

In vivo* anti-inflammatory and anti-platelet aggregation activities of longissiminone A, isolated from *Usnea longissima

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Abstract: Secondary metabolite, longissiminone A (**1**) was isolated from a lichen, *Usnea longissima*. It was screened for its *in vivo* anti-inflammatory and anti-platelet aggregation activities. Compound **1** showed moderate *in vivo* anti-inflammatory activity as well as moderately active against the aggregation induced by arachidonic acid at different doses.

Keywords: Usneaceae, longissiminone A, *Usnea longissima*, lichen, *in vivo* anti-inflammatory activity, anti-platelet aggregation activity.

INTRODUCTION

Usnea (Usneaceae) is usually found in the northern temperate zones, especially the sub-arctic and coastal rainforests areas of Asia, North America and Europe. It is a lichen with large hanging hair, used traditionally as antimicrobial by Venezuelan Andes (Marcano *et al.*, 1999). *Usnea* species have also been found effective for lowering respiratory infections (Hobbs, 1986), fever control and pain relief (Okuyama *et al.*, 1995).

Inflammation is a protective response of the body against various types of physical injuries, attack of pathogens and contact with chemicals. It is also a process of repairing against the damage caused by multiple factors. Such mechanisms involve dilatation of capillaries, arterioles and venules with exudation of fluids and increased vascular permeability, including leukocyte migration and plasma proteins into the inflammatory area. Inflammatory response removes the pathogenic infections, induces physiological adaptations to limit and confine the tissue damage. This process was recognized as body's natural defensive and healing process (Hurley, 1972). The entire process of inflammation is now recognized as a complex cascade in which several factors are involved, starting from the initiation of response, through the development of cardinal signs, followed by healing and restoration of normal morphology and function of the inflamed tissues or organs. Chronic inflammatory disorders include multiple sclerosis, atherosclerosis, rheumatoid arthritis, psoriasis, osteoarthritis, retinitis and inflammatory bowel disease (Neville *et al.*, 2004).

Based on chronic complications, associated with inflammation, extensive efforts have been made to identify and develop new effective and safe anti-inflammatory agents. Better understanding of

inflammatory process at the molecular level led to a new interest in this field.

Platelets are unique anucleated blood cells produced by bone marrow. They are specialized cells, which perform crucial roles in host integrity, defence, and repair (Weyrich and Zimmerman, 2004). They rapidly manifest their protective effects at the site of injury and infections. The process in which certain stimuli trigger the platelet-platelet interactions to form aggregate is known as platelet aggregation. This is an important event during the inflammation process. Convincing evidences have been presented to explain the role of platelets in signalling pathways, release of mediators and chemokines during inflammation (Herd and Page, 1995; Klinger, 2002; Nathan, 2002; Issekutz *et al.*, 1983; Boehlen and Clemetson, 2001; Weyrich, 1996). These mediators play a pivotal role in clotting and immune cascade (Patrono *et al.*, 1985; Patrono, 2002). Activated platelets rapidly release thromboxane A₂, which is a prothrombotic eicosinoid, produced from arachidonic acid. This reaction is catalyzed by enzyme cyclooxygenase. This is a key step in the treatment of atherosclerotic vascular diseases (Weyrich, 1996).

Previous phytochemical studies on *Usnea longissima* Linn. resulted in the isolation of longissiminone A (**1**), longissiminone B (**2**) and glutinol (**3**) (Choudhary *et al.*, 2005). Their cytotoxic and *in vitro* anti-inflammatory activities were also studied (Choudhary *et al.*, 2005). Now we are going to report here the *in vivo* anti-inflammatory and anti-platelet aggregation activities of longissiminone A (**1**) (fig. 1).

MATERIALS AND METHODS

Anti -platelet aggregation assay

In this study, the anti-platelet activity of compound **1** was obtained according to the modified method of Connor *et*

