antioxidant activity followed by ethyl acetate and water-acetone. This positive reaction of the samples to the DPPH assay would highly suggest the mechanism of action through which the wound healing activity.

CONCLUSION

Tensile strength was seen greatest in *Glycine soja* followed closely by *Dolichos lablab*. Histopathological studies support the wound contraction rate results in which there were increased deposition on the wounded site making faster wound healing. However, *Dolichos lablab* had the highest amount of hydroxyproline in both incision and excision wound models, thus had the highest collagen content. Further, *Dolichos lablab* showed the highest antioxidant activity to the DPPH assay which highly suggest the wound healing mechanism.

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