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SEASONAL VARIATION OF BIOACTIVE ALKALOID CONTENT IN *ACONITUM SPP.* FROM MANIPUR, INDIA

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ABSTRACT
Present study was carried out to investigate the seasonal variation in content of bioactive alkaloids including aconitine in different plant parts of two species of *Aconitum*: *A. nagarum* and *A. elwesii* collected from Manipur. The total alkaloid content of these two species was not found to be significantly different. However, thin layer chromatography (TLC) profiling of crude alkaloid extracts from roots and leaves indicated high aconitine content in *A. nagarum*. Further, alkaloid content was determined in terms of aconitine equivalent (AE mg/g) using high-performance liquid chromatography (HPLC). The results indicated that the crude alkaloid content was found to be significantly higher in roots as compared to leaves in both these species. The biosynthesis of alkaloids including aconitine in root was found to be highest in the month of November in both *A. nagarum* (6.50 AE mg/g) and *A. elwesii* (5.54 AE mg/g), respectively. The alkaloid content of leaves was highest in the month of August, (5.44 and 4.84 AE mg/g in *A. nagarum* and *A. elwesii* respectively) which is the pre-flowering season in both these species. However, aconitine content decreases significantly (at 5% level of significance) during October–November in *A. nagarum* leaves. Such change was not observed in *A. elwesii* leaves. Present study revealed the species and plant part based biological variation in distribution of bioactive alkaloid including aconitine in *Aconitum* species of the region.

INTRODUCTION

The genus *Aconitum* comprises approximately 400 species which are widely distributed throughout the northern hemisphere region, such as Asia, Europe and North America (Xiao *et al.,* 2006). About 28 species have been reported in India from sub-alpine and alpine regions of Himalayas between the altitudes of 2000 - 4800 m above mean sea level. Botanical Survey of India reported two species namely, *Aconitum nagarum* Stapf. and *Aconitum elwesii* Stapf. from Manipur, India (Singh *et al.,* 2002). Recently these species have been explored thoroughly for clinical gains (Sinam *et al.,* 2010 data unpublished). About 54 species of *Aconitum* have been chemically investigated (Rana, 2006). *Aconitum* species have tremendous use for the treatment of several human diseases. Bioactive alkaloids, responsible for therapies, are mainly accumulated in the roots, commonly known as aconites or monkshood. The root is ground into a paste and is spread on the skin as a remedy for neuralgia and other painful affections (Chopra *et al.,* 1958). Internally it is used in the treatment of fever, rheumatism, cough, asthma and snake bite (Chopra *et al.,* 1958). Some species are also reported to possess antibacterial and antifungal properties (Bessonova *et al.,* 1990; Kovtunenko *et al.,* 1997). Many *Aconitum* species are found to be insecticide and rodenticide (Smith and Secoy, 1981; Anon, 1979; Deshmukh and Borle, 1976). An aconitine type diterpenoid alkaloid, such as aconitine, which was isolated as the major component from the poisonous plant, *Aconitum napellus*, was probably the first diterpenoid alkaloid to have been studied pharmacologically. Aconitine containing liniments like Aconitysat™, Brinpax™, Etermol™ and Pectovox™ have been used in modern medicine for the treatment of rheumatism, neuralgia and sciatica. (Schemeller,1998). It is used as a cardiac depressant in high arterial tension of cardiac origin. (Chopra *et al.,*1958). Aconitine containing herbal extract were found to possess antitumor activity (Solyanik *et al.,* 2004).

Recently Sinam *et al.* (2010; Data unpublished) reported, alkaloids extracts from *A. nagarum* and *A. elwesii* root and leaf possess antibacterial properties against different bacteria including human pathogens namely *Bordetella bronchiseptica* and *Staphylococcus aureus*, further the compound responsible for antibacterial property was isolated from *A. nagarum* and charecterised as aconitine. Hence the present study was undertaken to ascertain the best harvest period to get the maximum yield of the bioactive molecule out of the two *Aconitum* spp. available in this region.

