Current Botany 2021, 12: 85-96
doi: 10.25081/cb.2021.v12.6892
http://updatepublishing.com/journal/index.php/cb/index

Research Article

ISSN: 2220-4822

Assessment of threatened status, phytochemical composition and biological properties of three *Aconitum* species from Kashmir Himalaya - India

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ABSTRACT

Genus *Aconitum* (Ranunculaceae) is represented by 6–8 species from Kashmir Himalaya. Traditionally *Aconitum* species are used to treat a wide array of diseases, but their ethnopharmacological validation and phytochemistry are hitherto unreported from Kashmir Himalaya. The present study was undertaken to bring insights into the traditional use and distribution pattern of three *Aconitum* species from the region. An ethnobotany-directed approach was employed to study the conservation status of three *Aconitum* species. Their phytochemical profiles and biological properties were screened under *in vitro* conditions. Folin–ciocalteu and Aluminium chloride assays were employed to measure their total phenolic and total flavonoid contents, respectively. Plant extracts were evaluated for antioxidant, antimicrobial and anti-inflammatory activities. Three *Aconitum* species, viz. *Aconitum heterophyllum* Wall. ex Royle, *Aconitum violaceum* Jacquem. ex Stapf and *Aconitum chasmanthum* Stapf. ex Holmes showed dwindling conservation status in Kashmir Himalaya. *Aconitum* extracts showed significant variations in total phenolic and flavonoid contents. Antioxidant activity of *Aconitum chasmanthum* methanolic extract was studied to be comparatively higher (80.115%). *Aconitum chasmanthum* DCM & methanolic extracts showed a good MIC value of 0.125 mg/ml against *Candida albicans* and *Streptococus pyogenes*, respectively. The percent inhibition of NLRP inflammasome was found significant in *Aconitum violaceum* ethyl acetate extract (74.61%). The present study revealed that *Aconitum* species are constantly declining at least in investigated habitats of Kashmir Himalaya and hence need strategic conservation planning. The results also emphasized the utility of *Aconitum* species as antioxidant, antimicrobial and anti-inflammatory agents that could be used to manage various health problems.

KEYWORDS: *Aconitum*; Kashmir Himalaya; Conservation status; Antioxidant activity; Antimicrobial activity; Anti-inflammatory activity

Received: February 23, 2021
Revised: March 21, 2021
Accepted: March 25, 2021
Published: April 15, 2021

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INTRODUCTION

Genus *Aconitum* (Ranunculaceae) is represented by approximately 300 species worldwide (Utelli *et al.*, 2000). About 166 species of *Aconitum* have been reported from Southwest China and Eastern Himalaya (Liangqian & Kadota, 2001) and 11 species from Pakistan (Qureshi & Chaudhri, 1988). In India, the genus is represented by 27 species distributed in alpine and sub-alpine zones of Himalaya extending from Kashmir to Uttarakhand and to the hills of Assam (Balakrishnan *et al.*, 1993). Previously, 6–8 species have been reported from Kashmir Himalaya (Conventry, 1930; Jabeen *et al.*, 2013). *Aconitum* is one of the most precious natural sources of highly useful bioactive metabolites such as aconitine, mesaconitine and hypaconitine possessing significant antitumor and immunostimulant properties. They are used to cure nervous system disorders, rheumatism, diabetes (Chhetree *et al.*, 2010; Yang *et al.*, 2017) and are effective against multi-drug resistant cancers (Wada *et al.*, 2015). Traditionally, the leaves of *Aconitum* species are used to treat common ailments like cough, cold, fever, diarrhoea and vomiting (Shah, 2005). The rhizomes of *Aconitum* species are used in combinatorial form with other medicinal herbs for curing kidney and bone-joint discomforts, dyspepsia, bronchitis and skin diseases. Traditionally, *Aconitum* has been used as

an integral medicine in treating nervousness, oral infection, heartburn, abdominal pain and also consumed to control blood glucose levels and taken as tonic by malaria patients to increase body vigour (Dar & Naqshi, 2001). Earlier it has been reported that decoction of *Aconitum* species was used to treat urinary tract infections (Lone *et al.*, 2012) and abdominal disorders (Khan *et al.*, 2004). *Aconitum* has been used as febrifuge to treat throat infections, dyspepsia and diabetes (Tantray *et al.*, 2009). Since *Aconitum* has been used in different systems of medicine for curing various diseases; as such the increasing demand of *Aconitum* species has led to overharvesting of its tubers resulting in rapid depletion of its natural stocks. In the last few decades, the genus *Aconitum* has been exploited and the consequence is that many of its species have been listed as endangered in the Red Data Book (Dar & Naqshi, 2001). The findings regarding the distributional data of *Aconitum* species from North Western (NW) Himalaya is well recorded. *Aconitum violaceum* is considered as vulnerable, whereas *Aconitum heterophyllum* as critically endangered (Rana & Samant, 2010). As per IUCN Red List Categories and Criteria (2010), *Aconitum heterophyllum* is reported as endangered however, Kaul had assigned it as vulnerable in Kashmir Himalaya (Kaul, 1997). Over the years, the species density of *Aconitum* has substantially decreased while its commercial demand has increased. Additionally, adequate measures have not been taken yet to multiply and domesticate its species. In the present study, we attempted to characterize the conservation status of three *Aconitum* species in Kashmir Himalayan region. The occurrence of *Aconitum* species was carried out by monitoring the field data at different altitudinal zones. Also taken into consideration the vast number of ethanopharmacological properties, the present study was attempted to carry out antioxidant, antimicrobial and anti-inflammatory properties of these *Aconitum* species.

MATERIAL AND METHODS

Search Strategy

Identification of Localities

The findings concerning the studies associated with distribution and ethnopharmacological evidence of three *Aconitum* species was carried out by searching different electronic databases, viz. Scifinder, Science Direct, Research Gate, Google Scholar, BioOne, and PLOS. The published literature was reviewed to gather the information about the biodiversity of *Aconitum* species of Kashmir Himalaya. The information on occurrence data of *Aconitum* was collected from two herbariums [University of Kashmir & CSIR-IIIM, J&K, India], which made it easy to approach to the regions of interest. The locations were visited with special emphasis from vegetative phase to the harvesting period of the desired plants. To avoid erroneous identification, focused group consultations with many respondents having sound knowledge of *Aconitum*, discussions with occasional practitioners for verifying the plant samples were executed. The data collected was also re-examined from natives of different regions by providing different vernacular names of plant specimens. Specimen photographs were shown to the natives to notify the ethnopharmacological claims of *Aconitum* species.

