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SNAKE VENOM ENZYMES NEUTRALIZATION POTENTIAL OF PHYTOCHEMICALS EXTRACTED FROM *DATURA ALBA* SEEDS

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ABSTRACT

Datura is a flowering perennial herb, belonging to the family Solanaceae, is widely distributed in many countries including Pakistan, and other tropical and sub-tropical parts of the world. The present study explored the bioactivities of phytochemicals obtained from *Datura alba* n-hexane and n-butanol seed extracts. The seed extracts showed the presence of flavonoids, terpenoids and alkaloids while saponins were absent. The n-hexane extract showed 100% human salivary amylase and 19% snake venom PLA_2 ($svPLA_2$) inhibition activity at examined lowest and highest concentrations respectively. It also exhibited mild (24% RSA) antioxidant activity at highest concentration (600 µg/µl) analyzed. On the other hand, salivary amylase inhibition and potent (80% RSA) antioxidant activity at 100 µg/µl concentration was observed in n-butanol extract. Interestingly, n-hexane extract showed snake venom protease while n-butanol extract showed $svPLA_2$ enhancing activity and no venom anti-protease activity. The study demonstrated that both n-hexane and n-butanol extracts are natural sources of antioxidant and antidiabetic compounds. The n-hexane extract showed venom PLA_2 neutralization which is reported for the first time.

Keywords: Traditional medicine; *Datura alba*; Anti-phospholipase A_2; Anti-amylase; Antioxidant

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INTRODUCTION

Traditional medicine (TM) is an integral part of the healthcare system. It may serve as an alternate treatment and aid in the discovery and development of new medicines (Yuan *et al.,* 2016). The conventional drugs are excessively expensive and not readily available whereas, the herbal medicines are accessible, affordable, and have less or no known side effects (Karimi *et al.,* 2015). More than 80% or three quarters of the world's population relies on traditional medicines of plant origin for their primary health care especially in developing countries (WHO, 2019). About 84% of Pakistani population also depends on TM for their basic health care needs (Alamgeer *et al.,* 2018). Bioactive compounds include both phytochemicals and proteins/peptides, have been shown to possess therapeutic potential to treat various disease conditions (Samtiya *et al.,* 2021). In modern pharmacopeia, more than 25% of drugs are plant derived and some of them are synthetic analogs of these effective bioactive compounds (Amin *et al.,* 2009; Veeresham, 2012). These include all main classes of organic compounds which includes aliphatic, aromatic, hydro-aromatic and heterocyclic molecules (Rungsung *et al.,* 2015; Xiao and Bai, 2019).

Datura spp. is a flowering medicinal herb from the Solanaceae family that is found in Europe, Asia, America, South Africa and other tropical and subtropical regions. It is known due to its antibacterial, anti-diabetic, anti-asthmatic, anti-inflammatory, antioxidant, analgesic, insecticidal, cytotoxic, wound healing, larvicidal, neurological and anti-ophidic properties (Sharma *et al.,* 2021). The four species of *Datura* found in Pakistan are *Datura stramonium, Datura innoxia, Datura suavelons and Datura alba. Datura alba* is a flowering perennial herb, belonging to the family Solanaceae (Yousaf *et al.,* 2008). Its leaves, seeds, roots, flowers all are used traditionally for the treatment of different ailments such as anti-asthmatic, antitussive, antispasmodics, bronchodilator, antiseptic, sedative *etc.* (Sharma *et al.,* 2021; Islam *et al.,* 2023).

It also possesses antimicrobial, insecticidal, spasmogenic, neurological, and wound healing effects (Omara *et al.,* 2020). In the current study bioactivities of phytochemicals from *Datura alba* n-hexane and n-butanol seed extracts has been explored. Based on the broad-spectrum medicinal properties reported from *Datura*, in the current study bioactivities of phytochemicals from *Datura alba* n-hexane and n-butanol seed extracts have been extended. Particularly

evaluation of anti-amylase and antivenom activity, an aspect that has not been studied previously is explored.

