Original Research Article

Assessment Of Antivenom Potential Of *Anthocephalus cadamba* Leaves Extract: A Traditionally Used Medicinal Plants For The Treatment Of Snakebite Patients

Diganta Das, Md. Minhaj Ansari, Nima D. Namsa, Mukherjee, A.K and Robin Doley*

Department of Molecular Biology and Biotechnology, Tezpur University, Tezpur-784028, Sonitpur, Assam, India

***Corresponding author:** doley@tezu.ernet.in

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Abstract: Snakebite is a major socio medical problem and use of commercially available antivenom is only the source of treatment, which has its own limitations. In India, medical facilities are inadequate and people in rural areas mostly depend on traditional healers and herbal antidotes. We have previously documented the medicinal plants used by the traditional healers for treatment of snakebite patients in Morigaon district of Assam, India. However, the traditional use of these medicinal plants lacks scientific evidence to support this claim. In the present study we have screened these medicinal plants for their anti-snake venom property. Various extracts were prepared using aqueous and organic solvents from the plant parts, which are used for treatment of snakebite patients by local healers. The extracts were dissolved in a saline solution (0.9% NaCl) and tested for their anti-snake venom activities. Out of the 19 plants screened, only 5 plants were found to possess phospholipase A2 (PLA2) inhibition property. The methanol extract of leaf of *Anthocephalus cadamba* (ACME) showed the highest inhibition of crude venom PLA2 activity. ACME was also found to inhibit the direct and indirect haemolytic activity of crude venom. Other important properties of "Big four" crude venom such as proteolytic activity and anticoagulation were not inhibited. In-vivo- neutralization of lethality shows that ACME was ineffective against *Naja naja* and *Bungarus caeruleus* venoms but partially effective against *Daboia russelli* and *Echis carinatus* venoms at doses tested under a given experimental condition. The anti-snake venom properties of ACME may be attributed to the presence of bioactive compounds.

Key words: Snake venom, Snakebite therapy, Anti-snake venom, Big four, *Anthocephalus cadamba*

Introduction

Snakebite is a "Neglected Tropical Disease" which needs immediate attention (www.who.int/neglected_diseases/diseases/en). Globally 0.5-1.8 million people are affected and in India approximately 40-50 thousand people die annually due to snakes bite (Mohapatra *et al*., 2011). In India "Big Four" - the Spectacled cobra (*Naja naja*), Common krait (*Bungarus caeruleus*), Russell's viper (*Daboia russelii*) and Saw-scaled viper (*Echis carinatus*) are responsible for most of the fatalities. Antivenom therapy is the only effective treatment of snakebite patients, however, it has many limitations like serum sickness, pyrogenic reactions and anaphylactic reactions (Gilon *et al*., 1989; Russell *et al*., 1985). In India polyvalent antivevenom is raised against these four venoms for treatment of snakebite patients. Pharmacological affects like haemorrhage, necrosis, nephrotoxicity induced during envenomation are not neutralized (Stahel *et al*., 1985; Sutherland, 1992; Theakston *et al*., 2003; Thwin *et al*., 2010). Moreover the antivenom is effective only when the patients receive within 4-6 hrs post envenomation. It has been observed that the rural hospitals do not have adequate facility for storing

this life saving medication. Hence in most instances patients have to travel long distance for treatment. Therefore, more effective and readily available antivenom is required to tackle this "Neglected Tropical Disease".

North east India is an integral part of the Indo-Burma biodiversity 'hotspot' and home to many endemic and rare species of flora and fauna (Myers *et al.*, 2000). Many plants have been used by the local healers and traditional practitioners for the treatment of various diseases including snakebite. However, the lack of proper documentation and scientific validation on medicinal plants often prevent the discovery of novel plant based therapeutic molecules. In recent years, exploration on medicinal plants for identification and isolation of bioactive molecules have been perused in many parts of India and abroad against snakebite (Chatterjee *et al.*, 2006; Da Silva *et al.*, 2008; Mahanta and Mukherjee, 2001; Martz, 1992; Mukherjee *et al.*, 2008; Shirwaikar *et al.*, 2004). Recently we have documented the traditionally used medicinal plants for treatment of snakebite patients in Morigaon district of Assam (Kalita *et al.*, 2014).

