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DETERMINATION OF ANTI-INFLAMMATORY ACTIVITY OF CREAM CONTAINING SEMI-PURIFIED FLAVONOIDS FROM *LIMNOPHILA SESSILIFLORA* (SCROPHULARIACEAE) LEAVES IN *RATTUS NORVEGICUS* (SPRAGUE DAWLEY RATS)

Renebelle G. Poquiz, Angela N. Rayco, Erika Grace A. Yunson, Grace C. Rico, Honey Grace B. Torres and Jacqueline O. Vallester

College of Pharmacy, Our Lady of Fatima University, Quezon City, Philippines

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ABSTRACT

Objective*: The study aims to determine the anti-inflammatory activity of cream containing semi-purified flavonoids of Limnophila sessiliflora (ambulia) leaves in Rattus norvegicus (Sprague Dawley Rats) using three concentrations namely:* 1%, 2%, and 4%.
Methodology*: Limnophila sessiliflora leaves were macerated for three days and were further extracted to obtain the semi-purified flavonoids. After semi-purification, the extract was subjected to various tests to confirm the presence of flavonoids. Then, it was subjected to cream formulation and anti-inflammatory determination. Physical tests of the cream, followed by Dermal Irritation Test on rats, were also conducted prior to formulation and anti-inflammatory testing.*
Results*: The semi-purified extract of Limnophila sessiliflora (Ambulia) resulted positive to flavonoids. Also, results showed that the cream containing semi-purified flavonoids met the Physical Tests requirements; did not exhibit dermal irritation; and exerted only a minimal anti-inflammatory effect that is not comparable to the reference standard, Indomethacin.*
Conclusion*: The cream containing the semi-purified flavonoids of Limnophila sessiliflora did not exhibit an anti-inflammatory activity up to 4% of concetration.*

Keywords: *Limnophila sessiliflora*, semi-purified flavonoid, cream, anti-inflammatory

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INTRODUCTION

Inflammation is part of the body's immune response upon cell injury. It can be evoked by a wide variety of noxious agents such as infections, antibodies, or physical injuries. The inflammatory response of the host is critical for interruption and resolution of the infectious process but is also responsible for the signs and symptoms of disease (Anilkumar and Jibin, 2015).

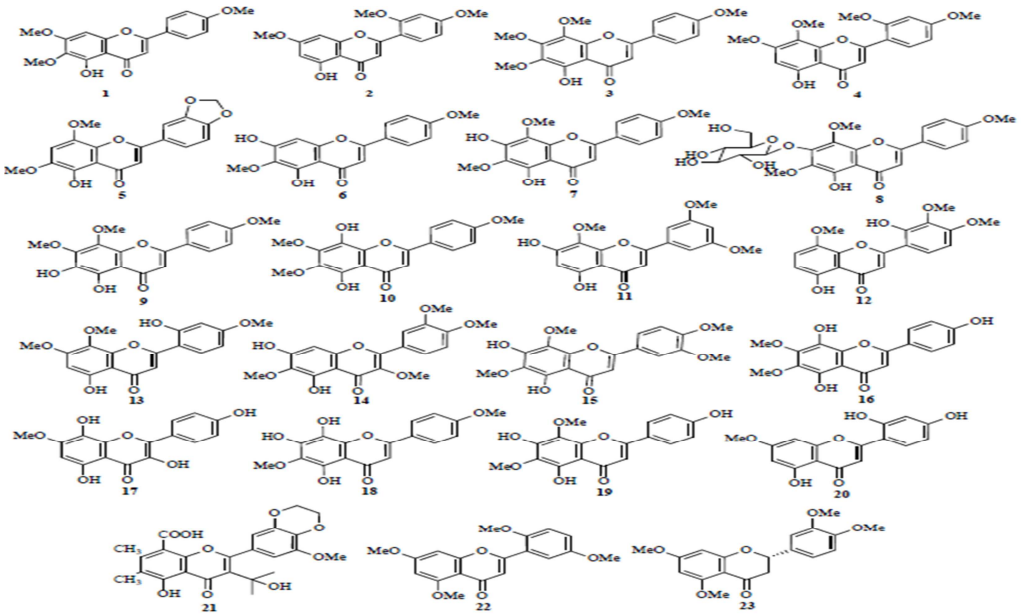
Limnophila, of the Scrophulariaceae family, originates from a Latin word which means pond-loving. Commonly known as Ambulia or Asian marsh weed, *Limnophila sessiliflora* is a perennial herb found in Southeast Asia including the Philippines, tropical to subtropical Africa, Australia, Pacific Islands, and North

