Lipoxygenase and Urease Inhibition of Extracts of *Polygonatum verticillatum* Rhizome: Augmented by its Isolated Compound, Santonin

[1,5] Haroon Khan*, [1] Muhammad Saeed, [2] Murad Ali Khan, [5] Naveed Muhammad, [3] Afsar Khan**
[4] Asmat Ullah and [1] Safiullah
[1]*Department of Pharmacy, University of Peshawar, Peshawar-25120, Pakistan.*
[2]*Chemistry Department, Kohat University of Science and Technology, Kohat, Pakistan.*
[3]*Department of Chemistry, COMSATS Institute of Information Technology, Abbottabad-22060, Pakistan.*
[4]*Gandhara College of Pharmacy, Gandhara University, Peshawar, Pakistan.*
[5]*Department of Pharmacy, Abdul Wali Khan University Mardan 23200, Pakistan*
hkdr2006@gmail.com*, afsarhej@gmail.com**

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Summary: The present study was designed to explore the enzyme inhibitory profile of extracts of rhizome of *Polygonatum verticillatum* against lipoxygenase and urease. When tested against lipoxygenase, ethyl acetate fraction was found the most potent (IC_{50}: 69 µg/ml) and the overall IC_{50} values of different extracts ranged from 69─174 µg/ml. In urease assay, *n*-butanol was the most potent fraction (IC_{50}: 169 µg/ml) while the overall IC_{50} values were in the range of 169─288 µg/ml. Bioactivity guided chromatography led to the isolation of compound **1** which was characterized as santonin on the basis of various spectroscopic techniques. When santonin was tested against lipoxygenase and urease, it showed potent inhibition of lipoxygenase (IC_{50}: 27.4 µM) but did not attenuate the urease activity. Our findings provided strong evidence for the enzyme inhibitory profile of the extracts of *P. verticillatum* rhizome and its isolated compound. Thus results are consistent with the traditional use of the plant as an anti-inflammatory agent.

Keywords: *Polygonatum verticillatum*, Rhizome, Extract, Lipoxygenase, Urease, Santonin.

Introduction

Arachidonic acid metabolism through LOX pathway produces various biologically active lipids which are chief mediators of inflammation including leukotrienes. Leukotrienes are considered as potent mediators of inflammation and allergy [1, 2]. The products of LOXs catalysed oxygenation (hydroperoxyeicosatetraenoic acids; HPETE, hydroxyleicosatetraenoic acids; HETE), leukotrienes, and lipoxins) are actually involved in the development of different disorders such as rheumatoid arthritis, psoriasis, asthmatic responses, and glomerular nephritis. Consequently, focus on the discovery of lipoxygenase inhibitors could be an ideal approach to deal with such disorders, especially of natural origin being considered safer than synthetic agents. Results of our study pointed out marked lipoxygenase activity of the extracts and fractions of rhizome of the plant and thus offered a natural lipoxygenase inhibitor.

Urease (urea amidohydrolase; EC 3.5.1.5) is usually found in different bacteria, fungi, algae, and plants. Infections induced by these bacteria such as *Helicobacter pylori* (*H. pylori*) and *Proteus mirabilis* usually have high urease activity. Urease is directly involved in the formation of infectious stones and the over expression of its activity contributes to the pathogenesis of urolithiasis, pyelonephritis, ammonia and hepatic encephalopathy, hepatic coma, and urinary catheter encrustation [3]. Urease catalyzes the hydrolysis of urea to produce ammonia and carbon dioxide, and to protect the bacteria (*H. pylori*) in the acidic environment through elevation in pH. In agriculture, high urease activity grounds environmental and economic problems [4]. Therefore, targeting urease for treating pathogenic disorders may open a new line of treatment for infections caused by urease producing bacteria. The rhizome of the plant *Polygonatum verticillatum* showed reasonable potential as urease inhibitor. The crude extract as well as its *n*-butanol and aqueous fractions showed strong potency against urease enzyme.

Polygonatum verticillatum (L.) All. (Nooreallam) represents the genus *Polygonatum* which comprises approximately 57 species of family Convallariaceae [5]. Several ethnobotanical uses of the plant are documented in literature. Pain, pyrexia, and burning sensation have been treated with syrup of fresh rhizome of *Polygonatum verticillatum* [5]. Rhizome of the plant is also used in combination with other herbs to promote urine discharge (diuretic) and to attenuate painful urination [6]. Of the various ethnobotanical uses, some of them have already been rationalized scientifically [7-18]. In continuation of

*To whom all correspondence should be addressed.

our exploration of different pharmacological activities of *Polygonatum verticillatum*, herein we are presenting the enzyme inhibitory profile of the rhizome of the plant against lipoxygenase and urease in well established *in vitro* protocols followed by bioactivity guided isolation.

