

Isolation of Anemonin from *Pulsatilla wallichiana* and its Biological Activities

¹Iftikhar Ali*, ²Sakeena Khatoon, ²Faiza Amber, ²Qamar Abbas, ¹Muhammad Ismail,

³Nadja Engel, and ⁴Viqar Uddin Ahmad

¹Department of Chemistry, Karakoram International University, 15100-Gilgit, Pakistan.

²Department of Biological Sciences, Karakoram International University, 15100-Gilgit, Pakistan.

³Department of Pediatric Surgery, University Hospital Marburg, Baldingerstraße, Marburg 35043, Germany.

⁴HEJ Research Institute of Chemistry, University of Karachi, Karachi-75270, Pakistan.
iftikhar.ali@kiu.edu.pk*

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Summary: The phytochemical work on *Pulsatilla wallichiana* Ulbr. (Ranunculaceae Juss.) afforded a dilactone, anemonin (**1**) isolated, purified and identified by spectroscopic methods and comparison of their spectral data with the reported data. To the best of our knowledge, this compound was isolated for the first time from *Pulsatilla wallichiana* Ulbr. Moreover, the aqueous (aq.) methanolic extract of the whole plant sample of *Pulsatilla wallichiana* Ulbr. (PWW) and the methanolic extract of the whole plant sample of *Physochlaina praealta* (Decne.) Miers (Solanaceae Juss.; PPM) were also investigated for several biological activities including antifungal, antiinflammatory, anticancer (Rh30, Hela, 3T3), cytotoxic, phytotoxic, brine shrimp lethality and insecticidal activities. PWW showed insignificant antifungal while higher anti-inflammatory activity as compared to PPM. In anticancer activity, PWW showed a concentration dependent effect on Rh30 cell viability while both samples exhibited moderate phytotoxic activity.

Key Words: Anemonin; *Pulsatilla wallichiana*; *Physochlaina praealta*; Bioassays; Gilgit-Baltistan.

Introduction

Medicinal plants are the source of therapeutic potential and it is reported that more than 10% of total 258,650 species of higher plants are used to treat ailments [1]. In Gilgit-Baltistan, the indigenous knowledge on therapeutic flora is totally in hold of old people while the young ones are unaware of such knowledge [2]. Plants and people interaction in Karakoram, Hindu Kush and Himalayan mountains has been documented recently [3-5]. Our present study is based on medicinally important plant species i.e. *Pulsatilla wallichiana* and *Physochlaina praealta*.

Pulsatilla wallichiana Ulbr. (Ranunculaceae Juss.; Syn. *P. duthiei* Gand., *Anemone albana* Hook. f. & Thomson, *A. wallichiana* Royle) [6] is commonly known as Ree-sman or Zgiongmonana Loqparimandoq in Baltistan region and its fruit has been reported to treat dysentery, dropsy [5] and dermatitis [7]. The therapeutic potential of *Pulsatilla* decoction, a classical prescription in Traditional Chinese Medicine, in the treatment of vulvovaginal candidiasis and murine ulcerative colitis has recently been reported [8, 9]. Certain chemical constituents isolated from *Pulsatilla* species have been reported as antischistosomal agent [10], antibacterial agent [11], and for the treatment of hepatitis B virus infection and hepatoma [12]. In literature, recently sufficient work has been reported on several other *Pulsatilla*

species e.g. *P. chinensis*, *P. koreana*, *P. nigricans* but however, *P. wallichiana* is not that much investigated. *Physochlaina praealta* (Decne.) Miers (Solanaceae Juss.; Syn. *P. grandiflora* Hook., *P. urceolata* Kuang & A.M. Lu, *Hyoscyamus praealtus* (Decne.) Walp., *Belenia praealta* Decne., *Scopolia praealta* (Decne.) Dunal) [6] is locally known as Luntung, reported as a poisonous plant and its leaves are used as antiseptic in cattle's sheds [13]. The seeds and flowers of *P. praealta* are reported for the treatment of toothache [14]. The phytochemical investigation of certain *Physochlaina* species e.g. *P. infundibularis* Kuang [15] and *P. physaloides* [16, 17] have been resulted in isolation of alkaloids. However, *P. praealta* is not scientifically investigated for the biological activities and/or phytochemicals.

