

Estimation of Alkaloids and Antibacterial Activity of *Aconitum spicatum* Bruhl Stapf from Manaslu Conservation Area

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Abstract

Aconitum spicatum is one of the deadly poisonous and highly valued medicinal plant. It is a national prioritized herb among 30 national priority herbs listed by the government of Nepal for their development, research and cultivation. The acute toxicity of the extract of this plant appeared to be directly related to the alkaloid content. The amount of total alkaloid of *A. spicatum* collected from Manaslu conservation area was calculated to be 1.7% by spectrophotometric method based on Dragendorff's reagent. *In-vitro* evaluation of the crude extract of plant rhizomes using agar well diffusion assay against eight bacteria, *Staphylococcus aureus*, *Escherichia coli*, *Schigella flexneri*, *Pseudomonas aeruginosa*, *Salmonella typhi*, *Salmonella paratyphi*, *Salmonella typhimurium* and *Klebsiella pneumonia*, displayed potential antibacterial activity. The diethyl ether fraction was the most effective against all pathogenic microbes with minimum bacterial concentration value 3.125-6.25 mg/ml.

Key words: *Aconitum spicatum*, antibacterial, Manaslu conservation area, total alkaloid

Introduction

Aconitum spicatum is one of the highly valued medicinal plants which is in trade from Nepal to India in a huge quantity (Olsen 1998). It belongs to the family Ranunculaceae and is widely distributed in the alpine and subalpine regions. The plants are usually perennial or biennial herbs, often with stout leafy stems, bulbs or creeping rhizomes. Leaves are mostly cauline, lobed, rarely divided and dentate. Flowers are simple or branched racemes. The tubers of *Aconitum* are used in the herbal medicines only after processing. The tuberous roots are commonly applied for various diseases, such as rheumatic fever, painful joints and some endocrinal disorders. *A. spicatum* as a traditional herb, its extracts have been employed in analgesic balms, sedatives and febrifuges in Tibet (FCCAS 1979). Considering its high medicinal value, *A. spicatum* was collected from Manaslu conservation area in 2010.

Manaslu Conservation Area is one of the most remote areas of Nepal, situated in upper region of Gorkha district. Nepal Academy of Science and Technology

(NAST) has initiated a long term research activities at Manaslu conservation area. It is resourceful in terms of the highly valued medicinal plants. As a part of ongoing investigation on the highly valuable medicinal plants, *Aconitum* was selected for determination of its alkaloid content and its biological activities. It mainly consists of C-20 diterpenoid and C-19 norditerpenoid alkaloids, bisnorditerpenoids and also phenolic and its glycoside compounds (Atta-ur-Rahman *et al.* 1995). The diterpenoid alkaloids can be divided into the following two broad categories on the basis of various substituents, which apparently affect both the chemical and pharmacological properties of these alkaloids. Alkaloids that have hexacyclic C19-skeleton and alkaloids that bear a C20-skeleton. C19-diterpenoid alkaloids comprise of the toxic ester moiety and can be further divided into four groups, aconitine, lycoctonine, pyrodelphinine and heteratisine classes on the basis of various carbon skeletons. C20-Diterpenoid alkaloids occur as esters but are relatively nontoxic. These alkaloids have been further classified into three basic types such as atisine, vetachine and

delnudine. Spicatine A and spicatine B were some reported norditerpenoids from *A. spicatum* by Chinese group (Gao *et al.* 2005). The determination of total alkaloid content is thus essential for standardization of this plant. It is determined by spectrophotometric method based on using Dragendorff's reagent. The toxicity of *Aconitum*, mainly derives from the diester diterpene alkaloids and can be decomposed into less or non toxic derivatives through different processing methods (Judith *et al.* 2009).

After processing, *Aconitum* species are usually mixed with other plants and used as a medicine in Ayurvedic and Traditional Chinese Medicine systems rather than the single component (Shyaula 2011). However, limited studies on antimicrobial activities have been done in *Aconitum* species (Ahmad *et al.* 2008). Even though pharmacological industries have produced a number of new antibiotics, resistance to these drugs by microorganisms has increased (Gold *et al.* 1996). The use of plant extract and its constituents can be of great significance in the therapeutic use. *Aconitum* is traditionally used for infected wounds to counteract the effect of edema which prompted us to carry out antimicrobial activity (Manandhar 2002). Evaluation of biological activities on *Aconitum* can have important role on discovery of the rational drugs. Here, the antibacterial property of *A. spicatum* (locally *Amchis* called *Chenduk*) collected from Prokh, Manaslu conservation area has been described.

Methodology

Equipment

For the evaporation process Hahn Shin Hahn vapor rotary evaporator (made in Korea) was used. The UV/Vis spectrophotometer 6715 was from Jenway/UK and Centrifuge FD 80-2 was from China.

Solvent and chemicals

The chemicals used were purchased from Merck, Qualigens, Himedia and S. D. fine-chem. All the chemicals used were of the analytical grade.