Herbarium Data Retrospection

The herbarium, viz. *KASH Herbarium*, University of Kashmir and *Janaki Ammal Herbarium*, CSIR-IIIM, J&K-India, were approached to collect the information of distribution patterns of all the available specimens deposited at respective places.

Collection and Sampling Method

The sampling of the plants was done by 'random approach' involving the collection of three *Aconitum* species found in the study area and 'ethnobotany-directed' sampling approach, based on traditional medicinal uses of *Aconitum* species.

Study Site Description

In the present study, the area comprises the accessible alpine and sub-alpine regions of the Kashmir Himalaya. Different locations were selected (Figure 1) and surveyed for the collection of different species of *Aconitum* according to the documented herbarium reports.

Distribution Pattern of *Aconitum*

The taxonomic relevance and distribution pattern of three *Aconitum* species was taken into account by surveying the selected belts frequently and contemplating the relevant literature and herbarium sheets at *KASH Herbarium* [Centre for Biodiversity and Taxonomy], University of Kashmir, Srinagar, J&K. The occurrence of different species of *Aconitum* was recorded by various aspects, viz. population density within 1 km^2 area, herbivory score, vulnerability score (0-3) and area of occupancy. The vulnerability assessment for *Aconitum* species in the region was followed by a standardized procedure (Wild & Mutebi, 1996; Uniyal *et al.*, 2011; Lone *et al.*, 2018).

Ethnobotanical Findings

The data acquisition based on ethnobotanical knowledge was compiled for documentation in the vegetative seasonal period of the three *Aconitum* species by following a standard prototype. To record the complete information of *Aconitum*, questionnaire was followed comprising the information about plant local names, vegetation season, medicinal uses, mode of consumption, dosages and diseases cured (Pardo-de-Santayana *et al.*, 2007). The information about the traditional medicinal knowledge of *Aconitum* species particularly from the tribal community of Gujjar & Bakkarwals was reported in order to make the findings more reliable. During the study period, the ethnomedicinally important sites, viz. Yarikhah, Gulmarg, Kangdoori, Apharwat, Khilanmarg, Hapatkhod, Dodpathri, Aharbal, Kausarnag, Dachigam, Naranag, Gurez, Kokernag, Ahlan, Verinag, Pahalgam, Aru, Sonmarg, Thajwas, Drass and Panikhar of different altitudinal ranges were chosen (Figure 1). The selected ranges were inspected routinely during harvesting

period of *Aconitum* species with special emphasis to collect different accessions. To avoid erroneous identification, focus group conversation with key respondents and herbal specialists (Hakeems) having good traditional knowledge of medicinal plants (Pardo-de-Santayana *et al.*, 2007) was executed to verify the plant samples and their ethnopharmacological claims.

Preliminary Phytochemical Screening

Preparation of Extracts

The plant specimens were collected, shade dried at room temperature and ground into fine powder using a mechanical blender. Dried powder was then packed in a soxhlet apparatus, extracted with different solvents for 6–8 hours and evaporated under reduced pressure at 40°C-50°C. The extracts were desiccated and stored in amber glass vials at 4°C for future experiments.

Total Phenolic Content (TPC) Analysis

An *in vitro* spectrophotometric assay was employed for phenolic determination (Blainski *et al.*, 2013) with few modifications. Folin–ciocalteu reagent was used to quantify total phenolic content of different extracts of three *Aconitum* species. 20 µL of each extract from the stock solution (10 mg/mL methanol) was dissolved to 180 µL with HPLC methanol. 200 µL of Folin–ciocalteu reagent was added to each sample, mixed thoroughly and incubated at room temperature for 5 min, followed by the addition of 400 µL of 20% (w/v) sodium carbonate. The mixture was then incubated for 20 min at room temperature in dark and spectral analysis for absorbance was detected at 760 nm. A calibration curve obtained from fivefold concentration of gallic acid was used to quantify the phenolic content of different samples (r^2=0.999). Total phenolic concentration was depicted as mg of gallic acid equivalent/g calculated from the calibration curve.

Total Flavonoid Content

For flavonoid determination, an *in vitro* spectrophotometric assay using aluminium chloride was used (Chang *et al.*, 2002) with few modifications. 30 µL of each extract from stock solution (10 mg/mL) was made up to the volume of 500 µL with distilled water. 30 µL of 5% $NaNO_2$ was added followed by the subsequent addition of 300 µL of 10% $AlCl_3$ solution. The sample mixture was incubated for 10 min at room temperature. With the subsequent addition of 200 µL of 1M NaOH solution, make up volume of the mixture was adjusted with distilled water. The mixture was allowed to stand for 10 min and spectral analysis for absorbance was detected at 510 nm. A calibration curve obtained from fivefold concentration of quercetin was used to quantify the flavonoid content of different samples (r^2=0.999). Total flavonoid concentration was depicted as mg of quercetin equivalent/g calculated from the calibration curve.

Antioxidant Potential by DPPH Assay

The DPPH antioxidant capacity of each extract was determined according to the modified method of Brand Williams (Miliauskas *et al.*, 2004). DPPH radicals have an optimal spectral absorbance at 517 nm, which diminishes by the addition of an antioxidant component. A methanolic solution (1 mg/25 mL) of the radical DPPH was freshly prepared and absorbance was recorded to