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al. (2001). Briefly, selected those healthy volunteers, who were not taken any medicine from last two weeks, and their heparinised venous blood was collected, and centrifuged for 15 minutes at 1000 rpm at 37 °C to obtain platelet rich plasma (PRP). Platelet poor plasma (PPP) was obtained by the centrifuge again of the remaining plasma for 10 minutes at 37 °C. PPP hardly contained any platelet and was used as a blank. For anti-platelet activity measurement, aliquots of PRP were taken in small glass cuvettes, containing various concentrations (300-500mM) of compound 1. Compound 1 was dissolved in DMSO (0.1% with normal saline) and cuvettes were incubated for 1 min before they were challenged with platelet aggregation agonist arachidonic acid. By using a Dual Channel Lumi-aggregometer, aggregation was measured against blank cuvette containing PPP for 4 additional minutes. % inhibitions were expressed in the dose-response curves between the % aggregation versus the time in minutes. 0.1% DMSO containing saline was used as control. The anti-platelet activity of the compound 1 is compared with the positive control in which various concentrations of arachadonic acid (100-25mM) were used to measure the inhibition of platelet aggregation. A dose dependant curved was obtained as a result of this treatment (figs. 2a and 2b).

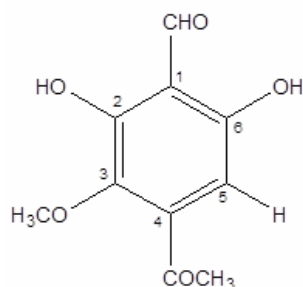


Fig. 1: Structure of longissiminone A (1)

In vivo anti-inflammatory activity in carrageenan-induced rat paw edema model

Plathysmometer (Model 76014, Ugo, Japan) was used to measure the paw volume. λ -Carrageenan (Sigma, St. Louis, MO, USA) dissolved in 0.9% saline solution prior to use. Compound 1 was dissolved in saline containing 10% DMSO. Aspirin was used as standard drug for the comparison with compound 1. Arachidonic acid, salts for buffer and sodium citrate (Sigma, St. Louis, MO, USA). Reagents of analytical grades were used. Deionized water was used in all experiments.

Animals

Wistar rats of either sex (180-210g) were obtained from the animal house of the International Center for Chemical and Biological Sciences, University of Karachi. These animals were kept on free excess of normal diet and water under the standardized conditions i.e. humidity 60%±4%, temperature 22±2°C, with 12/12 hrs. dark and light cycles. Animals were divided into three groups, each were

consists of 6 rats and were marked as control, test and standard groups.

Procedure

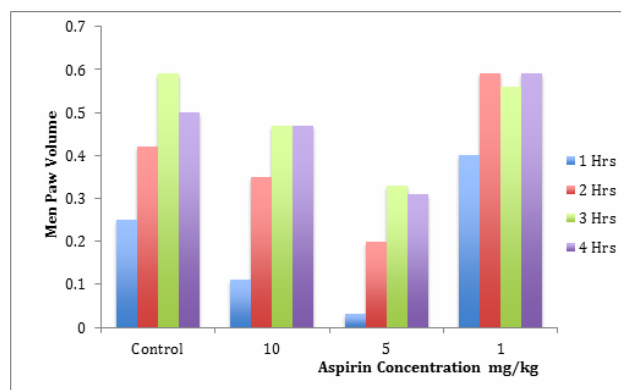
In vivo anti-inflammatory activity was obtained according to the modified method of Winter et al. (1962). Compound 1 and aspirin (three doses of 0.5, 1, 5 and 10 mg/ Kg of body weight) were injected in standard and test groups of mice intra-peritoneally (*i.p.*). Edema was induced by injecting 0.05mL of 0.1% carrageenan (dissolved in 0.9% saline) after 30 minutes in the sub-planter region of the right paw of each rat. Control group of animals received only the vehicle (10% DMSO with 0.9% saline). 0 hrs' measurement was taken prior to the *i.p.* injection of test compounds. Paw volume was measured after 30 min. of 1, 2, 3 and 4 hrs after the carrageenan injection. The percentage inhibitions in the test animals versus control group were determined by using following formula:

$$\text{Percentage inhibition} = (1 - V_t/V_c) \times 100$$

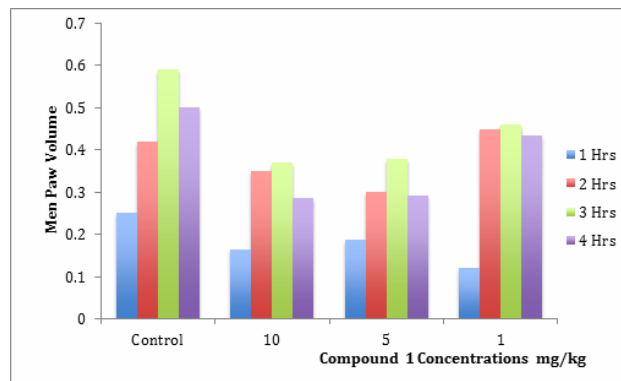
Where,

V_t = edema volume in the drug treated group

V_c = edema volume in the control group



Graph 1a: Showing anti-inflammatory activity of standard drug aspirin at various concentrations in rat paw edema model. Values are shown in mean paw volumes.



