

Antimicrobial and Antioxidant Role of the Aerial Parts of *Aconitum violaceum*

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Received July 27th, 2020; Accepted October 2nd, 2020.

DOI: <https://dx.doi.org/10.29356/jmcs.v65i1.1310>

Abstract. In the current studies, crude and subsequent fractions of *Aconitum violaceum* aerial parts were screened for their toxicity, antimicrobial effects as well as antioxidant potential. Phytochemically, the plant is enriched in alkaloids alongside anthraquinones (present in ethyl acetate fraction (AV₃) and saponins (detected in chloroform fraction (AV₂)). In Brine shrimp lethality assay, AV₃ was the most potent (59 %) in killing *Artemia naupili* larvae at a dose of 1000 µg/mL. AV₃ exhibited strongest antimicrobial effect against the bacteria *E. coli* (80 %) and *S. flexneri* (76 %) as well as against the tested fungi, *A. niger* (86 %) respectively. However, chloroform fraction (AV₂) was the most effective (almost 90 %) antifungal against *A. niger* and *C. albicans*. Overall, strong antioxidant activity was observed for AV₃ with IC₅₀ values of 120.04 ± 0.4 µg/mL (65.4 ± 0.01 standard) in DPPH and 125.1 ± 0.3 µg/mL (2.0 ± 0.03 standard) in ABTS free radical assays. AV₃ showed promising inhibition against acetylcholinesterase (AChE) 70 ± 0.8 % and butyrylcholinesterase (BChE) 57 ± 0.2 % at dose of 100 µg/mL thus confirming a potent invitro cholinesterase inhibitory effect. The overall results indicated strong biological potential of ethyl acetate fraction obtained from *A. violaceum* and a possible new therapeutic source could be formulated from its pure isolates.

Keywords: *Aconitum violaceum*; phytochemical; screening cytotoxic; antimicrobial; antioxidant; cholinesterase inhibition.

Resumen. En este estudio, se analizaron debido a su toxicidad, las fracciones crudas y posteriores de las partes aéreas de *Aconitum violaceum*, sus efectos antimicrobianos y potencial antioxidante. Fitoquímicamente, la planta está enriquecida en alcaloides junto con antraquinonas (presentes en la fracción de acetato de etilo (AV₃) y saponinas (detectadas en la fracción de cloroformo (AV₂)). En el ensayo de letalidad del camarón en salmuera, AV₃ fue el más potente (59%) para matar las larvas de *Artemia naupili* a una dosis de 1000 µg / ml. AV₃ mostró el efecto antimicrobiano más fuerte contra las bacterias *E. coli* (80 %) y *S. flexneri* (76 %), así como contra los hongos probados, *A. niger* (86 %) respectivamente. Sin embargo, la fracción de cloroformo (AV₂) fue el antifúngico más eficaz (casi el 90%) contra *A. niger* y *C. albicans*. En general, se observó una fuerte actividad

antioxidante para AV3 con valores de CI_{50} de $120.04 \pm 0.4 \mu\text{g} / \text{ml}$ (65.4 ± 0.01 estándar) en DPPH y $125.1 \pm 0.3 \mu\text{g} / \text{mL}$ (2.0 ± 0.03 estándar) en ensayos de radicales libres ABTS. AV3 mostró una inhibición prometedora contra la acetilcolinesterasa (AChE) $70 \pm 0.8 \%$ y la butirilcolinesterasa (BChE) $57 \pm 0.2 \%$ a una dosis de $100 \mu\text{g} / \text{mL}$ confirmando así un potente efecto inhibidor de la colinesterasa *in vitro*. Todos los resultados indicaron un fuerte potencial biológico de la fracción de acetato de etilo obtenida de *A. violaceum* y se podría formular una posible nueva fuente terapéutica a partir de sus aislados puros.

Palabras clave: *Aconitum violaceum*; fitoquímico; cribado citotóxico; antimicrobiano; antioxidante; inhibición de la colinesterasa.