MATERIALS AND METHODS

Crude extract preparation*: Datura alba* pods were collected from the vicinity of Dow University of Health Sciences, Karachi. Plants were get identified from Institute of Sustainable Halophyte Utilization, University of Karachi. The seeds were retrieved, dried and pulverized. These seed powder was soaked in methanol (Sigma-Aldrich) in the ratio of 1:5 (w/v) and were agitated at 125 rpm for 15 days at 25 ℃. The resulting crude methanolic extract (CME) was filtered using Whatman filter and concentrated on rotary evaporator (Heidolph Instruments, Germany) at 42 ℃. The procedure was repeated quadrice and all the extracts were combined (Toh *et al.,* 2023).

Solvent-solvent extraction: The concentrated crude methanolic extract (CME) was partitioned by solvent-solvent extraction method using organic solvents of increasing polarity. Briefly, crude extract was suspended in 20 ml of water and was partitioned between n-hexane (20 ml x 2; Sigma-Aldrich), dichloromethane (20 ml x 2; BDH), ethyl acetate (20 ml x 2; Sigma-Aldrich) and n-butanol (20 ml x 3; Sigma-Aldrich) successively. Organic and aqueous layers were separated, similar fractions were pooled together and concentrated (Maher *et al.,* 2018).

Qualitative analysis: The fractionated extracts (FEs) were tested for the presence of alkaloids (Pascale *et al.,* 2018), flavonoids (Jose *et al.,* 2020), terpenoids and saponins (Singh *et al.,* 2021). Also, CME and FE were screened by thin layer chromatography (TLC) using pre-coated silica gel 60, F_{254} TLC sheets (Merck). The developed TLC plates were observed under UV lamp at the wavelengths of 254 nm and 365 nm respectively (Maher *et al.,* 2018).

In-vitro biological activity assays

Phospholipase A_2 inhibition assay: Phospholipase A_2 inhibition activity was analyzed by agar egg yolk plate method with slight modifications (Giresha *et al.,* 2015). Agar (OXOID) and egg-yolk (final concentration 2% each) were prepared in 20mM phosphate buffer (pH 7.0) containing 1mM $CaCl_2$ and 0.01% sodium azide (VWR). The *Naja naja* venom that was previously collected, lyophilized and stored at -20 ℃ was taken as standard PLA_2 (0.25 µg/µl in water). Different concentrations of n-hexane (200, 300, 400, 600, 800 µg/µl) and n-butanol (25, 50, 100, 200, 400 µg/µl) extracts were prepared in 5% DMSO (Sigma-Aldrich), 95% MeOH, mixed with standard and incubated for 30 mins at 37 ℃ in a final volume of 20 µl. The test mixtures were then loaded into the wells and incubated overnight at 37 ℃ incubator (Innova 42, Marshall Scientific, USA). Next day, the zone of inhibition was measured using vernier caliper. Zone of standard was served as a positive control, zone of solvent (5% DMSO, 95% MeOH) and water were served as a negative control. All experiments were performed in triplicate on three independent days. Percentage inhibition of PLA_2 enzyme was calculated by the following formula.

$$\%\text{inhibition} = \text{Diameter (Control)} - \text{Diameter (Sample)} \times 100 / (\text{Diameter (Control)})$$

Protease inhibition assay: Protease inhibition assay was performed using skim milk agar plate method (Mohan *et al.,* 2018). The *Echis carinatus* venom that was previously collected, lyophilized and stored at -20 ℃ was taken as standard protease (50 µg/µl in water). The rest of the procedure was the same as used for PLA_2 inhibition assay.

Alpha-amylase inhibition assay: Amylase inhibitory activity was assessed by starch agar plate method with modifications (Jemaa *et al.,* 2017). Human saliva (3 µg/ml) was taken for standard amylase activity. The rest of the procedure was the same as used for PLA_2 inhibition assay.