Graph 1b: Showing anti-inflammatory activity of compound 1 at various concentrations in rat paw edema model. Values are shown in mean paw volumes.

Table 1a: Mean paw volumes in the carrageenan induced rat paw edema model

Compounds	Dose (mg/Kg)	Paw Volume $\pm$ S.E.M.			
		1 Hr	2 Hrs	3 Hrs	4 Hrs
Control	-	0.25 $\pm$ 0.03	0.42 $\pm$ 0.04	0.59 $\pm$ 0.05	0.5 $\pm$ 0.04
Aspirin	10	0.11 $\pm$ 0.09	0.35 $\pm$ 0.09	0.47 $\pm$ 0.16	0.47 $\pm$ 0.17
	5	0.03 $\pm$ 0.03	0.20 $\pm$ 0.12	0.33 $\pm$ 0.07	0.31 $\pm$ 0.10
	1	0.40 $\pm$ 0.10	0.59 $\pm$ 0.03	0.56 $\pm$ 0.24	0.59 $\pm$ 0.19
Compound 1	10	0.163 $\pm$ 0.03	0.35 $\pm$ 0.07	0.37 $\pm$ 0.05	0.286 $\pm$ 0.04
	5	0.186 $\pm$ 0.04	0.30 $\pm$ 0.33	0.38 $\pm$ 0.32	0.291 $\pm$ 0.11
	1	0.121 $\pm$ 0.04	0.45 $\pm$ 0.08	0.46 $\pm$ 0.07	0.433 $\pm$ 0.01

Note: All the result are expressed in term of Mean $\pm$ S.E.M., n=6 animals in each group; statistical significance was determined by ANOVA; $p < 0.05$

Table 1b: Percentage inhibition calculated in *in vivo* anti-inflammatory activity of aspirin and compound 1 in carrageenan-induced rat paw edema model.

Compounds	Dose (mg/Kg)	% Edema Inhibition			
		1 Hr	2 Hrs	3 Hrs	4 Hrs
Aspirin	10	64.23	52.06	51.37	1.43
	5	89.82	72.45	66.21	53.00
	1	25.26	19.17	42.38	28.80
Compound 1	10	33.78	45.57	30.18	43.42
	5	24.32	53.38	28.30	42.43
	1	50.67	29.94	12.89	14.47

Statistical analysis

Graphpad prism 4.0 software was used to analyse the data. The p values were calculated with less than significant difference ($p < 0.05$). One way ANOVA was performed for calculation of the p values. Each experiment was a replicate of 6 values ($n = 6$).

RESULTS

Longissiminone A (**1**) was screened for its *in vivo* anti-inflammatory and anti-platelet aggregation activities. It was observed that compound **1** have moderate *in vivo* anti-inflammatory as well as anti-platelet aggregation activities. Tables 1a and 1b showed all results of activities for compound **1**.

DISCUSSION

In vivo anti-inflammatory activity

Carrageenan-induced rat paw edema model is an acute model in which carrageenan was used to induce an acute inflammatory response. The mechanism involves the production of several mediators such as 5-HT, histamine, bradykinin and prostaglandins at the site of inflammation. The production of ROS is also mediated in this assay so this model is also useful for the comparison of the activity of respiratory burst inhibitors. During the current study the compound **1** exhibited inhibition potential during different time intervals (from 0-4th hour) of observation at initial acute inflammation phase (Letícia *et al.*, 2004). The

measurement of anti-inflammatory activity of test compound **1** was made during the initial 1st to 4th hours of edema progression. During the 1st and 2nd hr of carrageenan injection, there is a lag phase in which carrageenan initiate the production of several mediators, previously mentioned. At the 3rd hour of injection, the level of inflammatory mediators produced reaches to their highest concentrations and the marked inflammation on the paw of the rat can be seen. At the 4th hour of post carrageenan injection, the mediators began to reduce their levels. The measurement of the anti-inflammatory activity of the compound **1** was therefore performed during the initial four hours and the %inhibition was calculated.

