




# Anti-Nociceptive and Anti-Inflammatory Activity of *Hygrophila schulli* Leaves

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**Purpose:** The management of pain and inflammation with non-steroidal anti-inflammatory drugs and opioid analgesics are currently encountering severe adverse reactions. To overcome these problems, herbal remedies may offer new alternative medicines. *Hygrophila schulli* is a medicinal plant traditionally used for the treatment of pain and inflammation-related disorders; yet, these claims are not scientifically validated. Hence, this study was aimed to validate the traditional use of *Hygrophila schulli* leaves as anti-inflammatory and analgesic remedy.

**Methods:** In vitro anti-hyaluronidase assay and in vivo carrageenan-induced hind paw oedema model were used to evaluate the anti-inflammatory property of ethanolic leaf extract of *Hygrophila schulli*. Tail immersion and acetic acid-induced writhing tests were performed to determine the central and peripheral analgesic activity of the leaf extract, respectively.

**Results:** The ethanolic leaf extract exhibited significant anti-hyaluronidase activity ( $P < 0.001$ ) and significant inhibition of carrageenan-induced paw oedema ( $P < 0.05$ ) compared to untreated controls. Similarly, the extract significantly prolonged the reaction time of mice ( $P < 0.05$ ) for the hot-water stimuli. Furthermore, an oral dose of the extract showed significant inhibition ( $P < 0.01$ ) of acetic acid-induced abdominal contractions of mice. Besides, the ethanolic leaf extract did not cause any obvious sign of acute toxicity at a single oral dose of 2 g/kg.

**Conclusion:** The findings of this study may partially support the acclaimed traditional use of *Hygrophila schulli* leaves for the treatment of pain and inflammatory conditions.

**Keywords:** pain, inflammation, carrageenan, hyaluronidase, *Hygrophila schulli*

## Introduction

Pain and inflammation are health problems that affect economic development due to absenteeism from work, reduction of quality of life and eventually death.<sup>1</sup> Management of pain and inflammation by the conventional medicines is recently becoming problematic as the medicines are unaffordable and associated with adverse effects like tolerance, dependence and gastrointestinal problems.<sup>2-4</sup>

Over 80% of global societies are recently using traditional medicines for the management of mild to moderate health care needs.<sup>5,6</sup> Currently, medicinal plants are widely used as alternative remedies for the treatment of pain and inflammation. *Hygrophila schulli* M. R. Almeida & S. M. Almeida (Acanthaceae) is one of the traditionally used medicinal plants for the treatment of pain and inflammatory conditions in northern Ethiopia and India. It is an erect, flowering and fruiting annual herb commonly grown in wetlands or watery areas. This plant is widely distributed and used as a folk medicine in tropical Africa, India and China.<sup>7,8</sup> Moreover, previous studies

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show that the seed extract of *H. schulli* possessed significant anti-nociceptive and anti-inflammatory activities in a rodent model.<sup>9</sup> Besides, leaf and root extracts of *H. schulli* showed antibacterial activity against various bacterial strains<sup>10</sup> and the ethanolic extract of *H. schulli* seed was reported to demonstrate antioxidant properties against different free radicals.<sup>11</sup>

In north Ethiopia, leaves of *H. schulli* are macerated in alcohol overnight and used for the management of headache and pain secondary to physical damage. Also, fresh leaves of *H. schulli* are used against inflammatory skin conditions. However, scientific verification of this folkloric use is lacking. Hence, this study was aimed to assess the anti-inflammatory and anti-nociceptive property of ethanolic leaf extract of *H. schulli*.

## Materials and Methods

### Plant Material Collection

Leaves of *H. schulli* were collected in December 2018 from a locality called Grat-Kahsu (13.728820° N, 37.187930° E), Tigray, Ethiopia. The plant specimen was authenticated by Mr Shambel Alemu, the national herbarium at the Department of Biology of Addis Ababa University where a sample specimen (AD-001) was deposited. The collected plant material was then inspected to remove any debris or plant parts other than the leaves. Finally, the leaves were dried at room temperature in an open and shade area.