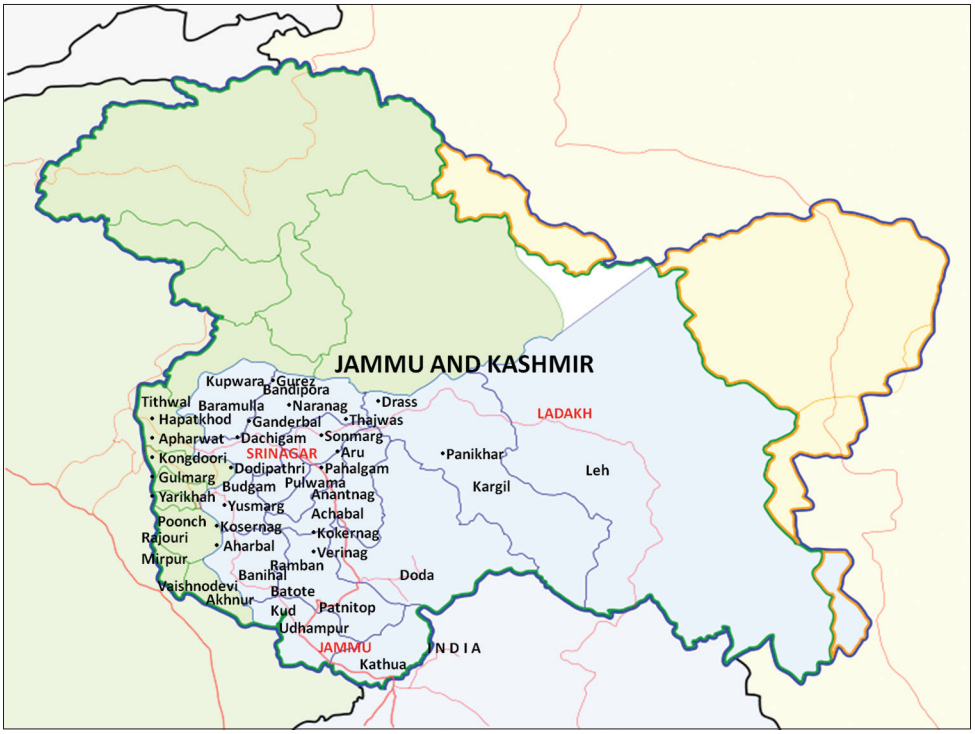

Figure 1: Distribution map of *Aconitum* species collected from different high altitudinal regions of Kashmir Himalaya

check the stability of the radical DPPH. Ascorbic acid used as standard was prepared in fivefold concentration to obtain the linear equation. Different concentrations of ascorbic acid were made up to the final volume of 1 mL with DPPH. The samples were incubated at room temperature in dark for 30 min; spectral analysis for absorbance was done in triplicates and recorded at 517 nm. A linear curve of concentration (ascorbic acid) was plotted against absorbance (r^2 = 0.994). Different concentrations of ascorbic acid were assayed in order to check the linearity response and to establish the antioxidant values in the adequate linear range. Same procedure was followed with each plant extract with the varying concentrations and the percentage of scavenging capacity of each extract was calculated from the formula:

$$\% \text{ inhibtion} = \frac{(AB - AE)}{AB} \times 100$$

Where, AB = absorbance of the blank sample, and AE = absorbance of the plant extract

The IC_{50} value for antioxidant activity was calculated by plotting the linear graph of percentage of inhibition against the concentration of each extract.

Antimicrobial Potential

The different extracts of three *Aconitum* species were screened against six bacteria *Klebsiella pneumoniae* (ATCC75388), *Staphylococcus aureus* (MTCC96), *Escherichia coli* (MTCC730), *Bacillus subtilis* (MTCC121), *Streptococcus pyogenes* (MTCC442), *Bacillus cereus* (IIIM25) and two fungi *Saccharomyces cerevisiae* (MRCJ-92) and *Candida albicans* (ATCC90028). The indicator microorganisms were maintained by periodic subculture on Tryptic Soy Agar (TSA) and Potato Dextrose Agar (PDA) and preserved at 4°C prior to use. The bacterial and fungal microorganisms were activated on TSA and PDA by incubating at 37°C for 18 h and 48 h, respectively. Inocula were prepared at 1.5×10^8 CFU/mL following 0.5 scale of McFarland turbidity standard. Suspensions were diluted at 1.5×10^6 CFU/mL and 2.5×10^4 CFU/mL for bacteria and fungi, respectively before use. The extracts were initially screened in the micro-well plates at higher concentration of 1000 µg/mL. Plates were then incubated at 37°C in shaker at 100 rpm for 18 h and 48 h for bacteria and fungi, respectively. 40 µL of an indicator dye, P-iodonitrotetrazolium chloride (INT), was added to each well containing the test microorganisms (bacteria) and incubated at room temperature for 30 min. The wells containing the test microorganisms and showing inhibitory effect did not affect the INT dye. While the wells which turned pink after treating with INT dye showed no inhibitory effect of extracts against the bacteria. The wells in which precipitation of cells was formed showed no inhibitory effect of extracts against the fungi.

In vitro Determination of Minimum Inhibitory Concentration (MIC)

The minimal inhibitory concentrations (MIC) of the plant extracts was determined in 96 micro-well sterile plates using microdilution method (Kuete *et al.*, 2008). To 150 µL of broth, 50 µL of each extract was added and followed by serial dilution with subsequent addition of 100 µL of test microorganism. Ciprofloxacin and clotrimoxazole were used as standard antibiotics against bacteria and fungi at concentrations ranging from 0.015 to 2 µg/mL and 0.003 to 0.5 µg/mL, respectively. The plates were further incubated at same conditions as previously mentioned. Inhibitory concentration of extracts and standard antibiotics was detected after addition of 40 µL of 0.2 mg/mL of INT dye for bacteria. For fungi, the bottom of the wells was observed for the presence of precipitation of the cells. MIC of extract was considered as the lowest concentration at which there was no visible colour change or no precipitation of cells was observed.

NLRP3 Inflammasome Activation Assay

The anti-inflammatory effect of different extract was determined by using NLRP3 inflammasome activation assay (Abdullaha *et al.*, 2019). The inhibition of NLRP3 Inflammasome was carried out in mouse macrophages (J774A.1 cells), which were seeded at a density of 0.4×10^6 cells/mL per well in 24 well plates overnight. The cells were primed with 1 µg/mL lipopolysaccharide (LPS) for 5 h and 30 min which were later treated with different extracts (10 µg) for 1 h in incomplete media. Finally, cells were treated with ATP (3 mM) as a second signal molecule for 30 min. The experiment was terminated by collecting supernatant for ELISA. Cell pellet was lysed with lysis buffer (0.2 N NaOH + 1% Triton X) for protein estimation. Level of cytokine, IL-1β, which is readout for NLRP3 inflammasome activation in cellular supernatant was checked by following mIL-1β ELISA kit (Invitrogen) protocol.

Statistical Analysis

Experiments were performed in triplicates and expressed as mean ± SD. Statistical analysis was performed using ANOVA and $P < 0.05$ was considered to be significant. Correlations among data obtained were calculated using the MS Excel software.

RESULTS AND DISCUSSION

Identification and Authentication

The plant specimens were deposited in the *Janaki Ammal Herbarium* [CSIR-IIIM, Jammu] and authenticated by comparing with the available specimens of *Aconitum* by a taxonomist. Proper voucher specimens with their respective accession numbers (*23006, 23007 and 23008*) were deposited for future reference and record. Some specimens of this genus were also deposited and identified in *KASH Herbarium*, University of Kashmir with their accession numbers (*43273, 43274* and *43275*) (Figure 2). Data was collected from different regions of major sampling sites. The survey was extensively conducted during the vegetative/growing period of *Aconitum* species (flowering to the seed development) based on reported retrospections from herbarium repositories, documented floras, research papers and web interventions.