DPPH (2,2-diphenyl-1-picrylhydrazyl) antioxidant assay: Antioxidant activity of n-hexane (100, 200, 400, 600 µg/µl) and n-butanol (12.5, 25, 50, 100 µg/µl) seed extract was evaluated by DPPH (Sigma-Aldrich) free radical scavenging assay (Petchiammal and Hopper, 2014). Ascorbic acid (25, 50, 100 200 µg/µl) was taken as standard antioxidant (Daejung). Absorbance was recorded at 517 nm in a microplate reader (BioTek Epoch 2, Agilent, USA) and percentage radical scavenging activity was calculated by the given equation. All experiments were performed in duplicate and on two independent days. The % inhibition is calculated as:

$$\%\text{inhibition} = \text{Absorbance (Control)} - \text{Absorbance (Sample)} \times 100 / (\text{Absorbance (Control)})$$

RESULTS

Phytochemicals identification: The appearance of yellow color followed by color loss upon addition of dilute acid and appearance of reddish-brown layer indicated the presence of flavonoids and terpenoids respectively. Formation of red orange precipitates indicates the presence of alkaloids while absence of foam formation indicates absence of saponins in n-hexane and n-butanol extracts. Overall, flavonoids and terpenoids are found in n-hexane extract while n-butanol extract showed presence of alkaloids along with flavonoids and terpenoids. The presence of saponins was not observed in either of the extracts.

Phospholipase A_2 inhibition assay: The n-hexane and n-butanol extract were analyzed for phospholipase A_2 inhibitory activities on egg-yolk agar plates. *Naja naja*

venom (0.25 µg/µl) that is rich in phospholipases A_2s (svPLA_2s) is used as standard enzyme. The n-hexane showed highest inhibitory activity (19 %) at 800 µg/µl concentration while n-butanol extract rather enhanced the activity instead of inhibition (Figure 1A and 1B). The extract could contain molecules that stabilize enzyme structure such as its Ca^{+2} binding loop which resulted in more enhanced and potent activity.

Protease inhibition assay: Extracts were analyzed for protease inhibitory activity on skim milk agar plates. *Echis* venom that is rich in proteases is used as standard protease. The n-hexane showed an activity enhancing effect, while, on the other hand n-butanol extracts exhibited neither inhibition nor enhancing effect on snake venom protease activity (Figure 1C and 1D).

Alpha-amylase inhibition assay: Among crude extracts of *Datura alba,* n-hexane showed inhibition of salivary amylase activity as visualized on starch agar plate. Complete inhibition was observed even at lowest concentration used i.e.100 µg/µl. The n-butanol extract showed inhibition of activity which increases with decreasing the extract concentration (Figure 2A and 2B).

DPPH antioxidant assay: Both the extracts showed increasing antioxidant activity with increasing concentrations. The highest concentration of n-hexane extract (600 µg/µl) showed only 24% radical scavenging activity (RSA) that demonstrated mild antioxidant activity by the extract. The n-butanol extract showed strong antioxidant activity with 80% RSA at 100 µg/µl concentration (Figure 3).