America (Sharma et al, 2016). As an aquatic or nearly aquatic herb, it is seen as submersed, emergent, and amphibious stem plant. The submerged stems are smooth and have feathery leaves with 30 millimeters long. These stems differ from the emergent stems, which are covered with flat shiny hair and have lance-shaped leaves, up to 3 cm long with toothed margins. The emergent stems are usually 2-15 centimeters above the water surface. The flowers can be white, pink, purple or blue to lavender, and are axillary and solitary or in axillary or terminal spikes or racemes, sessile, or pedicellate. The sepals have five, green, hairy lobes, each 4-5 millimetres long. The upper portion is purple and composed of five fused petals forming a tube with two lips—adaxial lip (dorsal) is 2-lobed, while abaxial lip (ventral) is 3-lobed. The fruit is capsule-containing up to 150 seeds. Below is the taxonomical classification of the plant (Brahmachari, 2014):

Kingdom: Plantae
Subkingdom: Tracheobionta
Division: Magnoliophyta
Class: Magnoliopsida
Subclass: Asteridae
Order: Scrophulariales
Family: Scrophulariaceae
Genus: Limnophila
Species: S*essiliflora*

Limnophila heterophylla, which belongs to the same Scrophulariaceae family, was reported as a source of flavonoids, terpenoids, and among other phytochemical constituents. The isolated phytochemicals as well as different extracts were already reported to exhibit various biological activities such as anti-inflammatory, antimicrobial, and wound healing (Gorai et al, 2014). Also, the essential oil, crude extract, and nevadensin (5,7-dihydroxy-6,8,4'-trimethoxy-flavone) from *Limnophila conferta* were reported to be effective in acute and chronic inflammatory model. Nevadensin was said to have a weak inhibitory activity against cyclooxygenase-1 and 2 (COX-1 and COX-2) in vitro as studied in COX-catalyzed prostaglandin biosynthesis assay (Brahmachari, 2014).

Flavonoid is one of the most important phytoconstituents shown to have a wide spectrum of biological activities: anti-inflammatory, anti-oxidant, immune stimulating, anti-cancer, cardio, radio, hepatic-, and gastro protective, anti-thrombotic, anti-allergic, and anti-viral effects (Dmitrienko et al, 2012). The figure below shows the structures of 23 flavonoids from the genus of Limnophila (Brahmachari, 2014):



On the other hand, creams are semisolid preparations containing one or more medicinal agents dissolved or dispersed in either a water-in-oil emulsion or an oil-in-water emulsion or in another type of water-washable base (Ansel, 2011). Many patients and physicians prefer creams than ointments because of its ease in spreading and removing.

In this study, the flavonoid of *Limnophila sessiliflora* (Scrophulariaceae) was formulated into a cream and was tested for potential anti-inflammatory use in *Rattus norvegicus* (Sprague Dawley Rats).

MATERIALS AND METHOD

Limnophila sessiliflora was gathered from Lagro Subdivision, Novaliches, Quezon City, Philippines (14.7260° N, 121.0665° E). The plant was authenticated at the Institute of Biology Jose Vera Santos Herbarium (PUH), College of Science, University of the Philippines, Diliman, Quezon City, Philippines. Healthy *Rattus norvegicus* weighing 150-200g were obtained from a registered veterinarian and were maintained according to the Guidelines for the Housing of Rats in Scientific Institution. The rats were acclimitized for a week in a controlled temperature and a well-ventilated animal housing with the guidance of a veterinarian. All tests were conducted at Our Lady of Fatima University, Quezon City.

Extraction and Semi-purification of the Plant Sample

Limnophila sessiliflora leaves were cleaned, air-dried at room temperature away from direct sunlight, and ground to a course powder (200 grams). The powder was then extracted using 20% aqueous methanol for 3 days and was filtered using Whatman filter paper (no. 1). The filtrate was collected in a round bottom flask and was dried using a rotary evaporator. The dried extract weighed 36 grams (18% yield). It was subsequently dissolved in 125 mL of hot methanol. The solution was mixed with 5g of celite and filtered through a Buchner funnel. The celite-residue material was suspended in 50mL of hot methanol and was filtered again. The two filtrates (about 300mL including washings) were combined, set aside overnight, and refiltered. About 250mL of the clear filtrate was mixed with activated charcoal (commercial type) using mechanical stirrer. Charcoal was added in portions until supernatant liquid showed no flavonoids as determined by polyamide TLC. A total of 80g of charcoal was added, two 20-g and four 10-g portions. The charcoal-flavanoid material was filtered through a small Buchner funnel, and the residue was washed with 2L of boiling methanol. The methanol filtrate eventually yielded 16.3g of semi-purified flavonoid.