### Chemicals, Reagents, and Equipment

Ethanol, glacial acetic acid, sodium chloride, dimethyl sulfoxide, bovine serum albumin, sodium acetate, sodium phosphate hyaluronidase (Sigma-Aldrich, Bangalore), hyaluronic acid (Sigma-Aldrich, Bangalore), carrageenan, normal saline, electronic weighing balance (Adventurer ohaus, China), drying oven (Genlab, England), double beam spectrophotometer (PG instrument Ltd, China), oral gavage, orbital shaker (Bibby sterling Ltd S01, UK), and digital plethysmometer (PLM-02 plus, India) were used in this experiment.

### Experimental Animals

Six- to eight-week-old Swiss albino mice of either sex weighing 20–30 g were obtained from the animal house of School of Pharmacy and acclimatized to the laboratory environment for 1 week prior to the experimentation with a regular supply of standard pellets and water ad libitum. Animals were kept in a transparent cage and maintained

under standard housing conditions with a 12 h light and dark cycle.<sup>12,13</sup>

### Preparation of the Extract

110 g weighed dried and powdered leaves of *H. schulli* were macerated with 70% ethanol for 3 days with intermittent shaking using an orbital shaker. Muslin cloth and then Whatman filter paper number 1 were used to filter the extract. The marc was then re-macerated twice to maximize the amount of extractable constituents in the extract. Then, the collected filtrates were dried in a drying oven at  $40 \pm 1$  °C. The dried crude extract was stored in an amber glass container in a refrigerator at  $-4$  °C until use. The percentage yield was determined using the equation below.

$$\% \text{Yield} = \frac{\text{Amount of crude extract obtained (g)}}{\text{Total weight of the powdered plant material (g)}} \times 100$$

### In vitro Anti-Inflammatory Study Hyaluronidase Inhibition Assay

The ethanolic leaf extract of *H. schulli* was investigated for its in vitro anti-hyaluronidase activity before the in vivo study according to Sigma protocol with slight modifications.<sup>16</sup> Concisely, the assay medium containing 3–5 U hyaluronidase in 100  $\mu$ L of 0.02 M sodium phosphate buffer (pH 7.0) with 0.077 M sodium chloride and 0.01% bovine serum albumin was pre-incubated with 5  $\mu$ L of different concentrations (10  $\mu$ g/mL, 50  $\mu$ g/mL and 100  $\mu$ g/mL) of the extract as well as a standard drug (indomethacin) for 15 min at 37 °C. The test was initiated by adding 100  $\mu$ L hyaluronic acid to the incubation mixture and incubated again for 45 min at 37 °C. The undigested hyaluronic acid was then precipitated with 1 mL acid albumin solution (made up of 0.1% bovine serum albumin in 24 mM sodium acetate and 79 mM acetic acid, pH 3.75). After standing at ambient temperature for 10 min, the absorbance of the reaction mixture was recorded at 600 nm. The absorbance in the absence of enzyme was used as the reference value for maximum inhibition. The activity of the enzyme was checked with a blank experiment conducted simultaneously, in which the enzyme was pre-incubated with 5  $\mu$ L of dimethyl sulfoxide. Percentage inhibitory activities of the test samples were determined using the formula given below:

$$\% \text{Hyaluronidase inhibition} = \frac{\text{Abs}_{\text{test}}}{\text{Abs}_{\text{ref}}} \times 100$$

where  $Abs_{test}$  refers to the absorbance in the presence of crude extract or standard drug and  $Abs_{ref}$  stands for the absorbance in the absence of enzyme.

## In vivo Anti-Inflammatory and Anti-Nociceptive Study

### Acute Oral Toxicity Study

The acute oral toxicity of the crude extract was performed as per the procedure for the limit test described in the Organization for Economic Co-operation and Development (OECD) 425 guidelines.<sup>13,14</sup> Briefly, five female mice (aged 8–9 weeks and weighing 30–35 g) were withheld from food with free access to water for 3 hours prior- and one-hour post-administration of extract. A single dose of the extract at 2 g/kg body weight was given orally to the first mouse and followed for 2 days to observe any signs of toxicity or mortality. Then, the remaining four mice were given the extract orally at the same dose as described for the first mouse. All mice were closely observed for the first 30 min, intermittently every 30 min for 4 hours and then daily for 14 days for the presence of changes in skin and fur, hyperactivity, tremors, sedation, convulsions, lethargy, and coma. Bodyweight of each mouse was also recorded before extract administration and weekly.