Results and Discussion

The lipoxygenase (LOX) inhibitory activity of rhizome of the plant is depicted in Fig. 1 and Table-1. It is evident from the results that the crude extract of rhizome possessed notable activity; the IC_{50} value was 102 µg/ml. profound changes in the activity of rhizome were recorded upon fractionation. All the fractions had reasonable activity and potency except the *n*-hexane fraction. The ethyl acetate fraction was the most dominant in terms of potency; the IC_{50} value was 69 µg/ml followed by *n*-butanol and chloroform fractions with IC_{50} values of 76 µg/ml and 79 µg/ml, respectively. Nevertheless, aqueous fraction possessed significant antagonistic activity against lipoxygenase (IC_{50} = 174 µg/ml).

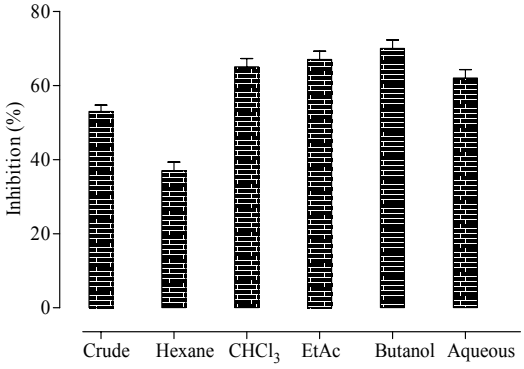

Fig. 1: Lipoxygenase inhibition (%) of rhizome of *Polygonatum verticillatum* at 100 mg/ml. Data are mean ± S.E.M. of three independent experiments.

Table-1: *In vitro* quantitative inhibition of lipoxygenase by crude extract and various fractions of *Polygonatum verticillatum.*

Extracts/Fractions	IC_{50}±S.E.M. (µg/ml)
Crude extract	**102±0.19**
***n*-Hexane**	**-**
Chloroform	**79±0.44**
Ethyl acetate	**69±0.09**
***n*-Butanol**	**76±0.17**
Aqueous	**174±0.32**
Baicalein	**22.6±0.09**

IC_{50} values are the mean±S.E.M. of three assays
Control = Ethanol
Standard = Baicalein

The urease inhibition of rhizome of the plant is presented in Fig. 2 and Table-2. The crude extract of rhizome exhibited significant attenuation of enzyme (IC_{50} = 209 µg/ml). When different fractions were tested for the activity, the *n*-butanol fraction was the most potent fraction (IC_{50} = 169 µg/ml) followed by chloroform fraction (IC_{50} = 197 µg/ml). However, the *n*-hexane and ethyl acetate fractions were inactive in urease inhibition assay.

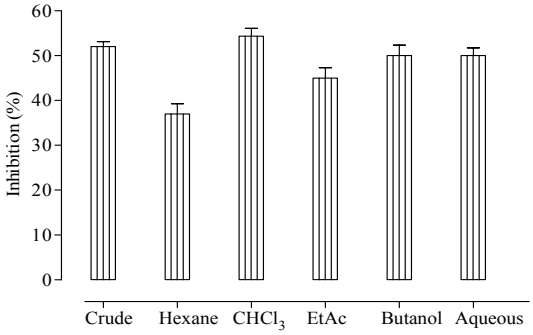

Fig. 2: Urease inhibition (%) of rhizome of *Polygonatum verticillatum* at 100 mg/ml. Data are mean ± S.E.M. of three independent experiments.

Table-2: *In vitro* quantitative inhibition of urease by crude extract and various fractions of *Polygonatum verticillatum*

Extracts/Fractions	IC_{50}±S.E.M. (µg/ml)
Crude extract	**209±0.54**
***n*-Hexane**	**-**
Chloroform	**197±0.48**
Ethyl acetate	**-**
***n*-Butanol**	**169±0.34**
Aqueous	**288±0.48**
Thiourea	**15.06±0.72**

IC_{50} values are the mean±S.E.M. of three assays
Control = DMSO
Standard = Thiourea