Introduction

Medicinal plants have been used traditionally as “folk medicine” in basic health care and have been well documented from centuries [1]. The biologically active constituents also known as “natural products” obtained from medicinal plants play a vital role in the treatment of various human ailments including cancer, diabetes, Alzheimer’s and Parkinson diseases as well as in neurological disorders [2]. Plants of genus *Aconitum* (family Ranunculaceae) also known as “the queen of poisons” have been used as nerve tonic, against heart diseases as well as in high fever. Owing to the poisonous nature, it has also been used against venom poisoning. Most of the natural products from the plant include diterpene alkaloids (tetracyclic and pentacyclic) of aconitine derivatives as well as resins [3-5]. The plant possesses strong anti-proliferative activities against ovarian cancer cell lines and colon adenocarcinoma [6, 7]. Owing to high therapeutic importance, the current study has been carried out to evaluate the phytochemical and biological potential of crude methanolic extract and various fractions of *Aconitum violaceum* (aerial parts).

Experimental

Plant material (identification and collection)

A. violaceum plant was identified by Dr. Ali Hazrat, plant taxonomist and Associate Professor, Botany Department, University of Malakand where a voucher specimen (Accession number A-032) was deposited in the herbarium. After identification, the leaves and bark of *A. violaceum* were collected from Kashoora, Dogdara valley, Dir-Kohistan, Pakistan ($35^{\circ}.422744$, N latitude and $71^{\circ}.943561$ E longitude) in July 2018.

Preparation of plant extract and fractionation

The plant material was dried under shade followed by chopping and grinding to fine powder using electrical blender. The powdered material (1.2 kg) was soaked in methanol (10 L) for seven days in a glass container and was shaken time to time so that maximum extraction should be achieved. After seven days, the combined extract was filtered through Whatman filter paper (No.42) to remove the residue and dust particles. The extract was concentrated on a Rotary evaporator (Heidolph, Germany) at 40°C yielding a dark green colored crude (330 g). The crude was successively fractionated into various fractions in polarity order by pooling separately with n-hexane (3 L), chloroform (3 L), ethyl acetate (3 L) and finally with n-butanol (600 mL) to yield AV1-AV4 sub-fractions. Each sub-fraction was flittered before drying on rotary and stored at 4°C for further process.

Preliminary phytochemical screening

Preliminary phytochemical tests were performed for the crude and all the fractions using simple chemical testes [8]. The following procedures were adopted.

Test for alkaloids. Wagner’s test was performed for the detection of alkaloids in our samples. Briefly, 2 g of iodine was mixed with 6 g of potassium iodide in 100 mL of water to produce wagner’s reagent. The

samples were prepared as in methanol (0.2 g/ 5 mL) in small test tubes, followed by the addition of 3 drops of this solution to each of the sample. Appearance of red color in the bottom of test tubes indicated the presence of alkaloids.

Test for glycosides. A small amounts of 5 % HCl was added to each sample (0.2 g/ 5 mL in methanol) followed by neutralization with 5% NaOH solution. To the mixture, Fehling solutions A and Fehling solution B were added (few drops). The presence of glycosides was confirmed by the appearance of orange-red color/precipitate.

Test for saponins: Each sample (0.2 g) was taken in a test tube and shaken with distilled water (5 mL) and was then boiled. The presence of saponins was indicated by the appearance of frothing (creamy miss of small bubbles).

Test for flavonoids: Each extract about 0.2 g was taken in a test tube and dissolved in diluted NaOH. Then Hydrochloric Acid (HCl) was added drop-wise to the mixture. The presence of flavonoids was indicated by the turning of a yellowish solution to colorless.

Test for terpenoids: Each extract about 0.2 gm was taken and then mixed in a test tube with 2 mL of CHCl₃ (chloroform) and with a great care, 3 mL of Sulphuric acid (H₂SO₄) were added from a layer to it. The formation of a reddish-brown coloration of the interface indicates positive results, thus the presence of terpenoids were confirmed.