### Sample Size Determination

Resource equation method, based on the law of diminishing return, as described by Charan and Kantharia (2013) was applied to determine the sample size used in the present study as multiple endpoints were measured.<sup>15</sup> Accordingly, the value of the degree of freedom of analysis of variance (ANOVA) (E) is suggested to lie in between 10 and 20 to obtain significant results from the study. The equation given below was used to calculate the value of E and the number of animals used was calculated at E value is 20 to have the highest possible significant results.

$$E = \text{Total number of animals} - \text{Total number of groups}$$

Five groups were used for each model. Therefore,  $20 = \text{Total number of animals} - 5$

$$\text{Total number of animals} = 20 + 5 = 25 \text{ animals}$$

Hence, 25 mice randomly divided into five groups (five in each) were used for each model to investigate the anti-nociceptive and anti-inflammatory activities of the leaf extract.

## Experimental Design and Treatment Regimes

Mice used in each model were grouped randomly into five groups each containing five mice. Group I was used as a negative control treated with distilled water (DW). Group II, III and, IV served as test groups treated with crude extract at doses 100, 200 and 400 mg/kg, respectively. Group V served as positive control and each mouse was administered with the standard drugs (Pethidine at 5 mg/kg in tail immersion test, acetylsalicylic acid/aspirin at 150 mg/kg in the acetic acid-induced writhing test, and 100 mg/kg aspirin in carrageenan-induced paw oedema model). All treatments were dissolved in distilled water (except pethidine as it was liquid preparation) and administered orally.

### In vivo Carrageenan-Induced Paw Oedema Model

In vivo carrageenan-induced paw oedema in mice model was followed as per the protocol described by Posadas et al (2004) to evaluate the in vivo anti-inflammatory property of the leaf extract of *H. schulli*.<sup>17</sup> Briefly, the mice groups were treated with their respective test samples orally as per the experimental design stated above. Then, one-hour post-treatment, each mouse has been injected 0.05 mL of carrageenan suspension (1%, w/v) into their dorsal region of the sub-plantar surface of the right hind paw. The initial paw volume of each mouse was recorded before the test sample administration (0 h). After 1 hour of carrageenan injection, right hind paw volumes were measured every 1 hour for 4 hours uninterruptedly using a plethysmometer. Percent of acute inflammation secondary to carrageenan injection was calculated based on the formula given beneath.<sup>18</sup>

$$\% \text{Inflammation} = \frac{V_t - V_o}{V_o} \times 100$$

where  $V_o$  is the normal paw volume prior to carrageenan injection and  $V_t$  is the paw volume post-carrageenan injection at different observation time points.

The inflammation developed in the test groups after carrageenan injection was compared with the inflammation produced on the DW-treated negative control group by calculating the percentage inhibition using the formula given below.<sup>18</sup>

$$\% \text{Inhibition} = \frac{\text{Mean } \% I_{\text{control}} - \text{Mean } \% I_{\text{test}}}{\text{Mean } \% I_{\text{control}}} \times 100$$

where Mean %  $I_{\text{control}}$  refers to the average percent inflammation of DW treated group and Mean %  $I_{\text{test}}$  refers

to the average percent inflammation of the test extracts and aspirin-treated groups.

### Acetic Acid-Induced Writhing Model

This test was performed to assess the effect of *H. schulli* leaf extract on acetic acid-induced peripheral pain in mice model according to the method described by Koster et al (1959).<sup>19</sup> Mice were fasted with free access to water for 16 h prior to experiment. Then, mice were treated orally as per the experimental design described above. After 45 min of the treatment, each mouse was injected 10 mL/kg body weight of acetic acid suspension (0.7% v/v, in normal saline) intraperitoneally. Then, the number of abdominal writhes of each mouse in each group was counted during a 15-min period, which was begun after 5 min of acetic acid injection. The peripheral analgesic activity is expressed in percentage inhibition of abdominal writhes using the following formula:<sup>20</sup>

$$\% \text{Inhibition} = \frac{\text{No. of writhes (negative control)} - \text{No. of writhes (test group)}}{\text{No. of writhes (negative control)}} \times 100$$

### Tail Flick Model

Central anti-nociceptive activity of the leaf extract was determined using the tail immersion test method as per the method of Toma et al (2003).<sup>21</sup> Concisely, hot water ( $55 \pm 1$  °C) was used as thermal stimuli. Before the experiment, all mice were screened for sensitivity to the stimuli by immersing two-third of the height of the tail of mice gently in the hot water and mice dislodged their tail from the water within 15 s (15 s was regarded as an endpoint to avoid injury to the mice) were included in this study. Mice were grouped and treated as designated earlier. A basal reaction time of each mouse in each group to the thermal stimuli was determined before treatment (0 min). Response latency of all mice was recorded every 30 min since treatment for 3 hours consecutively.