Bioactivity guided isolation led to the purification of compound **1** (Fig. 3) as a light yellowish powder. The compound was soluble in chloroform at room temperature. It was UV active and showed a molecular ion peak at *m/z* 246.1245 in HR-EI-MS. Thus, molecular formula of the compound was determined as $C_{15}H_{18}O_3$. ^{1}H-NMR spectra of compound **1** showed a doublet resonating at δ 6.65 (*J* = 10.0 Hz) due to olefinic C-1 methine proton and another doublet appeared at δ 6.19 (*J* = 10.0 Hz) due to C-2 methine proton. Both of these protons showed cross peaks to each other in the COSY plot. A doublet was resonating at δ 4.75 (*J* = 11.0 Hz) corresponding to C-6 methine proton, which showed ^{1}H-^{1}H COSY correlation with C-7 methine proton resonating at δ 1.77. The C-7 proton showed cross peaks with C-8 protons (δ 1.98, dddd, *J* = 13.0, 5.0, 5.0, 5.0 Hz; δ 1.65, dddd, *J* = 12.75, 12.75,

12.75, 3.5 Hz) and C-11 proton. The C-11 proton also had vicinal coupling with methyl protons of C-13 and thus gave a dq at δ 2.38 (J = 13.5, 6.5 Hz). ^{13}C-NMR analysis of compound **1** confirmed signals for 15 carbon atoms. These signals were resolved into three methyl, two methylene, five methine, and five quaternary carbon atoms on the basis of DEPT experiment. The C-6 signal was resonating at δ 81.3 showing that it was geminal to the lactone oxygen. The two olefinic resonances for double bond appeared at δ 154.9 and 125.7 due to C-1 and C-2 carbon atoms, respectively. The carbonyl groups in the compound were confirmed by the signals resonating at δ 186.2 and 177.5 due to C-3 and C-12, respectively [19, 20]. Based on various spectroscopic techniques, the structure of compound **1** was assigned as (6*S*,7*S*,10*S*,11*S*)-4,10,11-trimethyl-7,8,9,10-tetrahydronaphtho[16,12-*b*]furan-3,12(6*H*,11*H*)-dione (santonin) which is a known compound [19, 20].

Table-3: *In vitro* quantitative inhibition of urease and lipoxygenase by Santonin.

Compound	Activity in terms of IC_{50}±S.E.M. (µg/ml)	
	Lipoxygenase	Urease
Santonin	**27.4±0.34**	–
Baicalein	**22.6±0.09**	–
Thiourea	–	**15.10±0.66**

IC_{50} values are the mean±S.E.M. of three assays
Control = DMSO

The compound **1** inhibited lipoxygenase, it showed marked inhibition (IC_{50}: 27.4 µM). However, it did not exhibit any activity against urease (Table-3).

Experimental

General Experimental Conditions

The ^{1}H-NMR spectra were measured in $CDCl_3$ at 400 MHz on Bruker Avance spectrometer; none-deuterated residual solvent signal of $CDCl_3$ was used as an internal standard. The ^{13}C-NMR spectra were recorded in $CDCl_3$ at 125 MHz. The other related techniques like COSY, HMQC, NOESY, and HMBC were measured on Bruker Avance spectrometer at 400 MHz. Mass spectrometric analysis was based on electron impact mass spectrometry (EI-MS) and high resolution electron impact mass spectrometry (HR-EI-MS).

Plant Material

The whole plant, *Polygonatum verticillatum* was collected from District Swat, Khyber Pakhtunkhawa, Pakistan, in July-Aug 2007. The botanical identity of the plant was done by the Taxonomy Department of PCSIR Laboratories Peshawar and a specimen with catalogue No: 9970 (PES) was deposited in the herbarium of PCSIR Laboratories, Peshawar.

Plant Extraction and Fractionation

The shade dried rhizomes of *Polygonatum verticillatum* (8 Kg) were ground to fine powder. The powdered material was extracted by maceration with methanol at room temperature for 14 days with occasional shaking [21]. The methanol extracted residue was filtered off with a muslin cloth and the filtrate was concentrated under vacuum at low temperature (40 °C) using rotary evaporator; yielded a dark greenish semisolid material (2.2 Kg, 27.50% w/w).

Fractionation and Isolation

The crude methanol extract (1.6 Kg) was suspended in distilled water and sequentially fractionated with *n*-hexane, chloroform, ethyl acetate, and *n*-butanol, yielding *n*-hexane (258 g, 16.13% w/w), chloroform (219 g, 13.69% w/w), ethyl acetate (226 g, 14.13% w/w), *n*-butanol (265 g, 16.56% w/w), and aqueous (501 g, 31.31% w/w) fractions.

The ethyl acetate soluble fraction (80 g) was subjected to column chromatography (silica gel). The initial polarity of mobile phase (methanol/chloroform; 0.5:99.5) was increased up to 12:88 (methanol:chloroform) and yielded 9 subtractions (M1-M9). Subfraction M3 was subjected again to chromatography over silica gel using methanol:ethyl acetate (0.5:99.5) which resulted into the isolation of santonin (**1**) as yellowish powder.