Figure 2: *Aconitum* species collected from different high altitudinal ecozones of Kashmir Himalaya (a) *Aconitum heterophyllum;* (b) *A. chasmanthum;* (c) *A. violaceum;* (d-f):-Herbarium specimens of (d) *A.heterophyllum;* (e) *A.chasmanthum;* (f) *A. violaceum.*

Traditional Use

For the last few decades, the arena of herbal medicine has remarkably increased and therefore demands a proper quality control for various medicinal plants used in traditional system of medicine (Nasreen *et al.*, 2010). Traditionally, plants utilized for treating various diseases by folklores have been an ancient practice with traditional insights being transferred to the progenies (Vitalini *et al.*, 2013). Ethnobotanical surveys based on the traditional awareness of medicinal plants have played a vital role on the platforms of various systems of medicine (Heinrich *et al.*, 2009). Such surveys have boosted interest among traditional herbal specialists and research workers for promoting important herbal medicinal plants (Heinrich & Gibbons, 2001; Leonti, 2011; Pendry *et al.*, 2005; Watkins *et al.*, 2012).

Genus *Aconitum*, a rich source of diterpene alkaloids and flavanoids is reputed to possess significant pharmaceutical values (Jabeen *et al.*, 2006). The studies on traditional system of medicine have shown that the genus *Aconitum* has been used in treating various diseases and is considered as a valuable nervine tonic (Dar & Naqshi, 2001). It has been used as depressant in high arterial tension of cardiac origin (Chopra & Chopra, 1994). In the present study, ethnopharmacological survey from Kashmir Himalaya was conducted in order to collect traditional knowledge of three *Aconitum* species with a hope to explore some herbal recipes practised to treat various diseases. During the frequent interviews and questionnaire, it was found that different plant parts are consumed in numerous ways as per folklore practices. Young leaves of *Aconitum* are either freshly crushed or eaten raw to cure cold, fever, diarrhoea and vomiting. A mixture of rhizome powder and mustard oil is massaged on the forehead and chest to treat headache and cough. Flower decoction is given to infants to get rid of intestinal worms. The plant juice is consumed to treat urinary infection, respiratory disorders, chest infections and asthma. The rhizome powder is taken to ease joint pain. Roots are boiled in water and consumed to treat abdominal ailments, dysentery, dyspepsia, gastric disorders, intestinal inflammation, malarial fever, piles and throat infection. A cream base paste of crushed tubers and oil is applied to treat skin problems. The leaves of *A. heterophyllum* are consumed either as raw or in the paste form to treat the common health issues like cough, cold and fever, mostly in children. The rhizome parts of *Aconitum heterophyllum* are stored in the powder form and used either alone or in mixture with other medicinal plants for treating kidney and bone-joint discomforts, diarrhea, dyspepsia, bronchitis, skin diseases and taken as tonic to increase body stamina. By exploring some more information of the local usage of *Aconitum* species, it was observed to be the main ingredient of various decoctions and tonics, used by tribal communities of Jammu and Kashmir. The consultation in the wider context from the local Hakeem's of the valley confirmed its ethnomedicinal conventions for treatment of bone related disorders like rheumatism. During mid summer, the aerial parts of non-poisonous species of *Aconitum* like *Aconitum heterophyllum* are collected from high altitudes and used as fodder to feed cattle. However, it was found that certain locals from Gujjar and Bakkarwal communities of the areas like Aharbal, Gulmarg, Pahalgam and Gurez regions were quite reluctant to share its traditional usage because all the *Aconitum* species in the region have been banned for collecting from the wild sources by the State Forest Department.

Population Size and Distribution

Kashmir Himalaya is an arc-shaped continuous chain of mountains situated on the Northern boundary of the country, India, between 33°.20' and 34°.54' N latitudes and from 73°.55' and 75°.35' E longitudes (Jabeen *et al.*, 2013). Various research findings from past few decades regarding the occurrence of the genus *Aconitum* from Kashmir Himalayan range has been carried out by various workers with the recent update in the current study (Figure 3). In the present study, the sites of interest were selected by approaching the Herbarium section of Kashmir University, J&K, where hundreds of *Aconitum* specimens have been deposited. The locations of the available specimens were noted and accordingly the places of interest were visited for exploring the distributional pattern of genus *Aconitum* in the region. Various species of *Aconitum* have been reported from time to time from different ecogeographical ranges of Kashmir Himalaya. During our thorough surveys and investigations, we are reporting presence of three species from new sampling sites which include Yarikhah, Gulmarg, Kangdoori, Apharwat, Khilanmarg, Hapatkhod, Dodpathri, Aharbal, Kausarnag, Dachigam, Naranag, Gurez, Kokernag, Verinag, Ahlan, Pahalgam, Aru, Sonmarg, Thajwas, Drass and Panikhar (Table 1). The distribution pattern of different collected species was plotted by studying various aspects such as populations density, herbivory score, vulnerability score (0-3) and area of occupancy. In account of the ecological field observations, *Aconitum* was found to grow better in the regions of shady slopes. The species performance in account of the populations found in Gulmarg, Hapatkhod, Gurez and Naranag was found naturally adapted with good population size, while some species were found very scant from the regions like Kangdoori, Apharwat and Sonmarg. In the regions with high disturbance, these three species were found extensively declining (Aharbal, Ahlan, Pahalgam, Aru and Thajwas), while in some other regions like Yarikhah, Dodpathri, Kausarnag, Dachigam, Kokernag, Verinag, Dras & Panikhar, the species of *Aconitum* are critically declining (Table 1). Some species of *Aconitum* which have been examined in the previous studies are entirely vanished from the regions like Jawahar Tunnel, Upper Munda, Lower Munda, Chorwan, Achoor, Khupri, Dahinala, Pir Panjal, Nilnag and Doodganga.