Plant materials

The rhizomes of *A. spicatum* were collected from Prokh Village development committee at the altitudes of 3,800-3,900 m situated in Manaslu Conservation Area. The plant was identified at National Herbarium Laboratory, Godawari, Lalitpur, Nepal. It was chopped into small pieces and air dried. The air dried material was then grinded into fine powder for extraction.

Extraction process

The powdered sample (695 g) was then extracted with 1% HCl/Methanol. The solvent was completely evaporated from the crude extract and it was then subjected to solvent-solvent extraction process to obtain hexane, diethyl ether, dichloromethane (neutral, basic and acidic) and ethyl acetate fractions.

Spectrophotometric Method of Estimation of Alkaloids

Total alkaloid content in the extract was estimated by spectrophotometric method based on using Dragendorff's reagent. The amount of bismuth present was estimated after precipitating the alkaloids with Dragendorff's reagent (Sreevidya *et al.* 2003).

Preparation of Dragendorff's reagent: The Dragendorff's reagent was prepared by mixing solutions A and B. (solution A, 0.8 g bismuth nitrate pentahydrate in 40 ml of distilled water and 10 ml glacial acetic acid; solution B, 8.0 g potassium iodide in 20 ml distilled water).

Preparation of standard bismuth nitrate solution: Bismuth nitrate stock solution was made by dissolving 10 mg $\text{Bi}(\text{NO}_3)_3 \cdot 5\text{H}_2\text{O}$ in 5 ml of concentrated nitric acid and diluting to 100 ml with distilled water.

Thiourea: 3 g thiourea was dissolved in 100 ml distilled water.

Disodium sulfide: 1 g Disodium sulfide was dissolved in 100 ml distilled water.

Extraction of *A. spicatum*: 10 g coarsely powdered of root of *A. spicatum* was extracted with 50 ml, 2% methanolic acetic acid for 1.5 h and the extract was diluted to 100 ml with 2% methanolic acetic acid.

The calibration curve was obtained by preparing series of dilutions of bismuth nitrate pentahydrate stock solution and constant amount of thiourea solution. The absorbance value of the yellow solution was measured at 435 nm. For determination of total alkaloid content in plant material, the extract was first treated with Dragendorff's reagent. The precipitate thus obtained was then treated with disodium sulfide. The brownish black precipitate formed was then dissolved in concentrated nitric acid and further treated with thiourea and absorbance was measured at 435 nm. The amount of bismuth present in the solution was calculated by multiplying the absorbance values with the factor. The factor is obtained from the standard curve which is constant for different concentrations.

$$\text{Factor} = \text{Concentration} / \text{absorbance}$$

Bacterial strains

Eight bacterial strains, *Staphylococcus aureus*, *Shigella flexneri* (ATCC 25923), *Pseudomonas aeruginosa* (ATCC 27853), *Salmonella paratyphi*, *S. typhi*, *S. typhimurium* (ATCC 14038) and *Klebsiella pneumoniae* (ATCC 13883) were used for antimicrobial activity of *Aconitum* extracts.

Antimicrobial activity of various fractions

The antibacterial assay of different fractions of the plant extracts were evaluated by agar well diffusion method as given by Dingle (Dingle *et al.* 1953). The method evaluated the antibacterial activity of the plant extracts with the determination of zone of inhibition (ZOI). The human pathogenic bacteria used for the study were *Staphylococcus aureus*, *Escherichia coli*, *Schigella flexneri*, *Pseudomonas aeruginosa*, *Salmonella typhi*, *Salmonella paratyphi*, *Salmonella typhimurium* and *Klebsiella pneumoniae*. For antibacterial assay, the standard inoculums of each bacterial species were prepared and matched with Mac Farland 0.5. The wells were made in the swabbed media plates with the help of sterile cork borer (diameter 4 mm). Fifty microlitres of working solution (50 mg/ml prepared in dimethyl sulphoxide) of the different fractions of the plant extract were loaded into the wells. The plates were then left for half an hour so as to facilitate diffusion of the extracts in the media. The plates were then incubated at 37 °C for 24 hrs and observed for the zone of inhibition suggested by the clean area without growth around the well. The antibacterial tests of the extracts were further carried out by two fold broth dilution method so that the antibacterial activity could be quantitatively interpreted in the form of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC).

Results and Discussion

The plant material (695 g) extracted with 1% HCl methanol by soxhlet extraction method yielded 106.43 g (15.31%) of crude extract. The crude extract when treated with Dragendorff's reagent, it showed pink red color indicating the presence of alkaloids. On further fractionation with solvent-solvent extraction process, hexane (1.15 g), diethyl ether (4.28 g), neutral dichloromethane (2.71 g), basic dichloromethane (2.1 g), acidic dichloromethane (1.17 g) and ethyl acetate (1.2 g) were obtained. The water insoluble part of the

extract was 8.23 g and was found to be soluble in methanol.