MP: 171−172 °C; UV (MeOH): λ_{max} = 258 nm, HR-EI-MS: *m/z* 246.1245. (calcd. for $C_{15}H_{18}O_3$; 246.1251). ^{1}H-NMR ($CDCl_3$, 400 MHz): Table-4. ^{13}C-NMR ($CDCl_3$, 125 MHz): Table-4.

Lipoxygenase Inhibition Assay

The lipoxygenase (LOX) inhibition assay was conducted by using different dilutions of the crude extracts and fractions [22, 23]. Type I-B (Soybean) lipoxygenase (1.13.11.12) and linoleic acid were purchased from Sigma (St. Loius, MO) and were utilized without further purification. All other chemicals used were of analytical grade. 160 ml of sodium phosphate buffer, 0.1 mM (at pH 7.0), 10 ml of sample solution (for crude extract and fractions), and 20 ml of LOX solution were mixed followed by incubation for 5 min at 258 °C. The biochemical

reaction was initiated by the addition of 10 µl linoleic acid solution and substrate, and the absorption change with the formation of (9*Z*,11*E*)-13*S*)-13-hydroperoxyoctadeca-9,11-dienoate was followed for 10 min. The test sample and the control were dissolved in 50% aq. ethanol. All the reactions were performed in triplicate. Baicalein was used as positive control of lipoxygenase while ethanol as negative control. The IC_{50} values were calculated using the EZ-Fit Enzyme Kinetics program (Perrella Scientific Inc., Amherst, USA).

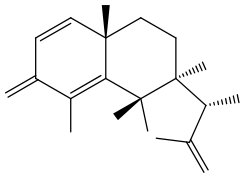

Fig. 3. Structure of santonin (**1**)

Table-4: NMR spectral data of santonin (**1**)

Position	^{13}C [δ]^a	^{1}H [δ]^b	Carbon type
1	154.9	6.65 d (*J* = 10.0 Hz)	CH
2	125.7	6.19 d (*J* = 10.0 Hz)	CH
3	186.2	-	C
4	128.5	-	C
5	150.9	-	C
6	81.3	4.75 d (*J* = 11.0 Hz)	CH
7	53.4	1.77 dddd (*J* = 12.0, 11.75,11.75, 3.5 Hz)	CH
8	22.9	1.98 dddd (*J* = 13.0, 5.0, 5.0, 5.0 Hz) 1.65 dddd (*J* = 12.75, 12.75, 12.75, 3.5 Hz)	CH_2
9	37.7	1.47 ddd (*J* = 13.5, 13.5, 4.5 Hz) 1.86 ddd (*J* = 13.5, 3.0, 3.0 Hz)	CH_2
10	41.2	-	C
11	40.9	2.38 dq (*J* = 13.5, 6.5 Hz)	CH
12	177.5	-	C
13	12.4	1.22 d (*J* = 6.5 Hz)	CH_3
14	24.4	1.27 s	CH_3
15	10.8	2.07 s	CH_3

[a]Bruker 125 MHz, $CDCl_3$, δ values are recorded in ppm.
[b]Bruker 400 MHz, $CDCl_3$, δ values are recorded in ppm.

Urease Inhibition Assay

For the study of urease inhibitory activity, standard documented assay was used [24]. Briefly, reaction mixtures comprising 25 ml (40 units/ml) of enzyme solution and 55 ml of buffers containing urea (100 mM) were incubated with 5 µl of the test samples at 30 °C for 15 min in 96-well plates. Ammonia production was measured as a urease activity by indophenol method [25]. Final volumes were maintained as 200 µl by adding 45 µl phenol reagent (1% w/v phenol and 0.005% w/v sodium nitroprussside) and 70 µl of alkali reagent (0.5% w/v NaOH and 0.1% active chloride NaOCl) to each well. Using a microplate reader (Molecular Devices, CA, USA), the increase in absorbance was measured at 630 nm after 50 min at pH 6.8. The results (change in absorbance per min) were collected using SoftMax Pro software (Molecular Devices, CA, USA). Thiourea was used as the standard inhibitor of urease while DMSO as negative control. The % inhibition was calculated from the following equation:

Statistical Analysis

Results obtained from various *in vitro* experiments are expressed as mean values ± S.E.M. (standard error of measurement). Experiments were carried out in triplicate.

Conclusion

The rhizome of *Polygonatum verticillatum* registered significant inhibitory profile against lipoxygenase and urease; over-expression of which is involved in the patho-physiology of different diseases. Compound **1** strongly augmented the lipoxygenase inhibitory profile of the extracts but not the urease reversal. However, further detailed studies are required for the isolation of other secondary metabolite(s) to provide better understanding of current show as well as lead compounds of clinical utility.

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