Test for anthraquinones: About 0.5 g of each extract was boiled with 10 % HCl for few minutes in water bath and filtered. Then it was filtered and allowed to cool. Equal volume of CHCl₃ was added to the filtrate. Then drops of 10 % ammonia was added to the mixture and heated. Rose-pink color indicated the presence of anthraquinones.

Cytotoxic bioassay

Modified brine shrimp lethality assay was used to find out the cytotoxic potential of all the samples [9]. Briefly, sea salt (3.8 g) was dissolved in distilled water (1 L), placed in a glass tank and 1 mg of *Artemia salina* (brine shrimp) eggs were added. The glass tank was incubated for 24 h at 25 °C which produced large number of larvae. Stock solutions of 20 mg/2ml (w/v in DMSO) of the crude and all the fractions was prepared and transferred to small vessels in 1000, 100 and 10 µL dilutions with addition of 5 mL sea water. 10 shrimps per vessel were also added into each vessel and incubated for 24 h. The number of survived shrimps were counted and the data was further analyzed for LD₅₀ determination.

Antimicrobial Bioassays

The antibacterial activity as performed according to the “Agar Well Diffusion Method” with some modifications [10]. Four bacterial strains including *Escherichia coli* (ATCC 15224), *Bacillus subtilis* (ATCC 6663), *Staphylococcus aureus* (ATCC 29213) and *Shigella flexneri* (ATCC 14028) were used in this assay while DMSO was used for the preparation of stock solution of crude as well as fractions (mg/mL). Concentration of 100 µL was added to the respective wells. For the positive control (10 µg/ml) and as a standard drug *Clarithromycin* was used. The plates were kept at room temperature to slow diffusion and then incubated for 24 hours at 37 °C. Inhibition zones were measured in mm.

Four pathogenic fungal strains including *Aspergillus flavus* (ATCC 32611), *Aspergillus niger* (clinically isolated), *Candida albicans* (ATCC 2091) and *Trichophyton longifusus* (ATCC 22397) and agar dilution method was used in antifungal assay [11]. Stock solutions were made in DMSO (mg/mL each sample) and 100 µg/mL concentration was used for measuring growth inhibition of tested fungi. Visual inhibition of fungal growth was observed after incubation of plates for 7 days at 37 °C. Media growth was obtained from the measurement of linear growth (mm) while the growth inhibition was calculated with reference to the inhibition of standard (miconazole, 10 µg/mL).

1,1-Diphenyl-2-hydrazyl (DPPH) method

DPPH method was used to determine the free radical scavenging assay of the crude and fraction of *A. violaceum* according to the procedure [12]. The stock solution was prepared using 100 µL of the test sample (mg/mL in methanol), mixed with 900 µL DPPH solution (1 mM in methanol) and various dilutions were prepared (100, 250, 500 and 1000 µg/mL). The reaction mixtures were mixed well and incubated in dark for 1

h at 37° C temperature. Absorbance of the reaction mixture was recorded at 517 nm. 3% methanol was used as blank sample and a mixture of 900 µL Ascorbic acid in 100 µL 3 % methanol was used as positive control. DPPH solution was used as negative control. The reduction in the absorbance was used as indicator of higher free radical scavenging activity. The percentage was calculated as:

$$\text{DPPH scavenging (\%)} = \left(1 - \frac{\text{Absorbance of sample} - \text{Absorbance of sample control}}{\text{Absorbance of blank}} \right) \times 100$$

(2,2'-Azino-bis-(3-ethylbenzothiozoline-6-sulfonic acid) (ABTS) method.