MATERIALS AND METHODS

Plant material

Leaves and roots of *Aconitum nagarum* Stapf. and *Aconitum elwesii* Stapf. at different time points were collected from Shirui peak situated in eastern part of Ukhrul, District of Manipur, India and its location falls at 94º27´37.8´ EL and 25º06´20´´NL, at altitudinal ranges from 2420 m to 2568 m above sea level. It falls under group 11 (Sub-Group 11 BC_1) of Eastern Himalayan Wet Temperate Forest type (Champion and Seth, 1968). Specimens were authenticated by comparing with the identified specimens deposited at Central National Herbarium, Indian Botanic Garden Kolkata, India. *Aconitum*

nagarum Stapf, Sirohee: 19.9.1948, Mukerjee, 3515. *Aconitum elwesii* Stapf., Sirohee : 19.9.1948, Mukerjee, 3518. Voucher specimens SYM -002201, *Aconitum nagarum*; SYM-002202, *Aconitum elwesii,* were also deposited at the herbarium of Department of Life Sciences, Manipur University, Imphal, Manipur, India.

Extraction of alkaloids

Extraction of alkaloid was carried out by the method described by Ohta *et al.* (1997). Dried leaves and roots were grounded into fine powder using a mixer grinder. The fine powder was homogenized with 1M HCl, in the ratio of 1:10 (w/v) in a mortar and pestle and filtered through double-layered cheesecloth. Filtrate was centrifuged at 12500 g for 20 minutes at ambient temperature (26 ± 2°C) and the supernatant was alkalized to pH 10 with ammonia solution (25%). The resulting suspension was extracted thrice with chloroform. The organic layer was pooled together and washed thoroughly with distilled water to remove ammonia. The organic phase was dried over anhydrous sodium sulfate to remove traces of water molecules, further evaporated with N_2 gas and quantified. Samples of same harvesting time were extracted to find out the yield of crude alkaloid in root and leaves of both the species. Following the same procedure samples collected at different harvest time were extracted.

Alkaloid detection

Mayer's reagent (K_2HgI_4) was prepared by dissolving mercuric chloride (1.36 g) and potassium iodide (5 g) in 100 mL of water. It was used for the detection of alkaloid in the plant extracts (Wangchuk, 2004). Wagner's reagent (Iodine-Potassium Iodide solution) was prepared by dissolving 1.27g of iodine and 2 g of potassium iodide in 5mL of water and diluted to 100 mL with distilled water and used as a specific reagent for aconitine test (Paech and Tracy,1979). Qualitative test for root and leaf of *A. nagarum* and *A. elwesii* were performed by transferring 1 mL of extract in petri dishes. Mayer's reagent and Wagner's reagent were added to these dishes separately and observed for the reaction. The extract was tested for the milky appearance on adding Mayer's reagent due to the formation of alkaloid salt precipitate. Also, the presence of aconitine in the extract was tested by adding Wagner's reagent which leads to brownish coloration.

Thin layer chromatography (TLC)

The crude alkaloids were separated by thin-layer chromatography (TLC) using silica-gel 60 F_{254} TLC plates (Merck, Germany). Aconitine from Sigma, USA was used as a standard. An aliquot of 100 μg of crude alkaloid extract was loaded for each sample. Plates were developed in solvent systems of diethyl ether - ethyl acetate in ratio 20:1 saturated with conc. ammonia) (Ohta *et al.,* 1997). Developed plates were visualized under UV lamp (Camag, Switzerland) at 254 nm.

High-performance liquid chromatography

The crude alkaloid preparation was analysed using HPLC (Jasco, Tokyo, Japan, (PU-980) HPLC pump; UV-975 UV/ Vis detector and Rheodyne injector 7725). ODS Hypersil (250 x 4.6 mm internal diameter, Thermo Hypersil- Keystone, PA, USA) was used as the stationary phase. The mobile-phase consisting of tetrahydrofuran (THF) and 0.2% trifluoroaceitc acid (TFA). HPLC was performed at the flow rate of 1mL min^{-1} and the alkaloid peaks were detected at 235 nm. The HPLC system was optimized with gradient system of increasing gradient of 20% solvent B to 40% solvent B in 20 min followed by decreasing gradient of 40% solvent B to 0% solvent B in 5 min, followed by re-equilibrating the column with 20% solvent B in 5 min.