For estimation of the total alkaloid content, 10 g coarsely powdered root of *A. spicatum* was extracted with 50 ml, 2% methanolic acetic acid for 1.5 h and the extract was diluted to 100 ml with 2% methanolic acetic acid. The alkaloids were precipitated as complex formation by Dragendorff's reagent. The bismuth from the alkaloidal complex was then completely released by disodium sulfide. Bismuth forms a yellow bismuth complex in nitric acid medium with thiourea. The calibration graph between concentration and absorbance was linear. Because the complex formed was 1:1, the amount of bismuth corresponds to amount of alkaloids present. The amount of alkaloid present is calculated to be 1.7%. The alkaloid content of *A. spicatum* from Manaslu Conservation Area was first time calculated and its content was found to be high in comparison to other species (Faugeras *et al.* 1973).

The antibacterial activity was expressed in terms of the diameter of zone of inhibition (in mm) and the results are summarized in Table 1. *S. aureus* causes urinary tract infections, wound infections and food poisoning while *P. aeruginosa* causes hospital acquired infections. *Salmonella* sp causes typhoid and paratyphoid fever. *S. flexneri* causes dysentery and *K. pneumoniae* causes respiratory tract infections. *E. coli* an intestinal opportunistic pathogen causes diarrhea and urinary tract infection. The results indicated that there was variation in the inhibitory activity among the different types of extracts on the pathogens. The extracts were effective against both the Gram positive as well as Gram negative bacteria (Fig 1). The diethyl ether fraction displayed the highest antibacterial activity in all the pathogens with the zone of inhibition varying from 17 to 20 mm. The acidic chloroform fraction was next effective fraction inhibiting five of the pathogens tested and the zone of inhibition ranged from 6 to 11 mm. This indicated non polar fraction of *Aconitum* extract has greater antibacterial property. *E. coli* was the most sensitive among the pathogens tested. The data obtained in this study demonstrate that the possibility of use of these extracts to lower the risk of microbial infections, particularly in the intestinal and respiratory tract.

Table 1. Antibacterial activity of different fractions of *A. spicatum* extract

Bacterial pathogens	Zone of inhibition given by the extracts (mm) (Diameter of well = 4 mm)						
	Hexane	Acidic chloroform	Basic chloroform	Neutral chloroform	Diethyl ether	Methanol	Ethyl acetate
<i>Staphylococcus aureus</i>	6	6	-	-	20	-	-
<i>Escherichia coli</i>	-	11	10	12	17	7	6
<i>Schigella flexneri</i>	-	10	9	-	20	-	-
<i>Pseudomonas aeruginosa</i>	-	-	-	-	17	-	-
<i>Salmonella typhi</i>	8	10	6	-	20	-	6
<i>Salmonella paratyphi</i>	-	-	-	-	19	-	-
<i>Salmonella typhimurium</i>	6	-	-	-	18	-	-
<i>Klebsiella pneumoniae</i>	7	6	-	-	18	-	-

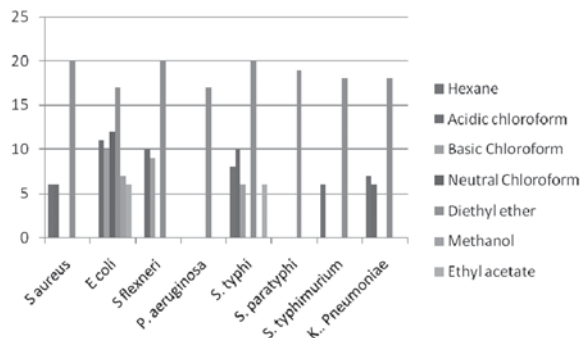


Fig. 1. Effect of different fractions of *Aconitum* extract on pathogenic bacterial strains

The diethyl ether fraction, being most effective one, was subjected for determination of MIC and MBC value. The extract being colored, MIC value for the tested pathogens could not be determined while the MBC

value ranged from 3.125–6.25 mg/ml (Table 2). This diethyl ether fraction was specifically effective towards *Salmonella typhi* with MBC value 3.125 mg/mL.

The determination of total alkaloid content of *A. spicatum* from Manaslu Conservation Area was found to be high and its further chemical analysis is essential to identify its potent bioactive constituents.

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Table 2. Minimum bactericidal concentration (MBC) of diethyl ether extracts of *A. spicatum*

Bacterial pathogens	Concentration of diethyl ether extract (mg/ml)						MBC (mg/ml)
	25.00	12.50	6.25	3.12	1.56	0.78	
<i>Staphylococcus aureus</i>	-	-	-	+	+	+	6.25
<i>Escherichia coli</i>	-	-	-	+	+	+	6.25
<i>Schigella flexneri</i>	-	-	-	+	+	+	6.25
<i>Pseudomonas aeruginosa</i>	-	-	-	+	+	+	6.25
<i>Salmonella typhi</i>	-	-	-	-	+	+	3.12
<i>Salmonella paratyphi</i>	-	-	-	+	+	+	6.25
<i>Salmonella typhimurium</i>	-	-	-	+	+	+	6.25
<i>Klebsiella pneumoniae</i>	-	-	-	+	+	+	6.25

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