Anthocephalus cadamba (Roxb.) commonly known as Kadam (Assamese) is a large size tree which is widely distributed throughout India. The bark, leaves and fruits are reported to be used in the treatment of fever, anaemia, uterine complaints, blood diseases, skin diseases, leprosy, dysentery, for improvement of semen quality, stomatitis, anti-fungal, antimalarial, antitumor, and anti-hepatotoxic activities, (Castillo and Hudgins, 1989; Mishra and Padhy, 2013; Mishra and Siddique, 2011; Slkar *et al.*, 1992). The traditional healers of Morigaon district of Assam extract the juice of the fresh leaves and administer to the snakebite patients orally (Kalita *et al.*, 2014). However, these unexplored plants claimed to be antidotes in folklore medicine lacks proper scientific validation. Therefore, in the present investigation, an effort was made to evaluate the anti-snake venom property of traditionally claimed snakebite plant extracts against the 'Big Four' venoms of India. Of the 19 plants screened, the methanol extract of leaf of *Anthocephalus cadamba* (ACME) exhibited the maximum inhibition of big four crude venom PLA_2 activity and was partially effective in neutralizing the lethality of *Daboia russelii*and *Echis carinatus* venoms.

Materials and methods

Chemicals

All chemicals and solvents used were of analytical grade and procured either from Merck or Sigma-Aldrich, (USA).

Snake venoms and animals

Crude lyophilized venom of *Naja naja, Daboia russelii, Bungarus caeruleus* and *Echis carinatus* commonly known as "Big Four" were procured from Irula Snake Catcher Industrial Co-operative Society limited Ind. 969 Vadanemmeli Village, District Kancheepuram, Tamil Nadu, India. Venoms were stored in -20°C until further use.Male Swiss albino mice of 40±3gm were obtained from central animal facility, University of Mysore were used for animal studies. All animals were housed in well ventilated cages with access to food and water *ad libitum*. The protocol for animal experimentation was approved by Animal Ethical Committee, University of Mysore, Mysore, India, (Proposal no. UOM/IAEC/25/2011).

Collection of medicinal plants and preparation of plant extract

Identified medicinal plants were collected from Morigaon district of Assam and Tezpur University campus and specimen were deposited in the departmentof Molecular Biology and Biotechnology, Tezpur university, Tezpur, Assam, India (Kalita *et al.*, 2014).

Plant extracts were prepared according to the previously published methods (Alam and Gomes, 2003; Mahanta and Mukherjee, 2001). Briefly, fresh leaves/roots/barks were thoroughly washed with tap water followed by distilled water and dried at room temperature. Dried plant parts were made to a coarse powder and extracted. For extraction, 2gm powder of either leaves or roots or bark was soaked with 100 ml of distilled water (aqueous extract) with continuous stirring for 2hr at room temperature. The extract was filtered through a muslin cloth and filtrate was freeze

dried. The residue were further extracted by 50% ethanol and then refluxed with methanol (60–80°C for 72hr) (methanol extract). Extract were concentrated and stored in a desiccator at room temperature for further use and named as *Anthocephalus cadamba* methanol extract (ACME). The dried extract was finally dissolved in normal saline (0.9% NaCl) at a concentration of 5mg/ml and centrifuged at 2000rpm (Thermo Scientific, USA, Heraeus Multifuge X1R) for 10mins and the supernatant was used for the neutralization studies.

Neutralization of phospholipase A_2 activity

Phospholipase A2 (PLA2) activity using egg yolk as a substrate was measured as described by Joubert and Taljaard (Joubert and Taljaard, 1980) and modified by Doley and Mukherjee (2003). For neutralization of PLA2 activity of snake venom by plant extracts, 1µg of crude venom was incubated with different amount (µg) of plant extracts (PE) for 30 min at 37°C. PLA2 activity without plant extract was treated as control (100% activity) and compared with other values. One unit of PLA2 activity is defined as decrease in 0.01 absorbance in10 min at 740 nm.

Neutralization of direct and indirect haemolytic activity

Fresh goat blood was collected in citrated tube (0.11M Tri sodium citrate) at 1:9 ratio (citrate: blood) and centrifuged at 3000 rpm for 15mins. The plasma was collected and used for anticoagulant assay and RBC pellet was washed 4-5 times with 0.90% NaCl by centrifugation (Mukherjee and Maity, 2002). The RBC pellet was resuspended in 0.9% saline to a final concentration of 10% (v/v). Various concentrations of plant extract was pre-incubated with crude venom for 60 mins at 37°C followed by addition of 150µl of 10% RBC to final volume of 2ml with 0.9% NaCl. This mixture of plant extract, venom and RBC was incubated for 60 minutes at 37°C. The tubes were centrifuged at 5000rpm for 10 min and supernatant was transferred to fresh tubes. Absorbance of the supernatants was measured at 540nm using a MultiSkan GO, UV-Vis spectrophotometer (Thermo scientific, USA). The haemolysis caused by distilled water (dH_2O) was considered as 100% haemolysis. For indirect hemolytic assay, 20µl of egg yolk substrate solution was added to the reaction mixtures at the time of incubation and hemolysis was measured as described for direct hemolytic assay. The activity without the plant extract was considered as 100% activity and accordingly the percentage (%) of inhibition was calculated.