Statistical analysis

Experiments were replicated thrice and IRRISTAT 4.0 programme, developed by the Biometrics unit, IRRI, Philippines was used for statistical analysis. Analysis of variance (ANOVA) was done for the experiments laid out in Completely Randomized Design (CRD). The treatment means were compared by using the Least Significant Difference (LSD) test. Statistical significance was set at the 5% level of significance.

RESULTS AND DISCUSSION

Alkaloid detection, quantification, seasonal and spatial distribution

Alkaloid extracts from roots and leaves treated separately with Mayer's reagent, turn milky due to the formation of alkaloid salt precipitate. This test is extensively used to detect the presence of alkaloid in the extract. Further, the addition of Wagner's reagent turns the solution brownish indicating the presence of aconitine in the extract. While these tests were performed, all tested samples turned milky indicating presence of alkaloid in the extract. We got brown ppt. in *A. nagarum* root, leaf and *A. elwesii* root extract. However, *A. elwesii* leaf extract did not show brownish colouration indicating absence of aconitine in this qualitative test.

TLC chromatogram of *A. nagarum* root and leaf showed spots with same R_f value with that of standard aconitine (0.39) indicating aconitine as one of the major compound (Fig.1), earlier work by Ohta *et al.* (1997) also showed R_f of aconitine to be 0.31.

The gradient elution of mobile phase, 80 - 60% (A) in 0 - 20 min; 60 - 100% (A) in further 5 min; 100 - 80% (A) in further 5 min. resulted in resolved peaks of alkaloids. Aconitine peak was observed at retention time of 10 min (Fig.2 a; 2b; 2c; 2d).

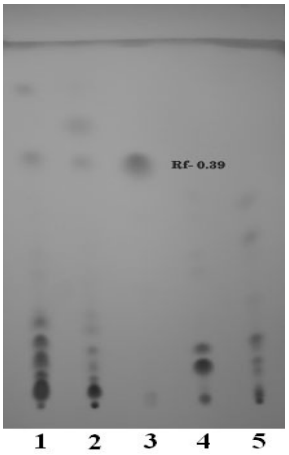

Figure 1: TLC profile of aconitum; 1- *A. nagarum root* , 2- *A. nagarum* leaf, 3- Aconitine ,4- *A. elwesii root,* 5- *A. elwesii* leaf