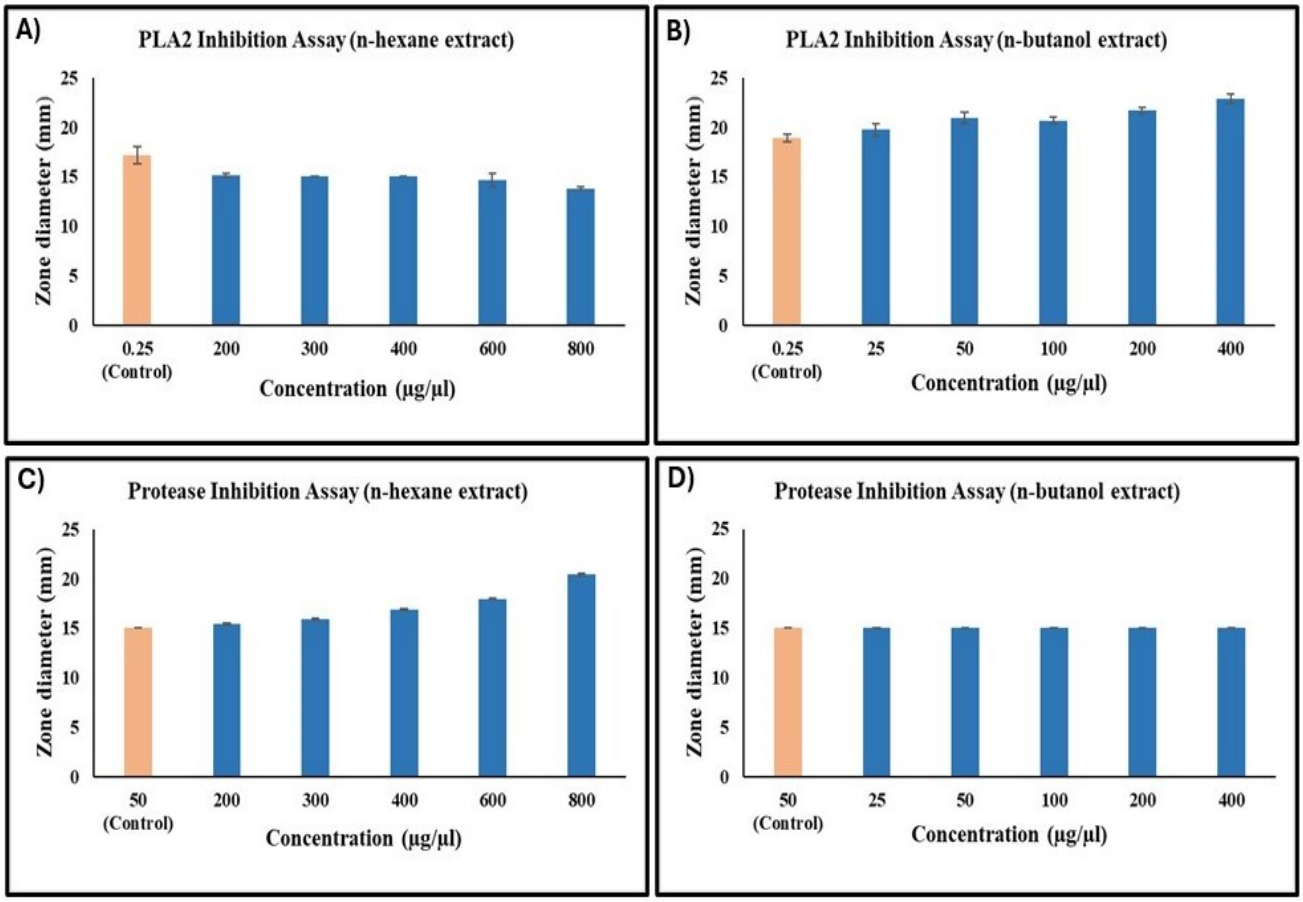

Figure 1 Phospholipase A_2 and Protease Inhibition Assay of Extracts: Graphical representation of PLA_2 inhibition assay at different concentrations of (A) n-hexane extract (B) n-butanol extract on egg yolk agar plate. (Control) *Naja naja* venom. Graphical representation of protease inhibition assay at different concentrations of (C) n-hexane extract (D) n-butanol extract on skim milk agar plate. (Control) *Echis carinatus* venom.