ABTS assay was performed according to the method described by Chen et al [13]. The antioxidant kit (cat. No. CS0790) was used in deionized water, ABTS as substrate while trolox as standard scavenger. Briefly, in step one, 3 % hydrogen peroxide solution (25 µL) was added into ABTS solution (10 mL) to obtain a standard curve within 10 minutes. Immediately after, in second step, 10 µL of trolox solution, 20 µL of myoglobin working solution and 20 µL of the samples were mixed and added to the respective wells (96 well plates) as positive control. In the next step, 150 µL of ABTS working solution was added to each well, incubated for 5 minutes at room temperature and the reaction was stopped by adding 100 µL of stop solution (Cat. No. S3446) to each well. The absorbance was determined at 405 nm using plate reader.

Acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) inhibition assay

The enzyme inhibition assays were carried out according to the method of Elman with slight modifications [14]. Briefly, 40 µL of sample solution (mg/mL) was first added into 200 µL of 5,5'-dithiobis-2-nitrobenzoic acid (DTNB, 0.2 mM) having pH of 8.0 maintained by the addition of 800 µL of sodium phosphate buffer (62 mM). Afterwards, both the enzymes were separately added into each solution (AChE/BChE, 40 µL each) and incubated for 15 minutes on room temperature. The reaction was initiated by the addition of acetylthiocholine or butyryl- thiocholine (40 µL each) which produced a yellow color anion (5-thio-2-nitrobenzoate). The absorbance for the product was measured by BMS spectrophotometer (USA) at 412 nm for 15 minutes.

Statistical analysis

All the tests/reactions were carried out in triplicate and analytical grade solvents/chemicals were used. The results were assessed using GraphPad Prism Software, expressed as mean ± SD. IC₅₀ values were calculated using ANNOVA method.

Results and discussion

Phytochemical analysis

Preliminary phytochemical screening resulted in the identification of various classes of phytochemicals in the crude as well as fractions (AV1-AV4). The ethyl acetate fraction (AV3) strongly showed the presence of flavonoids, alkaloids and anthraquinones while chloroform fraction (AV2) was enriched in alkaloids and saponins. Terpenoids were detected in *n*-hexane (AV1) and *n*-butanol fraction (AV4) showed the presence of glycosides (Table 1).

Table 1. Phytochemical tests of *A. violaceum* crude extract and various fractions.

S/NO	Class	Crude	AV ₁	AV ₂	AV ₃	AV ₄
1	Alkaloids	++	-	+	++	-
2	Glycosides	+	-	-	-	++
3	Saponins	+	-	++	-	-

4	Flavonoids	++	+	-	++	-
5	Terpenoids	++	++	-	-	-
6	Anthraquinones	+	-	-	++	+

Key: (++) Present in very Appreciable quantity; (+) = Present, (-) = absent

Cytotoxic investigations

Laboratory cultivated *Artemica naupili* shrimps have widely been used as part of pre-clinical tests to find out cytotoxicity of crude drugs [15]. In Brine shrimp lethality assay, concentration dependent cytotoxic effect was determined where the concentration dose of 1000 $\mu\text{g/mL}$ was effective (Table 2). The IC_{50} values however, remained lower unexpectedly. Moderate effect was reported for ethyl acetate (AV3) fraction ($\text{IC}_{50} = 0.51 \pm 0.08 \text{ mg/mL}$) as compared to the standard ($\text{IC}_{50} = 0.05 \pm 0.08 \text{ mg/mL}$). This cytotoxic effect may be caused due to the presence of alkaloids and anthraquinones in this fraction [16].

Table 2. Cytotoxic results of crude extract and various fractions of *A. violaceum*.

Concentration ($\mu\text{g/mL}$)	No of Shrimps died %					
	Crude	AV ₁	AV ₂	AV ₃	AV ₄	Control
100	15 \pm 1.12	10 \pm 1.32	13 \pm 0.05	17 \pm 0.09	9 \pm 0.01	100
250	27 \pm 0.51	13 \pm 1.55	23 \pm 0.12	25 \pm 0.01	20 \pm 0.03	100
500	35 \pm 0.29	19 \pm 1.35	37 \pm 1.82	40 \pm 1.19	34 \pm 1.34	100
1000	55 \pm 0.65	30 \pm 0.89	50 \pm 0.59	59 \pm 1.41	57 \pm 1.96	100
IC_{50}	0.62 \pm 0.12	-----	0.75 \pm 0.05	0.51 \pm 0.08	0.59 \pm 0.52	0.05 \pm 0.15

Values shown are mean \pm SEM, triplicate of experiments.