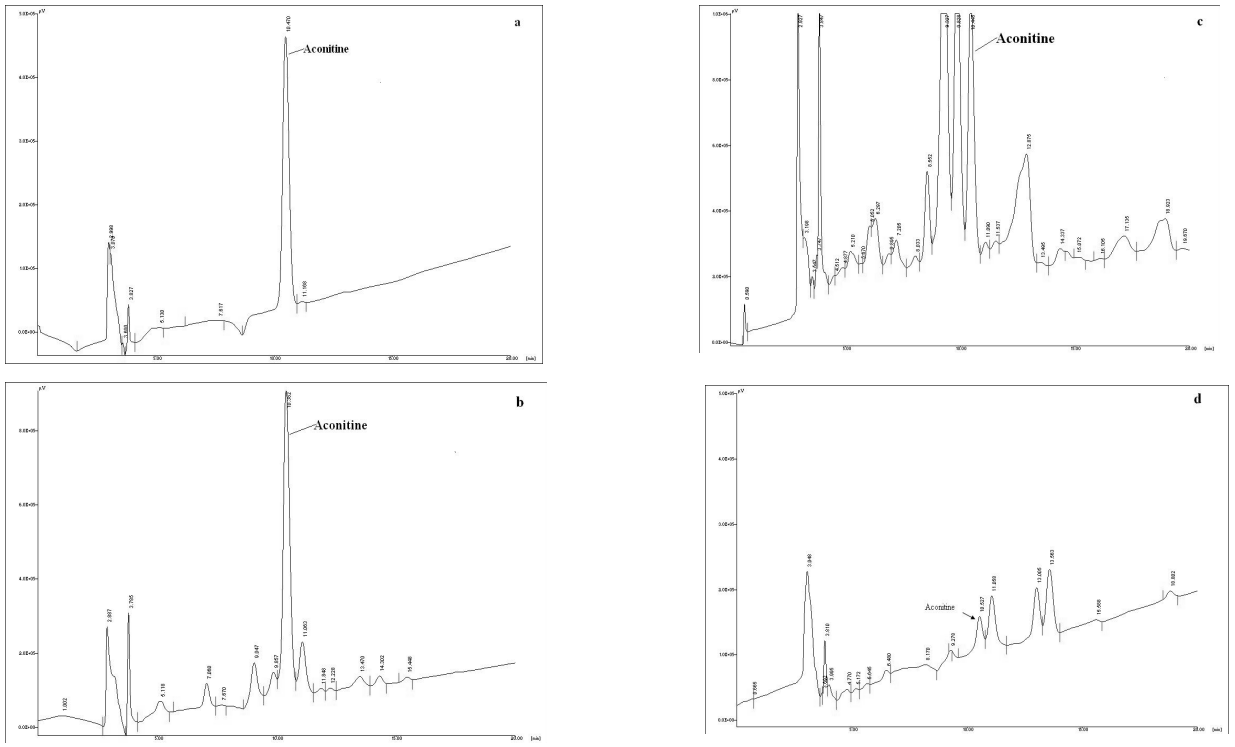

Figure 2: HPLC chromatograms of *Aconitum* alkaloid a- standard aconitine, b- *A. nagarum* November root c- *A. nagarum* August leaf, d- *A. elwesii* November root