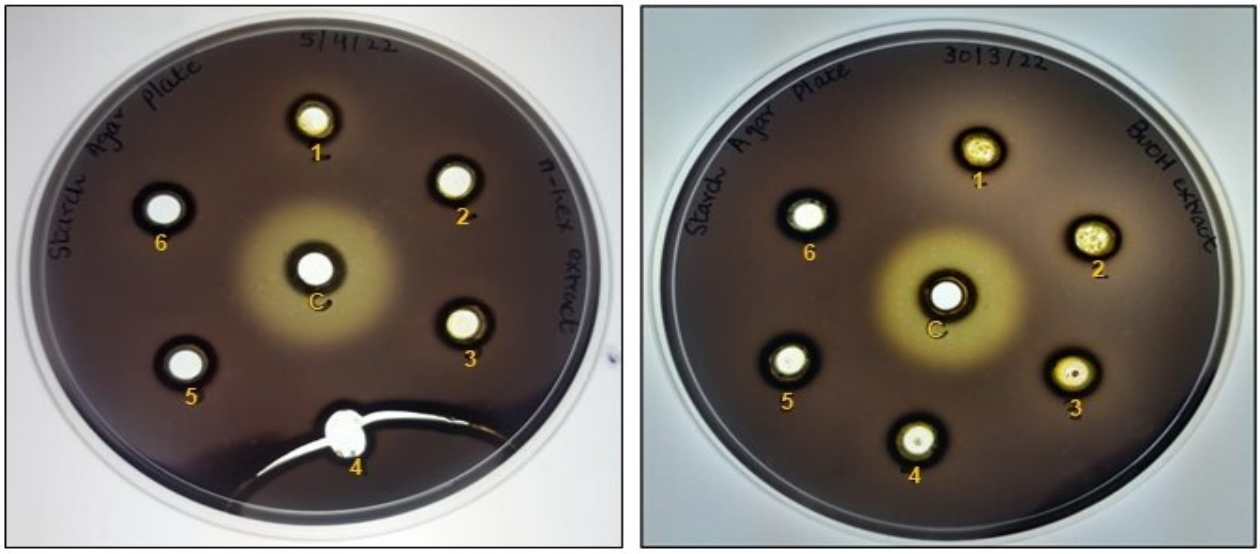

Figure 2 Amylase Inhibition Assay of Extracts: Starch agar plates showing inhibition assay at different concentrations of (A) n-hexane extract (B) n-butanol extract. 1-6 represents decreasing concentrations of extracts. (C) Human saliva (3 µg/ml).

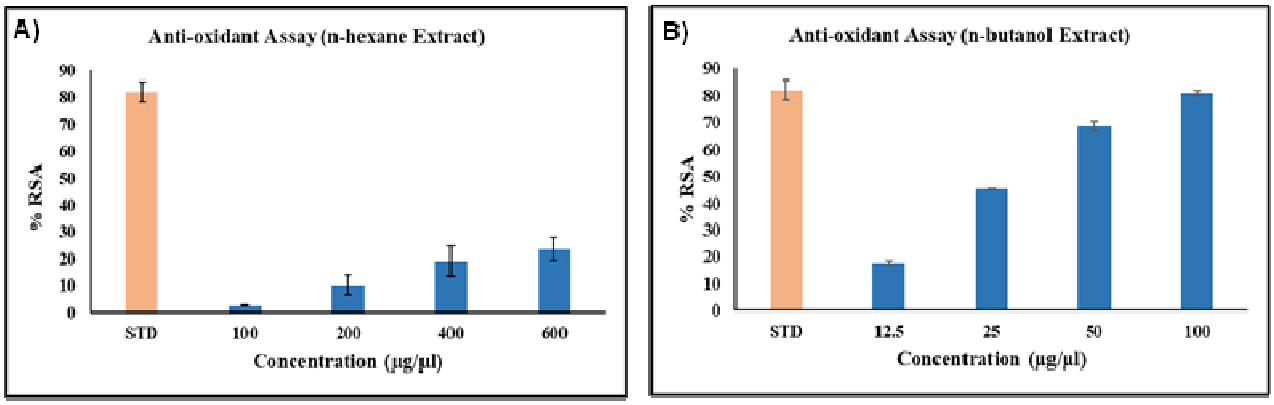

Figure 3 Antioxidant Assay of Extracts: Graphical representation of %RSA of (A) n-hexane (B) n-butanol extract. (STD) %RSA of Ascorbic acid at 25 µg/µl concentration.

DISCUSSION

In modern world, more than 25% of the drugs are plant derived or their synthetic analogs. Paclitaxel (Taxol), a potent anticancer drug, is a terpenoid isolated from Pacific Yew Tree bark (Barbuti and Chen, 2015). Resveratrol a polyphenol is an anticancer, anti-inflammatory, antioxidant, antibacterial, neuroprotective, and anti-hypertensive drug, which is extensively used worldwide (Zhang *et al.,* 2021). Artemisinin from *Artemisia annua* is an important natural sesquiterpene lactone which is used as an antimalarial drug (Ivanescu *et al.,* 2015). Different parts of *Datura* were used in traditional medicine for the treatment of rheumatism, burns, ulcer, wounds, inflammation, bronchitis, Parkinson disease, sciatica, bruises, asthma, sinus, allergies, fever, and toothache (Sharma *et al.,* 2021). The present study was conducted to relate *Datura alba* seed extract qualitative properties with their biological activities.