Antimicrobial effects

All the samples except hexane fraction (AV1) showed promising antibacterial as well as antifungal effects against the tested pathogens in dose of 100 $\mu\text{g/mL}$ (Table 3). Ethyl acetate (AV3) fraction was the most potent with inhibition zones of $24 \pm 0.45 \text{ mm}$ (80 %), $19 \pm 0.67 \text{ mm}$ (82 %), $16 \pm 0.66 \text{ mm}$ (76 %) against *E. coli*, *B. subtilis*, *S. aureus* while showed moderate inhibition against *S. flexneri* (18 ± 0.44 , 72 %). The percentage inhibition against all the bacteria has been provided in Fig. 1 which is relative to the inhibition of standard drug, clarithromycin at same dose which indicate the potency of our sample. The strains of *E. coli* and *S. flexneri* were inhibited by almost all our samples while the crude also showed almost similar effects to that of AV3 (Fig. 2). All these pathogens are responsible for many diseases such as diarrhea, respiratory and urinary tract infections. Parallel findings have been observed previously for various plants extracts and their antibacterial effects [17, 18]. Our results suggest that *A. violaceum* extracts contain potent bacterio-susceptible constituents generally as well as alkaloids, saponins and anthraquinones [19] in particular which are responsible for such action.

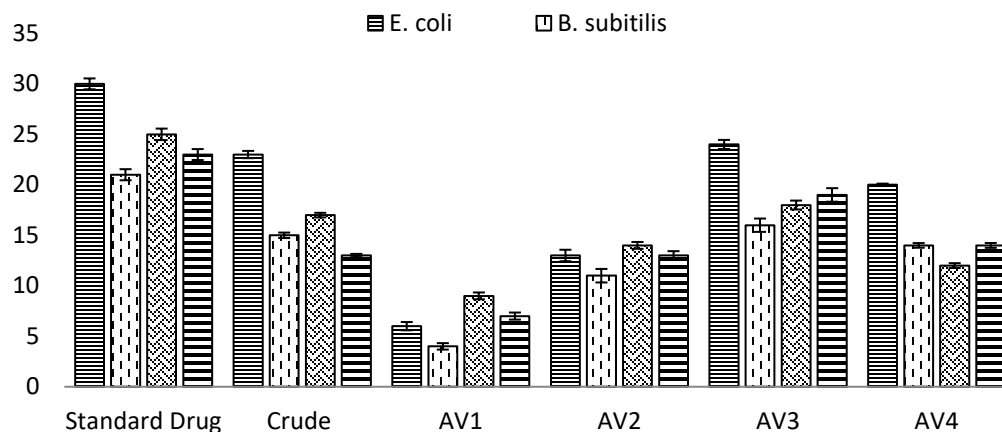


Fig. 1. Comparative zone of inhibition by the crude extract and all the fractions (AV1-AV4) of *A. violaceum* against all the tested bacteria.