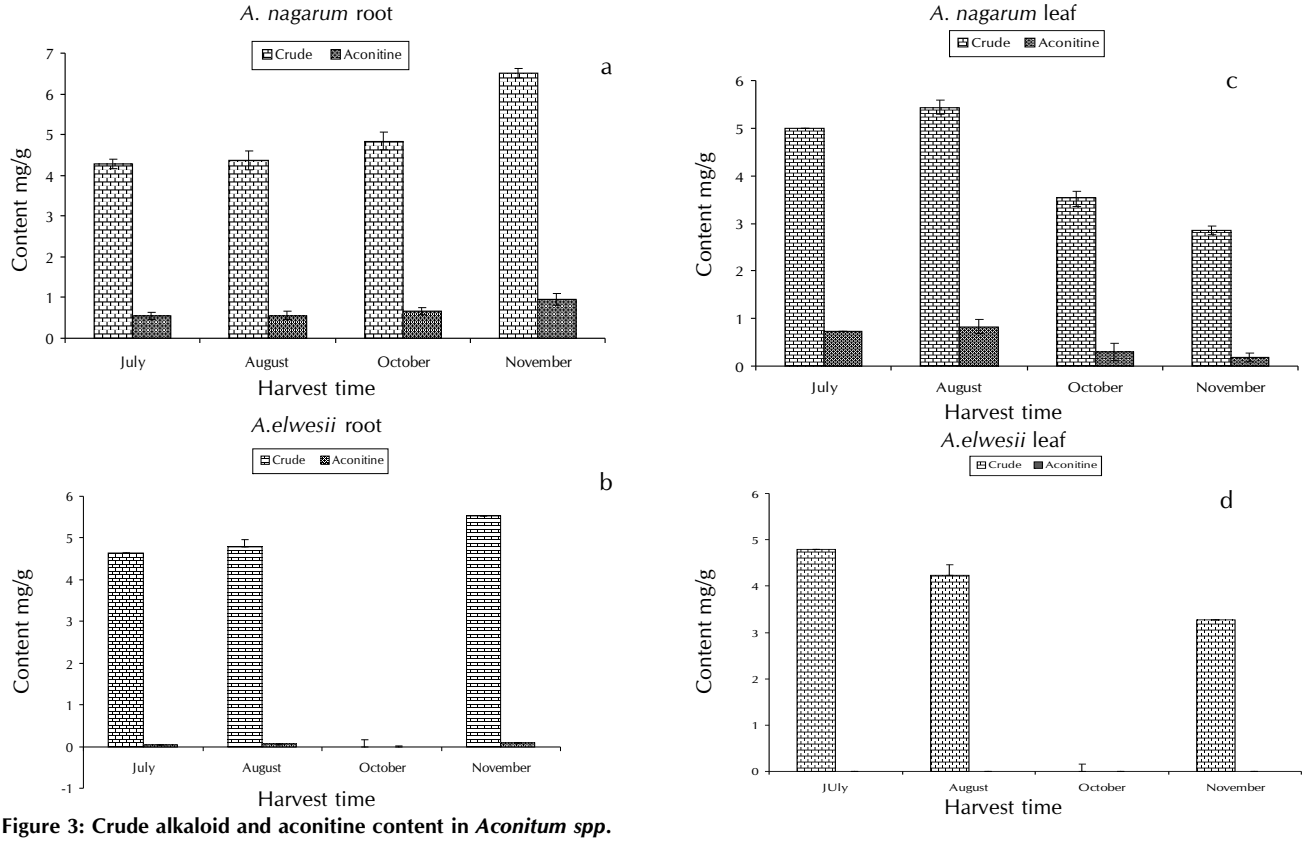

Figure 3: Crude alkaloid and aconitine content in *Aconitum spp*.

The alkaloid content was determined using HPLC and expressed in terms of aconitine equivalent (AE mg/g). Crude alkaloid content in *A. nagarum* root was 4.28, 4.37, 4.84 and 6.50 AE mg/g in July, August, October and November respectively (Fig.3a). It was significantly higher in the months of October and November when compared to July. It was

highest in November, which is the late flowering stage. Aconitine content was also highest in the month of November (0.96 mg/g) while in July, August and October aconitine content was 0.54, 0.56 and 0.67 mg/g respectively (Fig.3a).

In *A. .elwesii* root, crude alkaloid content was 4.65, 4.79 and 5.54 AE mg/g in July, August and November respectively (Fig.3 b) and significantly highest in November. Aconitine content was 0.05, 0.06 and 0.08 mg/g in July, August and November respectively. There was no significant difference in crude alkaloid content in roots of the two species. However there is significant difference in the aconitne content in root of the two species.

Crude alkaloid content in leaves of *A. nagarum* was 4.99, 5.44, 3.54 and 2.86 AE mg/g in July, August, October and November respectively. It was significantly highest in the month of August which is pre flowering season. Aconitine content was 0.72, 0.83, 0.29 and 0.19 mg/g in July, August, October and November respectively (Fig. 3c). The aconitine content in leaf during the vegetative stage (July - August) was higher. However, aconitine content decreases significantly during flowering stage (October – November). In *A. elwesii* leaf crude alkaloid content was 4.79, 4.84 and 3.27 AE mg/g in the months of July, August and November respectively (Fig. 3d), highest during pre flowering stage (August). Aconitine was not detected in *A. elwesii* leaf. As the plant passes different phanophases, alkaloids levels undergo changes, with their peak occurring during flowering. At the end of vegetation, alkaloids accumulate in seed, root (Gataulina, 2002; Brummund, 1988). Since alkaloid content in a plant constantly changes throughout the growth period, the maximum stocks of alkaloids in leaves are accumulated before flowering, and they gradually decline together with the qualitative composition of alkaloids with respect to the whole alkaloid complex. (Maknickiene, 2008). In both the species alkaloid content was significantly higher in root than leaf. In root of both the species crude alkaloid as well as aconitine content increases, as the plant progresses from vegetative to flowering stage, however, reverse is the case in leaves. Similar profiles were observed by (D'yachkovskaya, 1971) in *Aconitum* and *Lycoctonum* subgenera. In the early part of the vegetative period the alkaloid content is higher in leaves than roots and later on, during the flowering and fruiting stages, the alkaloid content is higher in roots. The amount of alkaloid in the leaves has fallen steeply and that in roots have risen (Bisset, 1981). Aconitine content in daughter tubers increased gradually during the bolting and flowering periods. In September, these aconitine alkaloid contents in daughter tubers in Ibaraki were higher than those in Hokkaido. In October, the alkaloid contents in the tubers in Hokkaido were almost equal to those in Ibaraki, but sucrose content in Hokkaido was higher than that in Ibaraki (Masanori *et al*., 2004). The *Aconitum* alkaloid contents can vary with the species, place of origin, time of harvest, and most importantly the method and adequacy of processing (Chan *et al.,*1994).

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