The aim of the present study was to investigate and isolate the bioactive constituents from *P. wallichiana*. Furthermore the extracts of *P. wallichiana* and *P. praealta* were studied for various biological activities e.g. antibacterial, antifungal, anti-inflammatory, anticancer, cytotoxic, phytotoxic, brine shrimp lethality and insecticidal properties. Because of the investigation, a dilactone namely anemonin (**1**) was isolated and identified for the first time from *P. wallichiana* Ulbr.

*To whom all correspondence should be addressed.

Experimental

Plant material

The plant samples of *Pulsatilla wallichiana* Ulbr. (Ranunculaceae; voucher specimen No. IS 167) and *Physochlaina praealta* (Decne.) Miers (Solanaceae; voucher specimen No. S-001) were collected from different areas in Gilgit-Baltistan in August 2016 and were authenticated and the voucher specimens were deposited in Department of Biological Sciences, Karakoram International University, Gilgit. The whole plant samples were collected and dried in shade at room temperature. The dried samples were crushed to powder using a grinder.

Extraction and Isolation

The powdered form of *Pulsatilla wallichiana* Ulbr. and *Physochlaina praealta* (Decne.) Miers were macerated and extracted with 90% MeOH (2 L) and 100% MeOH (4 L), respectively for 7 days at room temperature. The extracts were dried using rotary evaporator at 40°C under reduced pressure. Thus methanolic/aqueous (aq.) methanolic extracts were obtained (PPM, PWW). PWW was undertaken for phytochemical isolation in the present study.

PWW was then dissolved in 600 ml methanol and transferred to separatory funnel (2L) and 600 ml of *n*-hexane was added to the mixture. The separatory funnel containing the mixture was shaken well and left standby for 15 minutes. Two clear layers of methanol and *n*-hexane were observed and further collected in separate flasks. The methanol layer was further extracted with *n*-hexane and repeated the process for third time. The *n*-hexane fractions were combined and evaporated to obtain solid residue (11.5 g). The aqueous mixture of the defatted aqueous (aq.) methanolic extract was prepared by adding 500 ml distilled water and shaken well for few minutes with the addition of 500 ml Dichloromethane (DCM). The two clear layers of DCM and water were observed. The DCM layer was collected in a flask and this process was repeated three times to obtain the DCM fraction (8 g). The remaining aqueous extract was further fractionated into ethyl acetate fraction by using 500 ml ethyl acetate for three times in a separatory funnel to obtain the solid residue (4.5 g). The ethyl acetate fraction was further subjected to silica gel column chromatography, starting with *n*-hexane, gradually increasing the polarity by adding ethyl acetate in multiple of 5 as mobile phase. In the end column was

washed with ethyl acetate. Total 30 fractions were collected from the column (SH-1 to SH-30). The semi pure fraction SH-9, eluted with 45% EtOAc:Hex was further subjected to preparative TLC. Thus compound **1** was purified through preparative TLC on silica gel by using 15% ethyl acetate:*n*-hexane as mobile phase. The UV active band was marked under UV lamp (254 nm) and scratched with the help of spatula. The powder material was then soaked in 20 ml ethyl acetate and filtered through Whatman filter paper. The filtrate was evaporated at room temperature to obtain pure compound as white solid powder. The compound was identified as anemonin (**1**) (Fig. 1) on the basis of its spectral data including ¹H-NMR, ¹³C-NMR, HSQC, HMBC, COSY, and NOESY and by comparison with the related literature [18, 19] and its molecular formula was deduced as C₁₀H₈O₄ corresponding to molecular mass of 192 amu.

Anemonin (1)

White powder, M.P. 154°C; ¹H NMR (CDCl₃, 500 MHz) δ: 8.26 (2H, d, *J* = 5.6 Hz, H- 4, H-4'), 6.30 (2H, d, *J* = 5.6 Hz, H-3, H-3'), 2.61 (2H, m, H-6a, H-6a'), 2.24 (2H, m, H-6b, H-6b'). ¹³C NMR (CDCl₃, 125 MHz) δ: 23.8 (C6, C-6'), 90.3 (C5, C-5'), 121.1 (C3, C-3'), 153.2 (C4, C-4'), 170.8 (C2, C-2'). EI-MS (70 eV) *m/z* (%): 192 (5), 174 (5), 164 (68), 136 (58), 110 (41), 96 (69), 82 (78), 68 (78), 54 (100).