Phytochemical Profiling

Total Phenolic and Flavonoid Content (TPC & TFC)

To prevent the onset of many human ailments including cancer, diabetes and many other metabolic disorders, plant derived phytochemicals have played a significant role. Alone these phytochemicals or in combination with other drug elites, are believed to be bestowed with beneficial effects in treating various metabolic abnormalities, either by inhibiting lipid oxidation, or by exhibiting anti-inflammatory and antimicrobial activities. Antioxidant effect primarily is due to presence of polyphenols which are capable of neutralizing free radicals due to their redox reaction properties (Sen *et al.*, 2010). Polyphenolic compounds have greatly contributed to antioxidant properties but are also potent anti-microbials (Soobrattee *et al.*, 2005; Saleem *et al.*, 2010). The antimicrobial activity of plant phenolics has been extensively studied against human pathogens (Puupponen-Pimiä *et al.*, 2005). Polyphenols have good effects on degenerative diseases and also helps to inhibit the pro-inflammatory responses (Scalbert *et al.*, 2005). A plethora of flavonoids have been reported to contribute for different anti-inflammatory effects (Iacobellis & Barbaro, 2008). For discovery of novel anti-inflammatory drugs, phenolic acid derivatives have played an important role (Silva *et al.*, 2014). These derivatives have been better linked with health management envisaged by both antioxidant and anti-inflammatory effects (Liang & Kitts, 2016). For instance, ferulic acid has been widely diversified for anti-inflammatory potentials for inhibiting TNF-α and macrophage inflammatory protein-2 production (Sakai *et al.*, 1997; Yang *et al.*, 2013). Keeping in view the importance of phytochemicals of plant sources and their wide use by the local community and practitioners for treating variety of ailments, the present study was attempted to quantify the

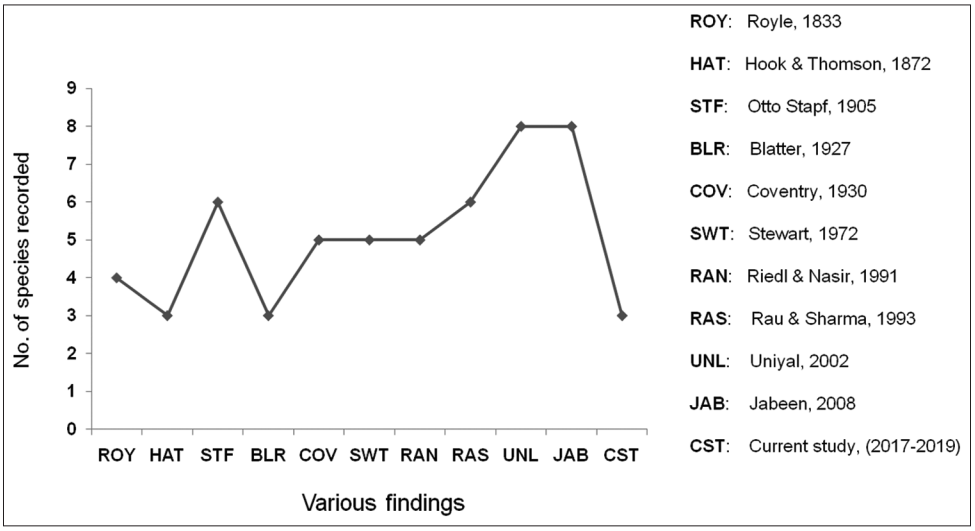

Figure 3: Various findings for occurrence of different species of *Aconitum* collected from different ecozones of Kashmir Himalaya with recent update

Table 1: Location, occurrence, co-ordinates, vulnerability-herbivory score of *Aconitum* species of Kashmir Himalaya; Parameters subjected as "0" = not vulnerable; 3"= highly vulnerable; A00=Area of Occupancy/km^2

Site	Locality or region	Code	Plant species	Climate zone	Latitude	Longitude	Altitude (m)	No of populations (1km^2)	Vulnerability score	Herbivory score	A00 Km^2
Yarikhah	Tangmarg Baramulla	YK	A.H	Sub alpine	33°54′ 40"	73°39′ 21"	2035	2-4	3	3	4
Gulmarg	Gulmarg Baramulla	GM	A.C	Alpine	33°02′ 56"	74°22′ 46"	2722	20-25	0	0	5
Kangdoori	Gulmarg Baramulla	KD	A.C	Alpine	34°01′ 34"	74°22′ 46"	3058	10-15	1	1	3
Apharwat	Gulmarg Baramulla	AW	A.V	Alpine	34°02′ 23"	74°22′ 09"	3644	12-15	1	1	12
Khilanmarg	Gulmarg Baramulla	KM	A.C	Alpine	34°02′ 51"	74°19′ 23"	2678	3-5	3	3	1-2
Hapatkhod	Yusmarg Budgam	YM	A.C	Sub alpine	34°02′ 56"	74°39′52"	2394	12-15	1	1	4
Dodpathri	Dodipathri Budgam	DP	A.H	Alpine	33°51′ 03"	74°33′47"	2787	2-6	3	3	4
Aharbal	Aharbal Kulgam	AB	A, H	Sub-alpine	33°38′ 43"	74°46′48"	2262	8-10	2	2	5
Kosernag	Kosernag Kulgam	KS	A.H	Alpine	33°30′ 02"	74°50′03"	3681	4-6	3	3	4
Dachigam	Harwan Srinagar	DG	A.H	Sub alpine	34°08′ 14"	75°02′15"	2913	4-8	3	3	5
Naranag	Naranag Ganderbal	NG	A.H	Sub alpine	34°21′ 16"	74°58′43"	2260	14-18	0	0	6
Gurez	Gurez Bandipora	GZ	A.C	Sub alpine	34°39′ 27"	74°44′06"	2656	15-20	0	0	7
Kokernag	Kokernag Anantnag	KN	A.H	Sub alpine	33°35′ 05"	78°18′14"	1939	3-6	3	3	4
Verinag	Verinag Anantnag	VN	A.H	Alpine	33°32′ 17"	75°16′19"	1878	4-6	3	3	3
Ahlan	Ahlan Anantnag	AL	A.H	Sub alpine	33°29′ 52"	75°23′00"	2387	8-10	2	2	2
Pahalgam	Pahalgam Anantnag	PG	A.H	Alpine	34°00′ 58"	75°18′41"	2178	7-9	2	2	6
Aru	Pahalgam Anantnag	AR	A.H	Alpine	34°05′ 44"	75°15′4"6	2512	6-8	2	2	4
Sonmarg	Sonmarg Ganderbal	SM	A.H	Alpine	34°18′ 10"	75°17′38"	2664	5-6	2	2	5
Thajwas	Thajwas Ganderbal	TJ	A.H	Alpine	34°16′ 37"	75°17′04"	2989	7-8	2	2	3
Drass	Drass Kargil	DR	A.V	Alpine	34°25′ 45"	75°44′53"	3085	2-3	3	3	3
Panikhar	Panikhar Kargil	PK	A.V	Sub alpine	34°07′ 16"	7557′ 21"	3246	2-3	3	3	3

Plant Species: A.H=*Aconitum heterophyllum;* A.C=*Aconitum chasmanthum* and A.V=*Aconitum violaceum*

phytochemicals (phenolics and flavonoids) from different extracts of three *Aconitum* species.