Test for Presence of Flavonoids

(a) Shinoda Test

The extract was added to pinch of magnesium turnings. Then 1-2 drops of concentrated hydrochloric acid was added. Formation of pink color indicated the presence of flavonoids (Bandiola and Ramos, 2017 & Banu et al, 2015).

(b) Lead acetate test

To the extract, a few drops of 10% lead acetate solution was added. Appearance of yellow color precipitate indicated the presence of flavonoids (Bandiola and Ramos, 2017 & Banu et al, 2015).

(c) NaOH test

The Extract was treated with a few drops of sodium hydroxide solution. Formation of intense yellow color, which became colorless upon the addition of dilute acid, indicated the presence of flavonoids (Bandiola and Ramos, 2017 & Banu et al, 2015).

(d) Ferric chloride test

The extract, when treated with a few drops of ferric chloride solution, resulted in the formation of blackish red color indicating the presence of flavonoids (Bandiola and Ramos, 2017 & Banu et al, 2015).

(e) Alkaline reagent test

The extract, when treated with sodium hydroxide solution, showed increase intensity of yellow coloration. Then when it became colorless upon the addition of a few drops of dilute hydrochloric acid, this then indicated the presence of flavonoids (Bandiola and Ramos, 2017 & Banu et al, 2015).

Solubility Tests for Semi-purified Flavonoids

(a) Water Solubility

Approximately (six) 6 drops of water was added to the test tube containing the sample. The tube was stirred with a stirring rod. Solubility was indicated by the formation of a homogenous solution.

(b) 5% HCl Solubility

Approximately 1 mL of 5% HCl was added in small portions to the test tube containing the extract. The test tube was shaken vigorously after the addition of each portion of solvent. Solubility was indicated by the formation of a homogenous solution, a color change, or the evolution of gas or heat.

(c) 5% NaHCO3 Solubility

Approximately 1 mL of 5% NaHCO3 was added in small portions to the test tube containing the extract. Test tube was shaken vigorously after the addition of each portion of solvent. Solubility was determined by the formation of a homogenous solution, a color change, or the evolution of gas or heat.

(d) 5% NaOH Solubility

Approximately 1 mL of 5% NaOH was added in small portions to the test tube containing the extract. Test tube was stirred vigorously after the addition of each portion of solvent. Solubility was determined by the formation of a homogenous solution, a color change, or the evolution of gas or heat.

(e) Acetone Solubility

Approximately 1 mL of acetone was added in small portions to the test tube containing the extract. Test tube was stirred vigorously after the addition of each portion of solvent. Solubility was determined by the formation of a homogenous solution, a color change, or the evolution of gas or heat.

(f) Alcohol Solubility

Approximately 1 mL of 70% ethanol was added in small portions to the test tube containing the extract. Test tube was stirred vigorously after the addition of each portion of solvent. Solubility was determined by the formation of a homogenous solution, a color change, or the evolution of gas or heat.

(g) Chloroform Solubility

Approximately 1 ml of chloroform was added in small portions to the test tube containing the extract. Test tube was stirred vigorously after the addition of each portion of solvent. Solubility was determined by the formation of a homogenous solution, a color change, or the evolution of gas or heat.

(h) Ether Solubility

Approximately 1 mL of ether in small portions of was added to the test tube containing the extract. Test tube was stirred vigorously after the addition of each portion of solvent. Solubility was determined by the formation of a homogenous solution, a color change, or the evolution of gas or heat.

Cream formulation

0.2g of methylparaben as a preservative, 7g cetylstearyl alcohol, 0.03g sodium lauryl sulfate, and 15 mL liquid paraffin were heated in a water bath at controlled temperature of 80°C. 60 mL of water was heated in a separate beaker using the same temperature of 80°C. Then, the hot water was added into the mixture with continuous stirring as 8 mL of propylene glycol and the respective amount (1g, 2g, or 4g) of semi-purified flavonoids from *Limnophila sessiliflora* were added until it was dissolved completely. The mixture was cooled down at room temperature and was weighed approximately 100g of *Limnophila sessiliflora* cream.

Physical test for Cream

The cream was evaluated for physical appearance, stability, pH, viscosity, uniform consistency, spreadability, and primary skin irritation test on experimental animals.

(a) Stability Test

This was determined by exposing the formulation to various temperatures for a specific period and if no change occured in the properties, it confirmed that the formulation was stable.