Biological Activities

Antibacterial Bioassay (96 Well Plate Method)

Organisms were grown in Mueller Hinton medium, inoculums were adjusted to 0.5 McFarland turbidity index. Stock solutions of each plant extract were prepared in DMSO (1:1). Media was dispensed to all wells and the work was triplicated. The test sample was added in wells, control wells were kept without any test. The volume of each well plate was made up to 200 µl and finally 5x10⁶ cells in all wells including both control and test were added. The plates were sealed using parafilm and incubated for 18-20 h. Alamar Blue Dye was dispensed in each well, covered with foil and shaken at 80 RPM in a shaking incubator for 2-3 h. The bacterial strains i.e. *Staphylococcus aureus*, *Escherichia coli*, *Bacillus subtilis*, *Salmonella typhi*, *Shigella flexenaria*, *Pseudomonas aeruginosa* were employed in our present study. The growth in bacterial strains was indicated by change in color of Alamar Blue dye from blue to pink. Absorbance was recorded at 570 and 600 nm by the ELISA reader [20, 21].

Antifungal Bioassay (Agar Tube Dilution Method)

The stock solution was prepared by dissolving 24 mg of crude extract and 12 mg of pure compound in 1 ml sterile DMSO. Sabouraud dextrose agar (SDA) was used for the growth of fungus. Media (pH 5.5-5.6) with high concentration of well dissolved glucose/maltose (2%) was prepared (32.5g per 500 ml distilled H₂O), and dispensed 4 ml into screw caps tubes and autoclaved at 121°C for 15 min. Tubes were allowed to cool (50°C) and non-solidified SDA was loaded with 66.6 µl of compound from the stock solution. The final concentrations of 400 µg/ml (crude extract) and 200 µg/ml (media for pure compound) were prepared. Tubes were solidified in slanting position at room temperature. Each tube was inoculated with 4 mm fungus piece (seven-day-old fungus culture). For non-mycelial growth, an agar surface streak was used. Other media supplemented with DMSO and reference antifungal drug were used as negative and positive control, respectively. The tubes were incubated at 27-29°C for 3-7 days. Cultures were analyzed twice in a week during incubation. Growth in the compound amended media was determined by measuring linear growth (mm) and growth inhibition was calculated with reference to the negative control [22, 23]. The fungal species *Candida albicans*, *Trichophyton rubrum*, *Aspergillus niger*, *Microsporum canis* and *Fusarium lini* were used in our present study. % Inhibition of fungal growth was calculated by the given equation:

$$\% \text{ inhibition} = \frac{100 - \text{linear growth in test (mm)}}{\text{linear growth in control (mm)}} \times 100$$

Anticancer activity

For anticancer screening the highly metastatic pediatric rhabdomyosarcoma cell line Rh30, DSMZ (Braunschweig, Germany) was chosen and cultivated in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Germany) with 10% fetal bovine serum (FCS; PAN Biotech GmbH, Germany) and 1% gentamicin (Ratiopharm GmbH, Ulm, Germany) at 37°C and in a humidified atmosphere with 5% CO₂. Prior to treatment cells were adapted to phenol-red-free DMEM (PAA Laboratories GmbH, Germany) with 10% charcoal stripped FCS (PAN Biotech GmbH, Germany) for 48 h to avoid unspecific stimulation of endogenous hormones in the serum (assay medium). Then, treatment with substances was carried out in the assay medium for 48 h.

MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-

tetrazolium) assay to determine cell viability was performed according to manufactures protocol (CellTiter 96® AQueous One Solution Cell Proliferation Assay; Promega Corp., Madison, WI, USA). Briefly, cells were seeded in 96-well plates at a density of 2000 cells/well in 100 µl medium and left to attach for 24 h. Treatment with PPM and PWV at final concentrations of 1, 10, 25, and 50 µg/ml was carried out as described previously [24]. In parallel, control approaches were carried out with medium only and 0.1 % of the solvent DMSO to calculate background absorbance. No background absorbance was obtained for PPM and PWV and MTS in the absence of cells, as some plant extracts are capable of reducing the MTS. After an initial incubation for 24 h cells were assayed with MTS. Colorimetric changes were measured at 490 nm and raw data was transferred to Microsoft Excel and analyzed. At least 8 replicates corrected with the background absorbance were performed. Reduction of cell viability at each concentration was plotted as a dose response curve. The IC₅₀ values were calculated using nonlinear regression to fit data to the dose-response. Data sets were expressed as means ± standard deviations (SD). Statistical significance was determined by the unpaired student's t-test (***) P < 0.001; ** P < 0.01; * P < 0.05).