The results of phytochemical quantification were retrieved with a wide variation of phenolic and flavonoid concentrations in different extracts of *Aconitum* species analyzed. The total phenolic concentration varied from 0.185 to 2.188 mg GAE/g of sample. Methanolic extracts of A. *heterophyllum* and A. *chasmanthum* showed a high phenolic content of 1.193 & 2.188 mg GAE/g, respectively. The total flavonoid concentration ranged from lowest in ethyl acetate extract of A. *chasmanthum* (0.144 mg QE/g) to the highest in methanolic extract of A. *chasmanthum* (2.196 mg QE/g). Determination of total phenolic and total flavonoid content was aided by using gallic acid and quercetin as standards. Calibration curve equations obtained were $y = 0.625 + 0.078$ ($r^2 = 0.999$) and $y = 0.017x + 0.044$ ($r^2 = 0.999$), respectively (Table 2). The results obtained during the study provided a scientific baseline in knowing the quantities of these phytochemicals (phenolics and flavonoids) in different extracts of three *Aconitum* species.

Antioxidant Capacity

Most of the plant species and herbs of medicinal importance have significantly contributed to the excellent antioxidant potentials globally (Krishnaiah *et al.*, 2011). The secondary metabolites such as phenolics and flavonoids play an important role as antioxidants in controlling different human diseases. These are a kind of natural products and antioxidant substances that protect human, animal and plant cells against damaging effects of free radicals (Kukić *et al.*, 2006). Nowadays most of the life threatening diseases including neurodegenerative and cardiovascular disease, oxidative stress have been considered as a main resourceful factor. Researchers have shown that the antioxidants of plant origin with free-radical scavenging properties could have enormous importance as therapeutic agents in diseases caused due to oxidative stress (Ramchoun *et al.*, 2009). In addition, the introduction of antioxidants have been proved to be an encouraging method to encounter the effects of oxidative stress (Kasote *et al.*, 2013).

In the present study, DPPH antioxidant assay was employed to evaluate the antioxidant potential of crude extracts of three *Aconitum* species. Determination of antioxidant efficiency was aided by use of ascorbic acid as standard, where the calibration curve equation obtained was $y = -112.6x + 0.942$ ($r^2 = 0.994$). DPPH radical scavenging activities of plant extracts varied from 25.09% to 80.115%, which approximately produced fourfold variation. Methanolic extract of A. *chasmanthum* represented significant antioxidant capacity (80.115%) in DPPH assay,

Table 2: Measurement of total phenolic and total flavonoid content and antioxidant activity in different extracts of three *Aconitum* species

Plant name	Acetone Extract				Ethyl Acetate Extract				Methanolic Extract			
	TPC (mg GAE/g dw	TFC (mg QE/g dw)	Antioxidant inhibition %	IC_{50} Values µg/mL	TPC (mg GAE/g dw	TFC (mg QE/g dw)	Antioxidant inhibition %	IC_{50} Values µg/mL	TPC (mg GAE/g dw	TFC (mg QE/g dw)	Antioxidant inhibition %	IC_{50} Values µg/mL
A. heterophyllum	0.450 ± 0.002	0.43 ± 0.13	25.090 ± 1.152	0.005 ± 0.000	0.185 ± 0.006	0.301 ± 0.193	35.850 ± 0.341	0.004928 ± 0.000	1.193 ± 0.035	0.425 ± 0.07	61.560 ± 2.999	0.004639 ± 0.000
A. violaceum	0.417 ± 0.053	0.275 ± 0.015	34.912 ± 1.218	0.026 ± 0.036	0.294 ± 0.004	0.203 ± 0.057	43.149 ± 0.320	0.004837 ± 0.000	0.836 ± 0.008	0.810 ± 0.09	60.845 ± 0.407	0.004656 ± 0.000
A. chasmanthum	0.520 ± 0.088	0.477 ± 0.15	36.639 ± 0.470	0.005 ± 0.000	0.300 ± 0.010	0.144 ± 0.111	55.054 ± 0.866	0.004734 ± 0.000	2.188 ± 0.028	2.196 ± 0.1	80.115 ± 1.333	0.004503 ± 0.000

followed by methanolic extract of A. *heterophyllu*m (61.560%). The acetone extract of A. *heterophyllum* showed the lowest antioxidant potential of 25.09% inhibition. The scavenging capacity of the DPPH radical by ethyl extract of A. *heterophyllum* was found to be higher [IC_{50} value of 49.28 ± 0.00 µg/mL].

Correlation between TPC, TFC and Antioxidant Potential

Regression analysis was performed by correlating the results obtained with different methods. Significant correlations were found between various quantification methods with respect to the antioxidant potential, expressed as linear calibration curve. Significant correlation of DPPH assay with respect to the total phenolic content was obtained ($r^2 = 0.976$). Likewise, a good correlation with respect to the total flavonoid content was also obtained ($r^2 = 0.914$). The variation in the chemical profiles of different extracts has accounted for significant antioxidant activities shown in Table 2.

Antimicrobial Potential

The tremendous throughputs of herbal drugs for treating the broad spectrum human ailments either directly or in the form of crude extracts or natural products or their semi-synthetic derivatives has indeed contributed to pharmacopeia extensively (Houghton, 2000). But somehow bioassay guided fractionation and isolation of active metabolites sometimes loses the synergetic effects of metabolites against various microbes. This demands standardization of plant extracts as an approach in treating various microbial infections (Njimoh *et al.*, 2015). Plant extracts have been widely explored as key element in traditional folk medicine to treat microbial infections and also constitute sources of conventional antimicrobials (Nawab *et al.*, 2011). The preliminary phytochemical screening of plant extracts have greatly contributed to the antimicrobial potentials (Tariq *et al.*, 2011; Malik *et al.*, 2011). Owing to the ability of phenolics and flavonoids to interact with cellular enzymes, destabilize cellular membranes and inhibit cell cycle progression, these phytochemicals often exhibit antimicrobial activity (Sen & Batra, 2012; Pisoschi *et al.*, 2018).