### Data Analysis

Results were analyzed by using SPSS version 21 and presented as a mean  $\pm$  standard error of the mean (M  $\pm$  SEM). Statistical difference between and within groups was tested using one-way ANOVA followed by Tukey's HSD post hoc multiple comparison method. Results were regarded as significant when  $P < 0.05$ . Graphs were done using GraphPad Prism version 8 software.

## Ethical Consideration

This experimental study was approved by the Health Research and Ethics Review Committee at Mekelle University with registration number ERC 1328/2019. Experimental animals were handled humanely according to the US guideline (Guide for the Care and Use of Laboratory Animals 2011) and were euthanized humanely using halothane overdose at the end of each experiment.<sup>12</sup>

## Results

### Extract Preparation

Dark green-colored crude extract was obtained after a cold maceration of *H. schulli* leaves using 70% ethanol. A total of 23.2 g (21.1%) extract were collected from three times maceration of 110 g of dried and powdered *H. schulli* leaves.

### Oral Acute Toxicity Test

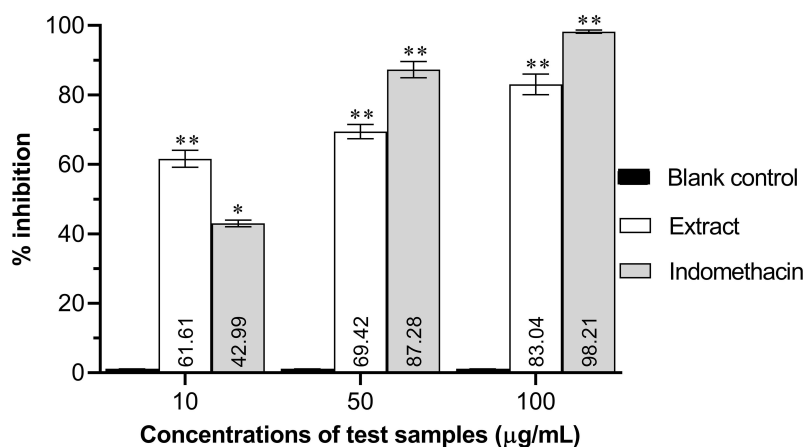
Neither sign of obvious toxicities nor mortality was recorded in the mice treated with the crude extract at a single oral dose of 2 g/kg body weight. Bodyweight of the test mice was increasing every week during the follow-up period like the normal mice do, which may indicate the absence of obvious toxicity.

### Anti-Inflammatory Activity

Significant ( $P < 0.001$ ) in vitro hyaluronidase inhibition was observed for treatments of the hydroalcoholic leaves extract of *H. schulli*. As can be seen from Figure 1, the crude extract demonstrated a dose-dependent in vitro anti-hyaluronidase activity. The anti-hyaluronidase property of the crude extract was found similar to the effect of the standard drug, indomethacin. Results of the in vivo anti-inflammatory activity of the extract against carrageenan-induced hind paw edema in mice model are presented in Table 1. The leaf extract of *H. schulli* exerted statistically significant ( $P < 0.05$ ) inhibition of carrageenan-induced hind paw edema in mice at the studied doses when compared to the distilled water-treated group. The in vivo anti-inflammatory property of this extract was observed to increase with dose.

### Analgesic Activity

As shown in Figure 2, the leaf extract of *H. schulli* possessed significant inhibition of acetic acid-induced writhes in mice in a dose-dependent manner. The oral dose of the ethanolic extract at doses of 200 and 400 mg/kg reduced the acetic acid-induced abdominal writhes by  $48.76 \pm$



**Figure 1** In vitro anti-hyaluronidase property of the 70% ethanolic leaf extract of *H. schulli*. The tests were conducted three times for each test sample concentrations; \* $P < 0.01$ ; \*\* $P < 0.001$  when compared to the blank control; the values presented inside the bars are mean of the respective test samples; the error bars represent the standard error of the mean of measurements.