(b) pH Determination

The determination of the pH value was carried out by measurement of the potential difference between electrodes immersed in standard and test solutions using a pH meter. The normal pH value of a cream is 4-6.

(c) Determination of physical appearance

Color, odor, and smoothness were determined using the organoleptic method.

(d) Viscosity Test

The viscosity of formulated o/w cream was measured by Brook field Viscometer (LVDV-III ultra-programmable Rheometer) using spindle CP-52 at varying speed and shear rates.

(e)Uniformity/Consistency
Grittiness and homogeneity were determined by rubbing the sample on the back of the hand. No solid components should be noticed.
(f) Spreadability Test
Three grams of sample was applied in between two glass slides and was compressed to uniform thickness by placing 1000-gram weight for five minutes. A shorter interval indicated better spreadability.

Primary Skin Irritation Test
The test animals were kept in different cages and were supplied with fresh food and water during the test period, 24 hours prior to test. The hairs from the neck and thigh region were shaved to expose sufficient large test area. The test site was cleaned with surgical spirit then with the formulated cream. The test site was observed for erythema and edema for 24 hrs; 48 hrs; and 72 hrs after application. This test was conducted to evaluate the irritancy of the prepared cream on the intact skin of the animals. None of the prepared cream showed any erythema or edema, indicating that the prepared formulation was non-irritant on the skin of animals. This test was carried out with the aid of a registered veterinarian.

Determination of Anti-inflammatory Activity
Male sprague dawley rats weighing 150-200 grams were divided into five (5) groups namely: Group I (using 1g/100g Indomethacin as positive control), Group II (using cream base or non-medicated cream as the negative control), Group III (using 1% formulated cream), Group IV (using 2% formulated cream), and lastly, Group V (using 4% formulated cream). The paw-volume were measured using a caliper at 0, 30, 60, 120, and 180 minutes after carrageenan was administered to induce inflammation. As a baseline, the test animals' paw were measured before carrageenan suspension (0.1mL of 1%) was administered.

The anti-inflammatory effect of the extract was calculated using the equation below:

$$\textbf{Anti-inflammatory activity (\%)} = (1 - D/C) \times 100\%$$

where D is the average paw volume after extract was administered to the rats and C was the average paw volume of the negative control animals. The percentage inhibition of inflammation was calculated using:

$$\textbf{\% inhibition} = D0 - Dt/D0 \times 100$$

where D0 was the average inflammation (hind paw edema) of the control group at a given time and Dt as the average inflammation of the drug treated (extracts or reference indomethacin) rats at the same time.

Statistical Analysis
The statistical package, Graph Pad Prism 5 was used to analyze all results. Values are expressed as mean ± S.E.M. One way ANOVA was used for analysis of data and for comparisons between treated and control groups. $P < 0.05$ was considered significant.

RESULTS

Test for Presence of Flavonoids
Results revealed that the semi-purified extract of *Limnophila sessiliflora* contained flavonoids.

Organoleptic Test for Semi-purified Flavonoids
The table below describes the plant sample *Limnophila sessiliflora* after it was semi-purified. Extract was clear white and odorless.

Table 1: Organoleptic Test for Semi-purified Flavonoids

Organoleptic Testing	Results
Odor	Odorless
Color	Clear white

Solubility Tests for Semi-purified Flavonoids

The table below shows the solubility of the semi-purified flavonoids with different solvents. The extract shows complete solubility with acetone, sodium bicarbonate, 0.5% hydrochloric acid, ethanol, sodium hydroxide and water while insoluble with chloroform and ether.

Table 2: Solubility Tests for Semi-purified Flavonoids

Solvent	Solubility
Acetone	Soluble
Sodium bicarbonate	Soluble
0.5% Hydrochloric acid	Soluble
Chloroform	Insoluble
Ether	Insoluble
Ethanol	Soluble
Sodium hydroxide	Soluble
Water	Soluble

Primary Skin Irritation Test

The table shows the skin irritation effects of the three formulated creams when tested in *Rattus norvegicus* (Sprague dawley). The test revealed that the creams did not cause skin irritation when compared with indomethacin cream as the reference standard.

Table 3: Skin Irritation Test

	Day 1		Day 2		Day 3	
	Erythema	Edema	Erythema	Edema	Erythema	Edema
1% semi-purified flavonoid cream	0	0	0	0	0	0
2% semi-purified flavonoid cream	0	0	0	0	0	0
4% semi-purified flavonoid cream	0	0	0	0	0	0
Indomethacine (reference)	0	0	0	0	0	0

Anti-inflammatory Activity

The table below shows the gathered data using caliper at 30mins, 60mins, 120 mins and 180 mins.