In the present study, antimicrobial activity of different extracts of three *Aconitum* species used in folk medicine were screened *in vitro* against eight microbial strains and their activities were determined by MIC values, depicted in Table 3. According to the results obtained, extracts of the plant species showed antimicrobial activities against one or more bacterial strains and the fungal species tested. The antimicrobial activities of plant extracts were correlated with standard antibiotics such as ciprofloxacin and clotrimoxazole, which were used as positive controls. It has been reported that activities of plant extracts are significant when MIC ≤ 0.1 mg/mL, moderate when 0.1 < MIC ≤ 0.625 mg/mL, weak when MIC > 0.625 mg/mL, according to the scale of sensitivity of plant extracts (Kuete *et al.*, 2008). The sensitivity of each individual microorganism depends on extract type and the solvent used for extraction. Each individual extract showed a moderate inhibition potential on at least one indicator microorganism. The methanol and dichloromethane extract of A. *chasmanthum* exhibited moderate antimicrobial activity than the other extracts with MIC value 0.125 mg/mL against *Candida albicans* and *Streptococcus pyogenes*, respectively. Methanolic and aqueous extract of A. *heterophyllum* and A. *violaceum* also showed similar activity against *Bacillus subtilis* and *Candida albicans*, (MIC = 0.125 mg/mL), respectively. Only *Bacillus cereus* was not inhibited by any of the extracts of A. *violaceum* while all the extracts from the three plant species showed moderate anti-fungal effect against C. *albicans*. Most of the extracts inhibited the growth at less than 1 mg/mL. However, none of the extracts inhibited the growth of *E.coli* and *Bacillus cereus* possibly due to the complexities in the cell wall (Li & Nikaido, 2009). The presence of cell wall in some microorganisms tends to block the passage of inhibitors and is generally more difficult to inhibit (Nikaido, 2003). In the present study, the methanolic extract of A. *heterophyllum* and A. *chasmanthum* have shown activity against the gram positive bacteria *Bacillus subtilis* and *Streptococcus pyogenes*, respectively. Whereas, extracts of A. *violaceum* and A. *chasmanthum* have shown antimicrobial activity against gram negative bacteria, *Klebsiella pneumonia* and *E.coli* at high concentration (1 mg/mL), respectively. None of the extracts of A. *heterophyllum* have shown antimicrobial effect against any gram negative bacteria. The results of the present study are in due agreement, demonstrating the antimicrobial potential of extracts of three *Aconitum* species and therefore, can be suitable candidates for discovering narrow spectrum antibiotics against different microorganisms. The specific indications claimed by the traditional healers are confirmed by the antimicrobial screening of three *Aconitum*

Table 3: Antimicrobial potential of different extracts of three *Aconitum* species (MIC in mg/ml)

Pathogen	Plant Name	Extract								Standard antibiotics	
		Acetone	Chloroform	Hexane	DCM	E. Acetate	Methanol	Ethanol	Aqueous	Ciprofloxacin	Clotrimoxazole
KP	*A.heterophyllum*	-	1.0	1.0	-	-	-	-	1.0	0.007	/
EC		-	-	-	-	-	-	-	-	<0.003	/
BS		-	-	0.250	-	-	0.125	-	0.250	0.062	/
SP		0.250	-	-	-	-	0.5	-	0.250	00.31	/
BC		-	-	-	-	-	-	-	0.250	0.062	/
SA		1.0	1.0	1.0	1.0	-	1.0	-	1.0	0.031	/
SC		0.5	-	0.5	-	-	-	1.0	-	/	0.140
CA		0.250	0.5	0.250	0.5	0.5	0.5	0.5	0.5	/	0.035
KP	*A.violaceum*	-	-	-	-	1.0	-	1.0	1.0	0.007	/
EC		1.0	1.0	-	-	1.0	-	-	1.0	<0.003	/
BS		0.5	1.0	-	1.0	1.0	0.5	1.0	-	0.062	/
SP		-	1.0	-	-	-	-	-	-	00.31	/
BC		-	-	-	-	-	-	-	-	0.062	/
SA		1.0	1.0	-	1.0	1.0	1.0	-	1.0	0.031	/
SC		-	0.5	-	-	-	-	-	-	/	0.140
CA		0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.125	/	0.035
KP	*A. chasmanthum*	-	-	-	-	-	1.0	1.0	-	0.007	/
EC		-	-	-	-	-	1.0	1.0	1.0	<0.003	/
BS		1.0	-	0.250	0.250	0.5	0.5	1.0	0.5	0.062	/
SP		-	-	0.5	0.5	-	0.125	0.5	0.5	00.31	/
BC		-	-	0.250	0.250	-	-	-	0.250	0.062	/
SA		1.0	-	-	1.0	1.0	1.0	1.0	1.0	0.031	/
SC		-	1.0	-	-	1.0	-	-	0.5	/	0.140
CA		0.5	0.5	0.5	0.125	0.5	0.5	0.5	0.5	/	0.035

KP= (*Klebsiella pneumoniae* (ATCC75388); EC= *Escherichia coli* (MTCC730); BS= *Bacillus subtilis* (MTCC121); SP= *Streptococcus pyogenes* (MTCC442); BC= *Bacillus cereus* (IIIM25); SA= Staphylococcus aureus (MTCC96); SC= Saccharomyces cerevisiae (MRCJ-92); CA= Candida albicans (ATCC90028)

species which could also provide the scientific basis in treating the microbial infections.

Anti-inflammatory Potential

The inflammatory process as a defense mechanism in the body is a beneficial immune response to foreign challenges or tissue injury, ultimately leading to the restoration (Lawrence *et al.*, 2002). During infection and disease development, macrophages are an important component and play important roles in human immune defense system (Schluger and Rom, 1998). Macrophages employed for inflammasome activation initiate the inflammatory responses by releasing the pro-inflammatory cytokines IL-1β which provides additional immune cells to the sites of infection (Boscá *et al.*, 2005). The inflammasome inhibition method has become the choice of the *in vitro* anti-inflammatory tests involving acetylcholinesterase, lipoxygenase and cyclooxygenase (Akula & Odhav, 2008; Rathinam & Fitzgerald, 2016; Oguntoye *et al.*, 2018; Abdullaha *et al.*, 2019). Inflammasomes are the most wide studied multimeric complexes involved in various auto-inflammatory and auto-immune diseases such as respiratory illness, atherosclerosis, Alzheimer's disease, diabetes, rheumatoid arthritis and skin diseases (Manzi & Wasko, 2000; Tak & Firestein, 2001; Heneka *et al.*, 2013; Simpson *et al.*, 2014; Fenini *et al.*, 2017). Inflammasomes upregulate the levels of cytokines (IL-1β) through activation of caspase-1 enzyme (Madouri *et al.*, 2015; Liu & Lin, 2017). The potential of plant extracts to serve as safer and effective therapeutic agents has gained attention to act as multi-effective anti-inflammatory leads. Therefore, the present study was aimed to gather information of *Aconitum* species used for treating human ailments mostly rheumatoid arthritis and thereby executing NLRP3 inflammasomes inhibitory properties of *Aconitum* species in the hope to suggest new baseline for the discovery of potential drug candidates.