12.39% and  $57.89 \pm 10.92\%$ , respectively, that were statistically significant ( $P < 0.01$ ) when compared to the negative control (DW-treated group). Acetylsalicylic acid (the standard drug) showed  $88.76 \pm 2.82\%$  inhibition of writhes which was statistically significant ( $P < 0.05$ ) as compared to the negative control, as well as the lower and medium dose of the extract. The extract at 100 mg/kg had declined the number of writhes in mice by  $29.07 \pm 9.36\%$ , though it was statistically insignificant. Similarly, the leaf extract was observed to prolong the reaction time of the treated mice for thermal stimuli in the tail immersion test. As detailed in Table 2, the extract at three different doses has possessed statistically significant ( $P < 0.05$ ) antinociceptive activity in mice against the thermal stimuli at different observation time points starting from 60-min through 180-min post-treatment relative to DW-treated group. The oral dose of mice with the reference drug, pethidine at 5 mg/kg, was found to significantly ( $P < 0.01$ ) increase response latency at all observation time points compared to the untreated group (distilled water-treated group). The lowest and middle doses of the extract were

observed to prolong the reaction time at 30- and 60-min observation time relative to the distilled water-treated group, though it was statistically insignificant ( $P > 0.05$ ).

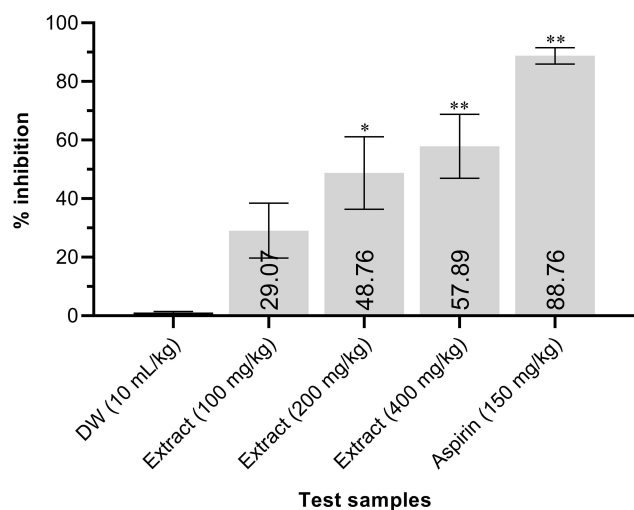
## Discussion

The anti-inflammatory property of *H. schulli* leaf extract was determined using in vitro hyaluronidase inhibition assay and in vivo carrageenan-induced paw oedema in mice model. The in vitro hyaluronidase inhibition assay is one of the commonly used, simple, validated, reliable, and well-established protocols to study an anti-inflammatory effect of substances. Hyaluronidase is an enzyme that degrades  $\beta$ -1,4 glycosidic linkage of hyaluronic acid which eventually leads to activation of pro-inflammatory cytokines release and immunity. Whenever there is inflammation, hyaluronidase enhances tissue permeability and the spread of the inflammatory responses around the affected organ.<sup>22–25</sup> Medicinal plants with anti-hyaluronidase activity may behave as anti-inflammatory remedies. In this investigation, significant and dose-dependent hyaluronidase inhibition property of the ethanolic extract of *H. schulli* leaf was observed.

**Table 1** Anti-Inflammatory Activity of 70% Ethanolic Leaf Extract of *H. schulli* in Carrageenan-Induced Paw Edema in Mice Model

Test Sample	% Inflammation (% Inhibition)			
	1 h	2 h	3 h	4 h
DW (10mL/kg)	$39.46 \pm 4.34$ (0.00)	$44.16 \pm 4.22$ (0.00)	$48.11 \pm 4.65$ (0.00)	$50.2 \pm 5.8$ (0.00)
Extract (100 mg/kg)	$25.41 \pm 3.84$ (35.61)	$26.23 \pm 2.33$ (40.6)*	$29.35 \pm 2.85$ (38.99)*	$32.03 \pm 5.38$ (36.2)*
Extract (200 mg/kg)	$17.97 \pm 4.76$ (54.46)	$24.12 \pm 5.69$ (45.38)**	$24.58 \pm 4.1$ (48.91)*	$16.8 \pm 2.53$ (66.53)***
Extract (400 mg/kg)	$19.02 \pm 8.28$ (51.8)	$12.1 \pm 1.57$ (72.6)***	$15.98 \pm 6.17$ (66.78)***	$14.25 \pm 1.65$ (71.61)***
Aspirin (100 mg/kg)	$8.08 \pm 2.97$ (79.52)**	$7.37 \pm 3.54$ (83.31)***	$13.67 \pm 4.32$ (71.58)***	$3.17 \pm 0.91$ (93.68)***