Table 4: Anti-inflammatory Activity

Group	Normal Paw Size	Carrageenan-induced Paw Size	30 min.	60 min.	120 min.	150 min.	180 min.
Group I(Indomethacin)							
1	0.312	0.424	0.351	0.345	0.331	0.316	0.312
2	0.316	0.413	0.363	0.343	0.330	0.321	0.316
3	0.315	0.355	0.347	0.331	0.326	0.318	0.315
Group II (Cream Base)							
1	0.314	0.627	0.476	0.492	0.559	0.578	0.482
2	0.318	0.411	0.408	0.357	0.412	0.491	0.527
3	0.308	0.565	0.489	0.497	0.508	0.499	0.574

Group III(1% Cream) 1 2 3	 0.322 0.318 0.315	 0.377 0.417 0.421	 0.356 0.392 0.411	 0.342 0.381 0.401	 0.331 0.342 0.342	 0.327 0.331 0.321	 0.323 0.318 0.315
Group IV(2% Cream) 1 2 3	 0.312 0.319 0.328	 0.456 0.432 0.465	 0.439 0.412 0.432	 0.389 0.343 0.399	 0.363 0.336 0.364	 0.351 0.325 0.332	 0.314 0.319 0.329
Group V(4% Cream) 1 2 3	 0.316 0.320 0.323	 0.424 0.452 0.549	 0.359 0.431 0.475	 0.351 0.420 0.386	 0.342 0.353 0.361	 0.333 0.336 0.345	 0.316 0.322 0.323

Results of Anti-inflammatory Activity

The table below shows the percentage of anti-inflammatory activity of semi-purified flavonoid from *Limnophila sessiliflora* using 1%, 2 %, and 4% concentrations.

Table 5: Anti-inflammatory Activity

Group	Result (%)
Group 3 (1% concentration)	29.81 %
Group 4 (2% concentration)	25.88 %
Group 5 (4%concentration)	25.80 %

Results of Percentage Inhibition

The table below shows the percentage inhibition of semi-purified flavonoid from *Limnophila sessiliflora* using 1%, 2 %, and 4% concentrations. The 4% concentration displayed the highest percentage inhibition of inflammation among the three (3) experimental controls.

Table 5: Percentage Inhibition

Group	Result (%)
Group 3 (1% concentration)	13.60 %
Group 4 (2% concentration)	19.48 %
Group 5 (4%concentration)	23.47 %

Statistical Treatment

Statistical treatment of the anti-inflammatory activity between the positive control (Indomethacin) and the experimental controls (1%, 2%, and 4% Creams) shows that the value of P (0.0434) is less than 0.05, rejecting the null hypothesis which means that positive control has significant difference when compared with the experimental controls.

Table 6: Statistical Treatment

Group	Mean	F-value	P-value	Decision	Interpretation
Grp 1 (Indomethacin) Grp 3 (1% FCLS) Grp 4 (2% FCLS) Grp 5 (4% FCLS)	.331 .34386667 .36313333 .36353333	4.32	0.0434	Reject H_o	There is a significant difference.

*If P-value > F-value =reject null hypothesis
*If P-value < F-value =accept null hypothesis

Also, the table below shows that the anti-inflammatory activity between the negative control (cream base) and the experimental control (1%, 2%, and 4% Creams) shows that the value of P (0.0004) is less than 0.05,

rejecting the null hypothesis which means that the negative control has significant difference when compared with the experimental control.

Table 7:anti-inflammatory activity between the negative control (cream base) and the experimental control

Group	Mean	F-value	P-value	Decision	Interpretation
Grp 2 (Cream Base) Grp 3 (1% FCLS) Grp 4 (2% FCLS) Grp 5 (4% FCLS)	.48993335 .34386667 .36313333 .36353333	20.56	0.0004	Reject H_o	There is a significant difference.

*If P-value > F-value =reject null hypothesis
*If P-value < F-value =accept null hypothesis

CONCLUSION AND DISCUSSION

Three cream concentrations (1%, 2%, and 4%) of the semi-purified flavonoids of *Limnophila sessiliflora* were tested for anti-inflammatory property in *Rattus Norvegicus*. Using One-way ANOVA, the three concentrations have significant difference compared to the standard reference, Indomethacin. Also, the three concentrations have signicant difference compared to negative control, cream base or non-medicated cream. Therefore, the medicated cream containing semi-purified flavonoids of *Limnophila sessiliflora* did not show anti-inflammatory activity up to 4% concentration and may exert such effect at higher concentrations.

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