Anticancer Bioassay (Hela cell lines)

Cytotoxicity was evaluated in 96-well flat-bottomed micro plates using the standard MTT (3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyl-tetrazolium bromide) colorimetric assay. Hela cells were cultured in minimum essential medium eagle, supplemented with 5% of fetal bovine serum (FBS), 100 IU/ml of penicillin and 100 µg/ml of streptomycin in 75cm² flasks, and kept in 5% CO₂ incubator at 37°C. Exponentially growing cells were harvested, counted with haemocytometer and diluted with a particular medium. Cell culture (6x10⁴ cells/ml) was prepared and introduced (100 µl/well) into 96-well plates. After overnight incubation, medium was removed and 200 µL of fresh medium with sample (1-30 µM) was added. After 48 h, 200 µL MTT (0.5 mg/ml) was added to each well and incubated (4 h). Subsequently, 100 µL DMSO was added to each well. The extent of MTT reduction within cells was calculated by measuring the absorbance at 570 nm, using a microplate reader (Spectra Max plus, Molecular Devices, CA, USA). The cytotoxicity was recorded as IC₅₀ for Hela [25]. The percent inhibition was calculated by using the following formula and % inhibitions were processed using Soft- Max Pro software (Molecular Device, USA):

$$\% \text{ inhibition} = \frac{100 - (\text{mean of O.D of test} - \text{mean of O.D of negative control})}{(\text{mean of O.D of positive control} - \text{mean of O.D of negative control})} \times 100$$

Cytotoxic Bioassay (3T3 Cells)

A standard MTT colorimetric assay was followed and the samples were evaluated in 96-well flat-bottomed micro plates. The standard fibroblast cell line 3T3 was cultured in Dulbecco's modified eagle medium, supplemented with 5% of FBS, 100 IU/ml of penicillin and 100 µg/ml of streptomycin in 75cm² flasks, and kept in 5% CO₂ incubator at 37°C. Exponentially growing cells were harvested, counted and diluted using particular medium. Cell culture (5x10⁴ cells/ml) was prepared and introduced (100 µl/well) into 96-well plates. After overnight incubation, medium was removed and 200 µl of fresh medium containing sample (1-30 µM) was introduced. After 48 h, 200 µl MTT (0.5 mg/ml) was added to each well and incubated (4 h). Subsequently, 100 µl of DMSO was added to each well. The extent of MTT reduction was calculated using a micro plate reader by measuring the absorbance at 540 nm (Spectra Max plus, Molecular Devices, CA, USA). The cytotoxicity was recorded as IC₅₀ for 3T3 cells [25]. The % inhibition was calculated by using the following formula and the results were processed using Soft-Max Pro software (Molecular Device, USA):

$$\% \text{ inhibition} = \frac{100 - (\text{mean O.D of test} - \text{mean O.D of neg. control})}{(\text{mean O.D of pos. control} - \text{mean O.D of neg. control})} \times 100$$

Anti-inflammatory/immunomodulatory Bioassay

Luminol-enhanced chemiluminescence assay was performed. Briefly 25 µl of diluted whole blood HBSS⁺⁺ [Sigma, St. Louis, USA] was incubated with 25 µl of three sample concentrations (1, 10 and 100 µg/ml), each in triplicate. Control wells were containing HBSS⁺⁺ and cells, but no samples. Test was performed in white half area 96 well plates [Costar, NY, USA], which was incubated at 37°C for 15 min in the thermostat chamber of luminometer [Labsystems, Helsinki, Finland]. After incubation, 25 µl of serum opsonized zymosan (SOZ) [Fluka, Buchs, Switzerland] and 25 µl of intracellular reactive oxygen species detecting probe, luminol [Research Organics, Cleveland, OH, USA] were added into each well, except blank wells (containing only HBSS⁺⁺). The level of the ROS was recorded in luminometer in term of relative light units (RLU). Ibuprofen (IC₅₀ = 11.2 ± 1.9 µg/ml) was used as standard [26].