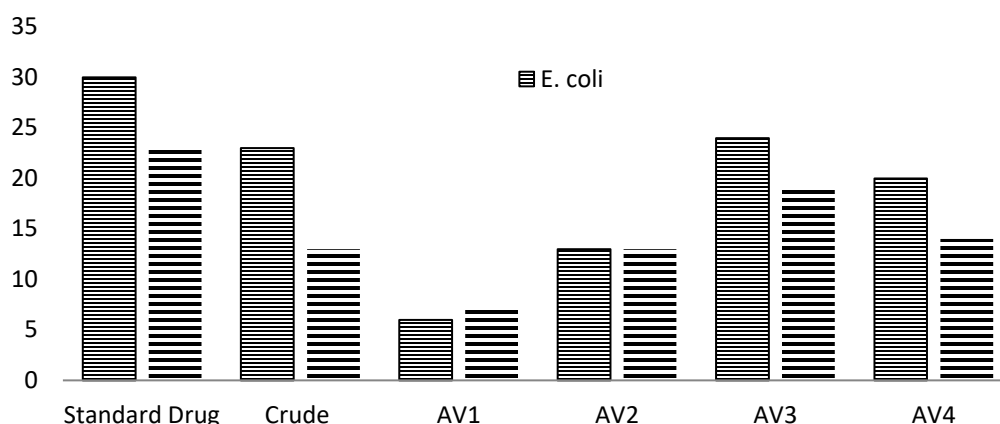


Fig. 2. Comparative zones of inhibition against *E. coli* and *S. flexneri* by the crude extract and all the fractions as compared to standard drug.

Table 3. Antimicrobial activities of *A. violaceum* crude extract and fraction (AV₁-AV₄).

Bacteria	Zone of inhibition in mm					
	Crude	AV ₁	AV ₂	AV ₃	AV ₄	Clarithromycin
<i>E. coli</i>	23±0.36	6±0.41	13±0.57	24±0.45	20±0.12	30±0.54
<i>B. subtilis</i>	15±0.27	4±0.32	11±0.67	16±0.66	14±0.23	21±0.55
<i>S. aureus</i>	17±0.23	9±0.34	14±0.34	18±0.44	12±0.24	25±0.57
<i>S. flexneri</i>	13±0.16	7±0.35	13±0.43	19±0.67	14±0.24	23±0.54
Fungi	Control Linear growth in mm					Control
<i>A. flavus</i>	15±0.36	7±0.33	16±0.36	18±0.38	16±0.22	19±0.38
<i>A. niger</i>	16±0.44	6±0.45	19±0.64	18±0.46	14±0.21	22±0.65
<i>C. albicans</i>	16±0.35	10±0.34	18±0.55	13±0.54	13±0.23	20±0.43
<i>T. longifusus</i>	12±0.32	7±0.55	15±0.56	14±0.34	13±0.25	18±0.34

Note: Values are mean inhibition zone (mm) ± S.D of three replicates.

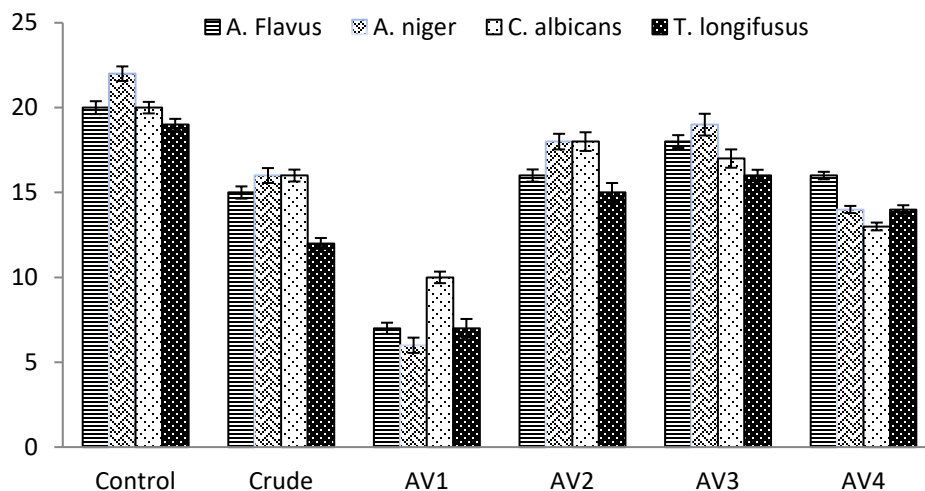


Fig. 3. Comparative zones of inhibition by the crude extract and all the fractions (AV1-AV4) of *A. violaceum* against all the tested fungi.