Given the importance of the NLRP3 inflammasome, we have established a screening assay for antiinflammatory potential of different extracts of *Aconitum* species that target the NLRP3 inflammasome signalling. LPS and ATP treatment of J774A.1 derived macrophages induce the activation of NLRP3 (Abdullaha *et al.*, 2019). After priming J774A.1 macrophage cells with lipopolysaccharides (LPS) and activation with ATP signal molecule and challenged against different extracts of three *Aconitum* species, the inhibition of NLRP inflammasome was found significant in the ethyl acetate extract of *A. violaceum* with the percent inhibition of inflammation as 74.61% followed by the ethyl acetate extract of *A. chasmanthum* (69.55%) at a concentration of 10 μg/ml each. The comparative inhibition of NLRP inflammasome by different extracts of three *Aconitum* species is depicted by an error bar graph (Figure 4)

During our study, the extracts exhibiting the strong potential combating against the inflammation can also prove as strong candidates for the discovery of new active compounds involved in the NLRP3 inflammasome regulation. Plant extracts of *Aconitum* source thus can be regarded to hit multiple targets which may result in better activities than the effect of individual metabolites, thereby reviving the synergism of the plant extracts.

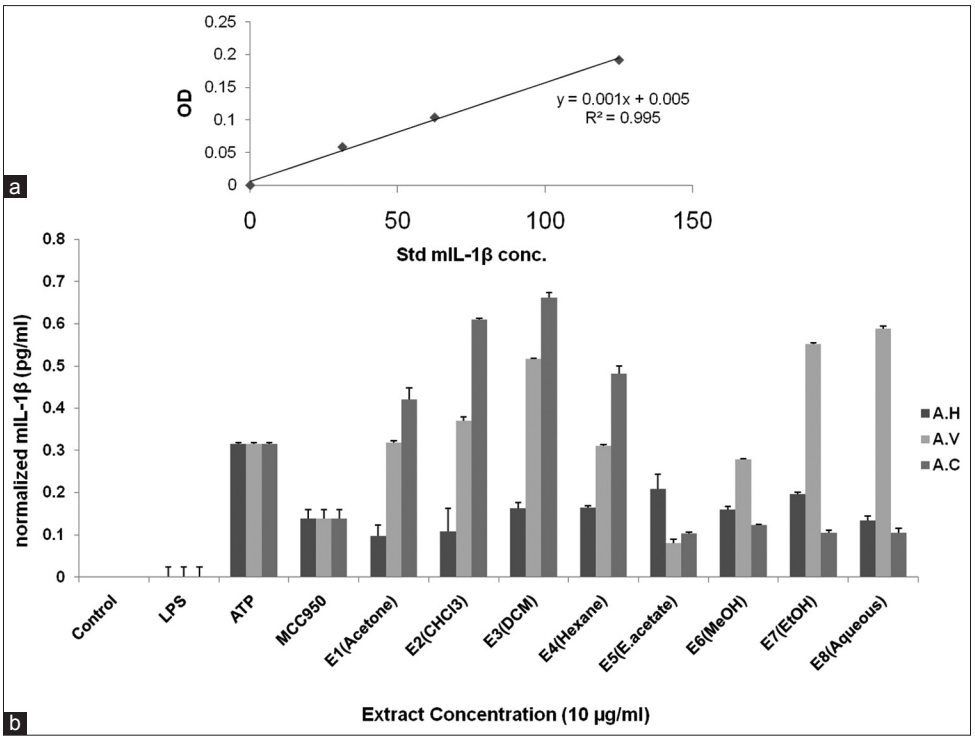

Figure 4: Inhibition of NLRP3 inflammasome: Anti-inflammatory effect of different solvent extracts of *Aconitum* species on IL-1β production and secretion in LPS-primed and ATP-stimulated (J774A.1) macrophage cells. (EI=acetone extract; E2=chloroform extract; E3=dichloromethane extract; E4=hexane extract; E5=ethyl acetate extract E6=methanol extract; E7=ethanol extract and E8=aqueous extract).

CONCLUSION

The present study provided an insight into the dwindling status of genus *Aconitum* from this region. The study revealed that *Aconitum* species are alarmingly declining from Kashmir Himalaya and some species being entirely vanished from the regions like Doodganga, Jawahar Tunnel, Upper Munda, Lower Munda, Chorwan Achoor, Khupri, Dahinala, Pir Panjal and Nilnag. As such the genus demands significant endeavours for its germplasm characterization, agrotechnology development and captive cultivation which are a prerequisite for its conservation. Our experimental investigation indicated that *Aconitum chasmanthum* could be useful to prevent free radical production which causes deadly diseases such as atherosclerosis, cancers and ageing. These species could also be used employed to treat microbial infections caused by *Streptococcus pyogenes, Bacillus subtilis and Candida albicans*. In addition, the results highlighted that *Aconitum violaceum* could be used as powerful anti-inflammatory agent to treat bone joint disorders such as rheumatoid arthritis. In conclusion, the present study could provide a scientific basis for the traditional use of *Aconitum* possibly leading to relatively inexpensive, effective, and safe therapies either as herbal/botanical products or through discovery of bioactive and safe natural products/semi synthetic derivatives.

ACKNOWLEDGEMENT

The work was a part of the Women Scientist-A project funded by Department of Science and Technology (DST), New Delhi, Govt. of India, awarded to SA. AN is thankful to TWAS-CSIR fellowship. DK is indebted to CSIR for SRF and NK is supported by ICMR for SRF. PS, SGG and QPH are thankful for funding from CSIR Phytopharma Mission (HCP0010).

AUTHOR CONTRIBUTIONS

SA has majorly contributed in executing the whole research work and writing the manuscript. AN helped in antimicrobial evaluation. DK and AK helped in inflammasome activation assay. PS, QPH and SGG has analyzed the results and proofread the manuscript and figures/tables.

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