Compound **1** was subjected to *in vivo* anti-inflammatory activity assay. Three different doses of compound **1** were injected intra peritoneally (*i. p.*) in rats, prior to the induction of edema. After the induction of edema on right paw of the rat by the injection of the carrageenan (0.1% w/v), the suppressive activity of the compound **1** towards the inflammation was measured. At the highest dose (10mg/kg), a maximum inhibition during the second hour after the edema progression (45.57%) was observed. Upon lowering the dose (1 to 5mg/kg) of compound, the inhibition persisted and 30-50% suppression of the edema was observed during all the observation points. At this concentration, compound **1** was initially showed a weak activity (at 1st hour), and then enhanced activity (53.38%) during the 2nd hour, which was then reduced to 28.30% and 42.43% during 3rd and 4th hour, respectively. At a much lower dose (1mg/kg), the significant suppressive

potential was observed with 50.67% inhibition at 1st hour (tables 1a and 1b). During the following 2-4 hours, a weak activity of compound 1 was observed. Graphical representation of the anti-inflammatory activity is shown in graphs 1a and 1b.

Platelet aggregation study

Compound 1 was found to be moderately active against the platelets aggregation induced by arachidonic acid at various concentrations (300-500mM) (fig. 2a). Results of compound 1 are presented in a dose-dependant curve (fig. 2b). At a dose of 300mM, compound 1 was exhibiting weak antiplatelet aggregation response. The potent activity was upon increasing concentration to 400mM where approximately less than 20% aggregation was observed. One more dose of 500mM showed further decrease in the platelet aggregation and it touched to the bottom value of the aggregation.

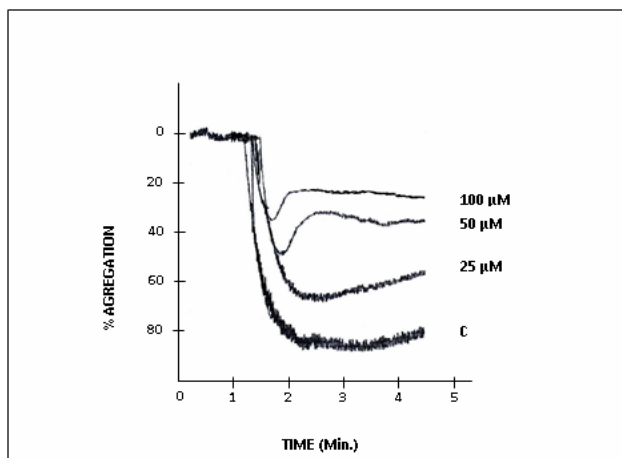


Fig. 2a: Effect of various concentrations of compound 1 on the arachidonic acid induced aggregation in the human platelets, where C stands for control in which 10% DMSO in saline was used.

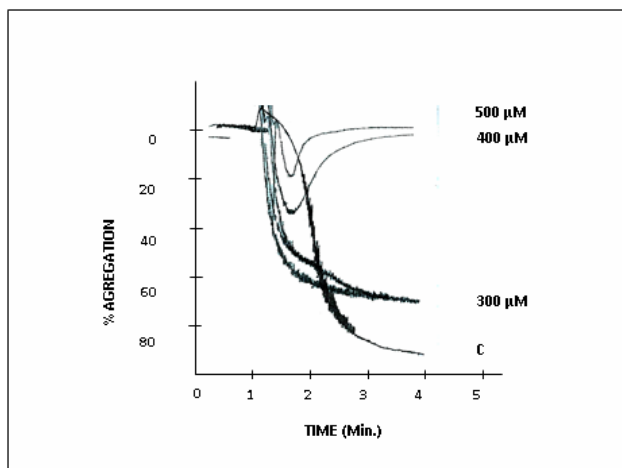


Fig. 2b: Effect of various concentrations of arachidonic acid on platelet aggregation, where C stands for control in which 10% DMSO in saline was used.

CONCLUSION

This study was based on the evaluation of our previous reported data on the compound 1 where we have been reported its *in vitro* activities. We have been used cell based and cell free biochemical assays to prove its potential as anti-inflammatory compound. In present study, we evaluated its anti-inflammatory potential by using *in vivo* acute model of inflammation and anti-platelet assay. In both cases, compound 1 was found to be showing promising results. Further detailed study on this molecule may reveal some interesting results, which might provide base to study this molecule for the extended drug discovery research.

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