The n-hexane extract exhibited svPLA$_2$ inhibitory activity with 19.075 % inhibition by the highest extract concentration of 800 µg/µl. Methanolic root extract of *Hemidesmus indicus* and leaves extract of *Leucas aspera* Linn has also shown PLA$_2$ inhibitory activity against viper and cobra venom. The inhibitory activity could be attributed to the presence of flavonoids and terpenoids in these extracts (Gopi *et al.,* 2014). A flavonoid (Quercetin-3-O-*α*-rhamnoside) isolated from *Euphorbia hirta* and *Cecropia peltata* extract showed *Naja naja* venom PLA$_2$ inhibiting capability (Singh *et al.,* 2017). Also, triterpenoid lupeol acetate isolated from Indian sarsaparilla *Hemidesmus indicus* (*L.*) neutralizes PLA$_2$ of *Naja kaouthia* venom (Chatterjee *et al.,* 2006). It has been observed that these phytochemicals make stable complexes with venom PLA$_2$ enzymes by forming

hydrogen bonds with protein hydroxyl groups thus inhibiting their activity (Samy *et al.,* 2012). The n-hexane and n-butanol extract showed venom protease and PLA_2 enhancing activities respectively. The extract must have micro/macro compounds like enzyme enhancers or ions that may aid in these enhancing activities. It has been observed that enzymes in presence of divalent ions like Ca^{+2}, Sr^{+2}, Ba^{+2} *etc* undergo conformational changes resulting in promotion of their biological activity (Marques *et al.,* 2018). The n-butanol extract showed neither inhibiting nor enhancing activity towards proteases from *Echis carinatus* venom. Metalloproteinases are the abundant enzymes found in Viperid venoms. It would be possible that this extract does not express inhibitors specific to these metalloproteases.

The inhibition of alpha amylases secreted from saliva and pancreas are important for the management of type 2 diabetes. In current study, human salivary amylase inhibitory activity was observed by both the extracts. It has been noted that presence of polyphenols, catechins, and procyanidins (flavan-3-ols) act as effective inhibitors of these enzymes (Yilmazer-Musa *et al.,* 2012; Mogole *et al.,* 2020). However, in the case of n-butanol extract, the inhibitory activity was increased by decreasing extract concentration. This could be explained by the phenomena of hormesis (Calabrese and Baldwin, 2001) or due to presence of positive allosteric modulator at higher concentrations that increases enzyme activity and render extract from inhibition of enzyme (Abdel-Magid 2015). DPPH has a free nitrogen radical with an unpaired valence electron. In antioxidant assay, flavonoids donate hydrogen and electrons by hydroxyl groups; DPPH loses its intensity and changes its color from purple to yellow when nitrogen atom accepts the electrons and forms stable radicals (Vuolo *et al.,* 2019). In our study, the n-hexane extract exhibited low while n-butanol extract exhibited high DPPH scavenging potential with reference to the standard ascorbic acid. Extraction of phytochemicals in different organic solvents depends on the polarity of solvents. The extract obtained in lower polarity solvent (n-hexane) has weaker antioxidant activity that could be due to low content of polyphenols as also shown by Khan *et al.,* 2019. On the other hand, strong antioxidant activity observed in presence of high amount of polar lipids and phenolics as extracted in n-butanol which were flavonoids and terpenoids (Bachheti *et al.,* 2018; Aryal *et al.,* 2019).

Conclusions: In summary, the fractionated extracts (FEs) of *Datura alba* seeds showed the presence of flavonoids, terpenoids while alkaloids were only found in n-butanol extract. Saponins were absent in both the n-hexane and n-butanol extracts. TLC spots indicated the presence of both polar and non-polar compounds in FEs. Both the extracts showed antioxidant and anti-amylase activity. The interesting observation was the inhibition of venom PLA_2 by n-hexane extract, an enzyme which is one of the major components and is responsible for toxicological effect caused by many snake venoms. This study therefore opens the potential to identify bioactive compounds from *Datura* seed extracts for neutralization of venom toxic effect along with other therapeutic applications.

Conflict of Interest: Authors declare no conflict of interest.

Co-authors contribution: SMF and WT executed all the experiments. MM supervised phytochemical extraction and analysis. HW conceived, designed the study and supervised *in vitro* biological assays. HK assisted in antioxidant assay. All these authors have substantial contributions to the final manuscript and approved this submission.

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