Phytotoxic Bioassay

E-medium was prepared in one liter dist. H₂O and pH was adjusted between 6.0 to 7.0 by adding KOH pellets (stock solution). Working E-medium was prepared by mixing 100 ml of stock solution and 900 ml of dist. H₂O. Stock solution was prepared by dissolving 30 mg of study sample in 1.5 ml of solvent (MeOH/EtOH etc.). Three flasks were inoculated with 10, 100 and 1000 µl of solution pipetted from the stock solution and were left overnight to evaporate the solvent. 20 ml (E. medium) was added and then *Lemna minor*, containing a rosette of 2-3 fronds, was added to each flask (total 20 fronds). Other flasks were supplemented with E-medium and reference (standard drug) plant growth inhibitors and promoters served as -ve and +ve controls, respectively. The flasks were placed in growth cabinet for seven days. Plants were examined daily during incubation. The number of fronds were counted and recorded per flask on day 7. Results were analyzed as growth regulation in %age, calculated with reference to the -ve control using the given formula [27, 28]:

$$\% \text{ regulation} = \frac{100 - \text{no. of fronds in test sample}}{\text{no. of fronds in negative control}} \times 100$$

Insecticidal Bioassay (Impregnated filter paper method)

The study sample was prepared by adding 3 ml solvent (EtOH, MeOH, Acetone etc.) in 200 mg test sample. The stored grain pests were reared in the laboratory under controlled conditions (temperature and humidity) in plastic bottles containing sterile breeding media. The test insects (*Tribolium castaneum*, *Sitophilus oryzae*, *Rhyzopertha dominica*, *Trogoderma granarium* and *Callosobruchus analis*) of uniform age and size were used for the experiment. In the first day, the filter paper was cut according to the size of petri plate (9 cm or 90 mm) and put them in the plate. The whole sample was loaded over the filter paper with the help of micropipette. The plates were left for 24 h to evaporate the solvent completely. In the subsequent day, 10 insects of each species were put in each plate (test and control) with the help of a clean brush. Healthy and active insects of same size and age were taken. The plates were incubated at 27°C for 24 h with 50% relative humidity in growth chamber. In the third day, the survival of the insects (count the number of survivals of each species) was assessed. The % inhibition or % mortality was calculated with the help of the following equation:

$$\% \text{ mortality} = \frac{100 - \text{no. of insects test sample}}{\text{no. of insects alive in control}} \times 100$$

The positive (standard insecticide, Permethrin) and negative (volatile solvent and test insect) controls were run with test sample [29].

Brine shrimp lethality bioassay

Artemia salina eggs were stored at low temperatures (4°C). The hatching tray, a rectangular dish (22x32 cm) was half filled with filtered brine solution then sprinkled (50 mg) eggs of brine shrimp and incubated at 37°C. The test sample (20 mg) was dissolved in 2 ml of respective solvent and from this solution 5, 50 and 500 µl were transferred to vials (3 vials/concentration; keeping 10, 100 and 1000 µg/ml, respectively). The solvent was allowed to evaporate overnight. After 2-days of hatching and maturation as nauplii, 10 larvae/vials were placed using a Pasteur pipette. The volume was made to 5 ml with seawater and incubated at 25-27° C for 24 h under illumination. Other vials were supplemented with solvent, and reference cytotoxic drug serving as negative and positive controls, respectively. The data was analyzed with Finney computer program to determine LD₅₀ values with 95% confidence intervals. [30-32].

Results and Discussion

Phytochemical investigation of *Pulsatilla wallichiana* resulted in the isolation of anemonin (**1**) and its structure was determined by means of 1D and 2D NMR spectra (Figure 1).

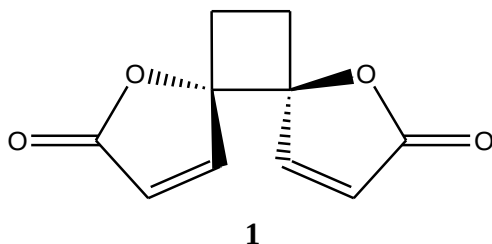


Fig. 1: Structure of Anemonin.

The methanolic extract of *Physoclaina praealta* (PPM) and aqueous (aq.) methanolic extract of *Pulsatilla wallichiana* (PWW) were evaluated for certain biological activities i.e. antibacterial, antifungal, anti-inflammatory, anticancer (Rh30, 3T3, Hela, MTT assay), cytotoxic, phytotoxic, brine shrimp lethality, and insecticidal activities.