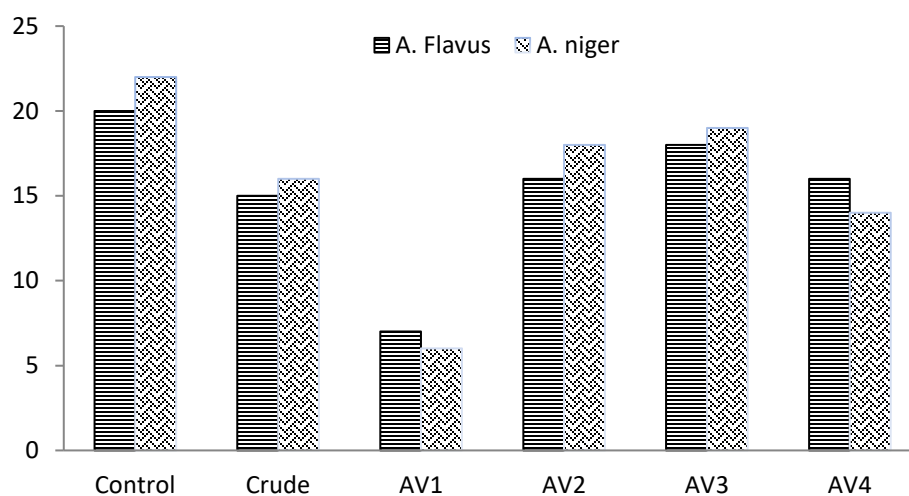


Fig. 4. Comparative zone of inhibition against *A. flavus* and *A. niger* by the crude extract and all the fractions as compared to standard drug.

Antioxidant potential

Concentration dependent antioxidant effect of crude and fractions of *A. violaceum* was determined against both DPPH and ABTS. Overall, strong antioxidant effect was observed for crude against DPPH ($IC_{50} = 87.4 \pm 0.03$) while AV3 scavenged DPPH with IC_{50} values of $120.04 \pm 0.4 \mu\text{g/mL}$ (65.4 ± 0.01 standard) and $125.1 \pm 0.3 \mu\text{g/mL}$ (2.0 ± 0.03 standard) against ABTS in free radical scavenging activities. The data is summarized in table 4. The results are similar to previous studies [22, 23] where various plant extracts were tested for antioxidant potential against free radical scavenging assays. The effect may be caused due to the presence of phenolic contents in plant extracts. These types of compounds could stabilize the DPPH radical in test solution or any free radical in solution by either absorbing or deactivating through bonding/coordination. Such compounds could also be able to protect membrane lipids from oxidation (lipid peroxidation) from any

peroxides formed in cells. [24]. Moreover, the oxidative stress could lead to many diseases especially diabetes hence *A. violaceum* may provide some sort of remedy for problems emerging due to overflow of reactive oxygen species.

Table 4. Concentration-dependent anti-oxidant effects of *A. violaceum* crude and its fractions.