**Notes:** Values are expressed as mean  $\pm$  standard error of the mean (M  $\pm$  SEM); values in parenthesis are percent inhibition of hind paw edema (% inflammation); n=5 for each group; \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$  when compared to DW; DW refers to distilled water-treated group.



**Figure 2** Mean percent inhibition of acetic acid-induced abdominal writhes in mice after treatment with 70% ethanolic leaf extract of *H. schulli*. The values presented inside the bars are mean of the respective test samples; the error bars represent the standard error of the mean of measurements; n=5 for each group; \*P<0.01; \*\*P<0.001 when compared to distilled water-treated group.

In the in vivo studies, three test doses were determined from the findings of the acute oral toxicity study as per the method described in OECD guideline.<sup>13</sup> Accordingly, one-tenth of the safe dose (2000 mg) in the acute oral toxicity test was used as a medium dose (200mg/kg) with half (100 mg/kg) and double (400 mg/kg) of the medium dose considered as a lower and higher dose, respectively. Furthermore, in vivo carrageenan-induced paw oedema model is the most common animal protocol frequently used to determine the anti-inflammatory and anti-oedema properties of natural remedies.<sup>26–28</sup> This model is principally based on the release of inflammatory mediators.<sup>29</sup> The ethanolic leaf extract of *H. schulli* exhibited significant ( $P<0.05$ ) anti-inflammatory activity against carrageenan-induced hind paw oedema in mice at two-, three-, and four-hours post-carrageenan injection. This anti-inflammatory activity was in a dose-dependent manner.

Even though statistically insignificant, the extract has shown suppression of paw oedema formation at the first hour observation time. The anti-inflammatory activity of the extract may be due to the inhibition of the secretion of inflammatory mediators. In line with the present study, previous reports indicated that extract from *H. schulli* seeds showed significant anti-inflammatory activity in carrageenan-induced paw oedema model.<sup>9</sup> Moreover, a study by Patra et al (2009) on a similar species reported that chloroform and alcoholic leaf extracts of *Hygrophila spinosa* demonstrated significant ( $P<0.05$ ) and dose-dependent in vivo anti-inflammatory activity against carrageenan-induced paw oedema to which findings of the current study are consistent.<sup>30</sup>

Different irritating chemical agents like acetic acid have been used to induce peripheral nociception in rodent models for the assessment of peripheral analgesic activity of medicinal plants.<sup>31</sup> Intraperitoneal injection of acetic acid causes writhes which are characterized by abdominal contractions, movement of the hind paws, inward rotation of one or more feet, twisting or turning of the trunk, and drawing-in of the abdominal wall.<sup>31–34</sup> In the current study, the acetic acid-induced writhing test was used to determine the peripheral anti-nociceptive activity of *H. schulli* leaf extract. The leaf extract exerted significant inhibition of peripheral nociception ( $P<0.05$  versus the negative control) at doses of 200 and 400 mg/kg. The lowest dose (100 mg/kg) has also reduced the number of writhes, though it was statistically insignificant. The analgesic effect of *H. schulli* leaf extract is consistent with the anti-nociceptive property of chloroform, alcoholic and aqueous leaf extract of *H. spinosa* against acetic acid-induced peripheral nociception.<sup>35,36</sup>

In addition to the writhing model, tail-flick model was also used to determine the central anti-nociceptive property of *H. schulli* leaves extract. The thermal-induced pain model can be conducted either by dipping the tail of mice