The methanolic extract of *P. praealta* (Decne). Miers exhibited the highest % inhibition against *S. aureus* (68.54%), *E. coli* (10.04%), *B.*

subtilis (06.96%) and *S. typhi* (01.04%) while it remained inactive against *S. flexenari* and *P. aeruginosa*.

The study samples PPM and PWW were investigated against the fungal species *Candida albicans*, *Trichphyton rubrum*, *Aspergillus niger*, *Microsporum canis* and *Fusarium lini* through agar tube dilution method. PPM did not exhibit any activity and PWW showed insignificant activity exhibiting 2.5% (MIC 97.5 µg/ml) inhibition at 97.5 mm linear growth against *Candida albicans*. Miconazole and Amphotericin B were employed as standard drugs.

Testing the samples for anti-inflammatory activity, PPM and PWW exhibited 17.6% and 42.6% inhibition at a concentration 25 mg/ml, respectively. Ibuprofen was used as standard drug that showed 73.2% inhibition at the similar concentration.

Studying the anticancer activity, the standard drug (doxorubicin) was used against Hela cell lines that showed 73% inhibition at 30 µg/ml concentration. PPM and PWW exhibited 30% and 24% inhibition and were considered inactive against Hela cell lines as compared to the standard drug.

To test the anticancer activity on highly metastatic cancer cells, the alveolar rhabdomyosarcoma cell line Rh30 was chosen. After treatment with 50 µg/ml PWW Rh30 were significantly reduced in cell viability up to 30%, measured by metabolic MTS assay (Figure 2A). In contrast, 50 µg/ml PPM slightly increased the cell viability up to 10%. However, PWW displayed a concentration dependent effect on Rh30 cell viability (Figure 2B). Calculated IC₅₀ value was 903.56 µg/ml, too high to consider PWW as a potential chemotherapeutic agent.

In determining the cytotoxicity, the samples PPM and PWW exhibited 22% and 27% inhibition respectively, and were considered nontoxic against 3T3 cell lines at the concentration 30 µg/ml. While the standard drug cycroamide was used against cytotoxic 3T3 cell lines that showed 70% inhibition on the similar concentration.

The standard drug parquet was used in studying the phytotoxicity. The activity was carried out at different concentrations i.e. 10, 100 and 1000 µg/ml. PPM and PWW showed moderate phytotoxic activity at highest concentrations as shown in the table.

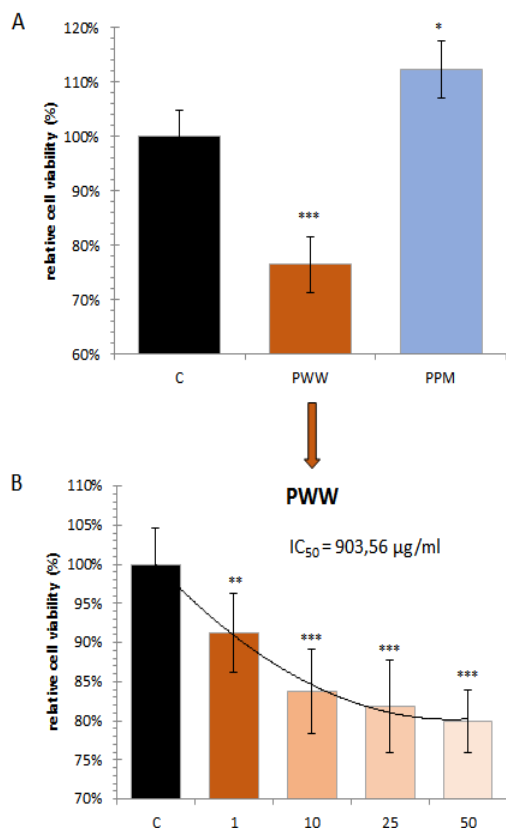


Fig. 2: Anticancer screening via MTS viability assay of PWW and PPM on highly metastatic alveolar rhabdomyosarcoma cell line Rh30. A: Initial screening at a high concentration of 50 µg/ml PWW and PPM in comparison with the vehicle control (C). B: Concentration series (1, 10, 25, and 50 µg/ml) of PWW to calculate the IC₅₀ value. Statistical significance was determined by the unpaired student's t-test (*** P < 0.001; ** P < 0.01; * P < 0.05)

Table-1: Results of phytotoxicity.