Conc (µg/mL)	Standard drugs	Concentration-dependent DPPH % Scavenging				
		Crude	AV ₁	AV ₂	AV ₃	AV ₄
50	38.2 ± 0.02	30.2 ± 0.2	08.2 ± 0.2	39.2 ± 0.1	21.2 ± 0.01	05.1 ± 0.02
100	88.3 ± 0.01	58.8 ± 0.01	09.4 ± 0.1	42.2 ± 0.01	39.6 ± 0.2	19.5 ± 0.3
250	100 ± 0.2	66.6 ± 0.11	15.6 ± 0.01	58.3 ± 0.83	55.4 ± 0.2	32.01 ± 0.05
500	-----	80.1 ± 0.41	20.3 ± 0.2	75.2 ± 0.01	82.8 ± 0.03	45.2 ± 0.08
(IC ₅₀ , µg/mL)	65.4 ± 0.01	87.4 ± 0.1	-----	130.2 ± 0.01	120.1 ± 0.8	-----
Trolox *		Concentration dependent ABTS % scavenging				
50	--	27.1 ± 0.15	05.3 ± 0.2	29.3 ± 0.03	37.2 ± 0.04	02.7 ± 0.1
100	--	44.5 ± 0.54	12.2 ± 0.09	40.2 ± 0.08	43.3 ± 0.05	09.7 ± 0.3
250	---	63.5 ± 0.12	32.3 ± 0.3	54.7 ± 0.13	59.1 ± 0.03	19.3 ± 0.04
500	---	79.3 ± 0.31	47.4 ± 0.6	71.3 ± 0.06	82.2 ± 0.07	30.1 ± 0.7
IC ₅₀ (µg/mL)	2.0 ± 0.03	120.04 ± 0.4	-----	158.7 ± 0.09	125.1 ± 0.3	-----

Values shown are mean ± SEM, No. of experiments = 3

Cholinesterase inhibition

The antioxidant effect was further correlated by performing cholinesterase inhibition assays for crude and fractions of *A. violaceum* against Acetylcholinesterase (AChE) and Butyrylcholinesterase (BChE) in concentrations dependent manner. The percentage inhibitions have been presented in table 5. Amongst all the samples, AV₃ showed highest inhibition of both the enzymes i.e. 70 ± 0.8 % against AChE (95 ± 0.8 for reference drug) and 57 ± 0.3 % against BChE (88 ± 0.3 for reference drug) at dose of 100 µL (mg/mL) (Table 4). The crude also remained effective with 60 ± 1.13 % and 55 ± 0.31 % inhibition of AChE and BChE. Allanzanthane was used as reference drug. The other fractions remained low inhibitors of both the enzymes.

The lack of acetylcholine neuro-transmittance is associated with memory loss leading to dementia and Alzheimer's disease (AD) could be encountered using natural antioxidants. These products eliminate the toxic effects of reactive oxygen species (ROS) which cause oxidative damage to cells causing cerebral dementia, AD and could result in diabetes. Various natural antioxidants have been found to treat AD like Vitamins (A & D), β-carotene as well as vitamin C, especially in old age patients [25,26]. The use of such medicinal plant extracts could lower the oxidative stress by scavenging any free radicals, especially ROS thus improve AD therapy and subsequently, can enhance memory [27]. Several cholinesterase inhibitors have been used as anti-AD drugs such as galantamine, donepezil, however, due to low effectiveness, there is a need of alternate drugs with minimum side effects and relatively more effective. [28, 29]. Our findings could lead towards *A. violaceum* based medication of AD and related diseases in particular.

Table 5. Cholinesterase inhibitory effect of *A. violaceum* methanolic extract and various fractions.

Sample	AChE (% Inhibition)	BChE (% Inhibition)
Crude	60 ± 1.13	55 ± 0.73
AV ₁	30 ± 0.11	19 ± 0.9
AV ₂	55 ± 0.17	43 ± 0.13
AV ₃	70 ± 0.8	57 ± 0.3
AV ₄	47 ± 0.14	38 ± 1.62
Allanzanthane	95 ± 0.8	88 ± 0.3

Conclusion

The current study was aimed in finding out the therapeutic potential of crude and subsequent fractions obtained from *Aconitum violaceum* aerial parts. Amongst all, ethyl acetate fraction showed better potential of as cytotoxic, antibacterial and antifungal, strong antioxidant and a potent inhibitor of acetylcholinesterase. These activities are surly based on the phytochemical profile of this fraction which include alkaloids as well as anthraquinones. Overall, the plant is highly toxic, but the toxicity could be minimized through extraction polarity-based extraction of these metabolites. The overall results indicated strong biological potential of ethyl acetate fraction obtained form *A. violaceum* and a possible new therapeutic source could be formulated into several concoctions.

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