**Table 2** The Analgesic Property of 70% Ethanolic Leaf Extract of *H. schulli* Using Tail Immersion Model

Test Sample	Reaction Latency (in Sec.)						
	0 Min	30 Min	60 Min	90 Min	120 Min	150 Min	180 Min
DW (10mL/kg)	2.08±0.21	2.18±0.11	1.51±0.14	1.71±0.19	1.81±0.12	1.61±0.18	1.71±0.14
Extract (100 mg/kg)	3.28±1.12	4.06±0.65	9.55±2.04**	8.23±1.92**	5.59±1.29*	4.06±0.60*	3.24±0.19*
Extract (200 mg/kg)	2.6±0.33	4.57±0.62	6.32±0.49*	6.53±1.67*	5.74±0.69*	7.26±0.43***	4.89±0.39***
Extract (400 mg/kg)	2.31±0.22	10.41±1.59***	8.01±1.05*	6.72±2.09*	5.25±0.53*	4.92±0.76**	5.67±0.42***
Asprin (100 mg/kg)	1.64±0.27	9.25±1.54**	9.67±1.67**	10.44±1.29**	8.55±1.03***	6.48±0.26***	7.94±0.5***

**Notes:** Values are expressed as mean ± standard error of the mean (M ± SEM); n=5 for each group; \*P<0.05; \*\*P<0.01; \*\*\*P<0.001 when compared to DW; DW refers to distilled water-treated group.

in hot water or placing the tail of mice on radiant heat.<sup>32,34</sup> Immersing tail of mice in hot water is linked with acute and quick tail movement and recoiling of the whole body which is a spinally mediated reflex to the stimuli.<sup>27,34</sup> In this study, an oral dose of an extract of *H. schulli* exhibited a significant prolongation of reaction latency ( $P < 0.05$  versus distilled water-treated group) to the thermal stimuli beginning from 60 to 180 min in a dose-dependent fashion. This central anti-nociception property is in agreement with previous studies, which reported potent anti-nociceptive activity of the aqueous extract of the aerial parts and roots of *Hygrophila auriculata*<sup>37</sup> as well as chloroform, alcoholic and aqueous leaf extracts of *H. spinosa*<sup>35</sup> against thermal stimuli in a dose-dependent fashion. Similarly, a significant increment of pain threshold to the thermal-induced pain has been reported from the extract of *H. schulli* seeds.<sup>9</sup>

The phytochemical work carried out on the experimental plant is inadequate. However, one previous study described the presence of stigmaterol (a phytosterol molecule) and the triterpenoid molecules lupeol and lup-20(29)-ene-3 $\beta$ ,23-diol in *H. schulli*.<sup>38</sup> Various sterols and triterpenoids were also reported from similar species such as *H. spinosa* and *H. auriculata*.<sup>39</sup> These triterpenoids and sterols were reported to possess anti-nociceptive and anti-inflammatory activities.<sup>39–41</sup> Specifically, stigmaterol and lupeol were reported to demonstrate significant anti-nociceptive and anti-inflammatory properties.<sup>42–45</sup> Similarly, lup-20(29)-ene-3 $\beta$ ,23-diol was reported to exhibit anti-inflammatory activity.<sup>46</sup> Yet, the anti-nociceptive activity of lupeol still needs further confirmation since other authors such as Geetha and Varalakshmi (2001) reported that it lacks anti-nociceptive activity.<sup>44</sup> Therefore, stigmaterol, lupeol, and lup-20(29)-ene-3 $\beta$ ,23-diol may contribute to the anti-inflammatory activity of leaf extract of *H. schulli* whereas, its anti-nociceptive property may be due to the presence of stigmaterol and/or other uncharacterized compounds.

## Conclusion

To conclude, the ethanolic leaf extract of *H. schulli* exhibited significant in vitro hyaluronidase inhibition, in vivo anti-inflammatory activity, and peripheral and central anti-nociceptive activities in mice model. Some of these activities, especially the anti-inflammatory activity may be attributed to the reported secondary metabolites such as stigmaterol, lupeol, and lup-20(29)-ene-3 $\beta$ ,23-diol. Besides, the extract was found to cause neither sign of

acute toxicity nor mortality at a single oral dose of 2 g/kg. Hence, the findings of the present study may justify and partly supports the traditional use of *H. schulli* leaf for the treatment of pain and inflammatory conditions. Further works to characterize the responsible bioactive molecules from the plant leaf extract and elucidate the mechanism of action may be suggested.

## Abbreviations

ANOVA, analysis of variance; DW, distilled Water; HSD, honestly significance difference; OECD, Organization for Economic Cooperation and Development; SPSS, Statistical Package for Social Science.

## Data Sharing Statement

The datasets of this study are available from the corresponding author on a reasonable request.

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## Disclosure

The authors declare that they have no conflicts of interest for this work.

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