Table 1: Results of phytochemistry.						
Name of Plant	Conc. of sample µg/ml	No. of Fronds		% Growth Regulation		Std. Drug µg/ml
		Sample		Control		
		PWW	PPM	PWW	PPM	
<i>Lemna minor</i>	10	38	48	48	20	0
	100	34	43	48	29	10.4
	1000	25	28	48	47.9	41.6

The brine shrimp lethality activity of the samples PPM and PWW was determined in 10, 100 and 1000 µg/ml concentrations. The standard drug etoposide was used having LD₅₀ of 7.4625 µg/ml.

The number of shrimps was kept 30 in each concentration. The number of survivors in case of PPM was 30, 26 and 24 at the 10, 100 and 1000 µg/ml concentrations, respectively. While in case of PWW, the number of survivors was 29, 27 and 27 at the given concentrations. The samples thus did not display any significant brine shrimp lethality activity.

The sample PWW was tested against different insects but however it did not exhibit any significant insecticidal activity.

P. praealta has been investigated for some chemical [33] and biological [34, 35] properties previously. But however, in our present study we hereby report, for the first time to the best of our knowledge, on the antibacterial, antifungal, anti-inflammatory, anticancer, cytotoxic, phytotoxic, brine shrimp lethality, and insecticidal activities. *P. wallichiana* has recently been reported for good antioxidant, antiglycation and immunomodulatory activities [36]. Pulsatilla species have been reported for its anticancer properties. The chemical constituents isolated from Pulsatilla species have exhibited such therapeutic potential. For example, saponin derivatives from *P. chinensis* have been reported as potent anticancer drug candidates [37, 38]. In addition certain other species have exhibited therapeutic properties against inflammation like *P. koreana* has been reported for herbal massage and to treat wounds [39]. In our present study, *P. wallichiana* exhibited 42.6% inhibition at a concentration 25 mg/ml in antiinflammatory activity and anemonin, a small natural product, was isolated from *P. wallichiana*. Anemonin is recently reported as a potent protective molecule for osteoarthritis [40] molluscicidal properties [41], neuroprotective effects [42], inhibition of melanin synthesis [43], inhibition of inducible nitric oxide synthases [19] and for alleviating intestinal inflammation [44]. Anemonin has been reported from *Clematis* species [19, 41, 45], *Drymaria* species [46, 47], *Ranunculus japonicus* [48], *Helleborus foetidus* [49] etc. This compound could be the reason behind the local use of the plant *P. wallichiana* for inflammation, dermatitis etc. [7]. However this plant could be employed for more scientific investigation.

Conclusion

In this study the extract of *P. wallichiana* Ulbr. was investigated to isolate the bioactive constituents and as a result anemonin (1) was isolated and identified for the first time from *P. wallichiana* Ulbr. In addition, the aqueous (aq.) methanolic extract of *Pulsatilla wallichiana* Ulbr. (PWW) and

methanolic extract of *Physochlaina praealta* (Decne.) Miers (PPM) were investigated for miscellaneous biological activities namely, antibacterial, antifungal, anti-inflammatory, anticancer (Rh30, Hela, 3T3), cytotoxic, phytotoxic, brine shrimp lethality and insecticidal properties. The sample PPM exhibited the highest % inhibition against *S. aureus* (68.54%), while it did not exhibit antifungal activity against any of the fungal species i.e. *Candida albicans*, *Trichophyton rubrum*, *Aspergillus niger*, *Microsporum canis* and *Fusarium lini* through agar tube dilution method. The other study sample PWW showed insignificant activity exhibiting 2.5% (MIC 97.5 µg/ml) inhibition at 97.5 mm linear growth against *Candida albicans*. Similarly PPM and PWW exhibited 17.6% and 42.6% inhibition at a concentration 25 mg/ml, respectively. PPM and PWW exhibited 30% and 24% inhibition and were considered inactive against Hela cell lines as compared to the standard drug. To test the anticancer activity on highly metastatic cancer cells, the alveolar rhabdomyosarcoma cell line Rh30, 50 µg/ml PPM slightly increased the cell viability up to 10%. However, PWW displayed a concentration dependent effect on Rh30 cell viability. The samples did not display any significant brine shrimp lethality, and insecticidal activities, while both plant samples, PPM and PWW exhibited moderate phytotoxic activity.

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