Neutralization of anticoagulant activity

Various amount of plant extract was incubated with crude venom in 50µl of PBS for one hour at 37°C. This pre-incubated mixture was incubated with 50µl of goat plasma at 37°C for 3 min and 50µl of 0.025M CaCl2 was added to initiate the clot formation. The clotting time was compared with crude venom's clotting time for its inhibition. The clotting time with PBS was considered as normal clotting time.

Neutralization of proteolytic activity

Various amounts of plant extract were incubated with crude venom (0.1mg) in 50µl of PBS for one hour at 37°C. Then, 1% (w/v) casein in 20mM Tris-Cl, pH7.4 was incubated with pre-incubated mixture. The enzymatic reaction was stopped by adding ice cold 10% (v/v) trichloroacetic acid and centrifuged for 10min at 5000rpm. The proteolytic activity of digested protein in the supernatant was determined according to Lowry's method.

Neutralization of lethality

To determine the effective dose (anti-venom potential) of ACME, twice the lethal potency dose of "Big four" venoms were pre-incubated with different doses of ACME (1:0.5 - 1:100) for 1hr at 37°C and injected intra-peritoneally to Swiss Albino mice (n=10). The animals (n=10) which received twice the lethal dose of venom, PBS and extract alone served as negative controls. After injection, mice were monitored up to 24 hr for any physiological changes and the survival time was recorded.

Results

Screening of medicinal plants for anti-PLA_2 property

The leaves extracts and crude venom was pre-incubated at the ratio of 1:1, 1:2.5, 1:5, 1:25 and 1:50 (plant extract: venom;

Table 1. Screening of medicinal plants leaf extracts for anti-PLA_2 activity

Sl. No.	Scientific Name	Inhibition of crude venom PLA_2 activity by various extracts		
		Water	50% ethanol	Methanol
1	*Acacia pannata*	Nil	Nil	Nil
2	*Acalypha indica*	Nil	Nil	Nil
3	*Amaranthus viridis*	Nil	Nil	Nil
4	*Antidesma acidam*	Nil	Nil	Nil
5	*Baccaurea ramiflora*	+	+	+
6	*Foeniculum valgare*	Nil	Nil	Nil
7	*Gloriosa superb l.*	Nil	Nil	+
8	*Glycosmis pentaphyla*	Nil	Nil	Nil
9	*Heterofragma adenoflora*	Nil	Nil	Nil
10	*Heteropanax pragus*	Nil	Nil	Nil
11	*Leucas linifolia*	+	+	+
12	*Mentha viridis*	Nil	Nil	Nil
13	*Opuntiadilleni L*	Nil	Nil	Nil
14	*Pogostemon parviflorus*	Nil	Nil	Nil
15	*Polygonum bracteatum*	Nil	Nil	Nil
16	*Piper longum*	Nil	Nil	Nil
17	*Sansevieria lourentii*	Nil	Nil	Nil
18	*Xanthium strumarium*	+	+	+
19	*Anthocephalus cadamba*	+	++	+++

+ sign indicated ability to neutralize the enzymatic activity of PLA_2 enzyme

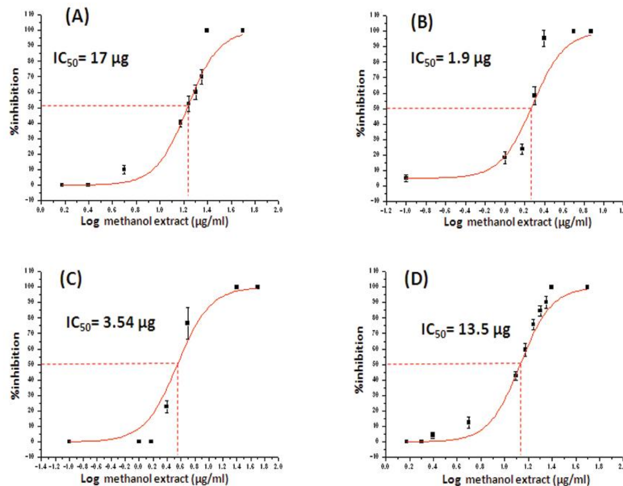

Fig. 1. Inhibition of crude venom PLA_2 activity by methanol extract of *A. cadamba* leaves. Panel (A) *E. carinatus*, (B) *D. ruselii*, (C) *N. naja* and (D) *B. caeruleus*. Results are means of three experiments. The PLA2 activity in absence of plant extract was considered as 100% activity.

w/w) prior to screening of anti-PLA2 compounds. Of the 19 plants tested, methanol extract of only four plants viz: *Baccaurea ramiflora, Leucas linifolia, Xanthium strumarium,* and *Anthocephalus cadamba* showed positive towards *in-vitro* neutralization of enzymatic activity of PLA2 enzyme of the crude cobra venom *(Naja naja)* (Table 1). The methanol

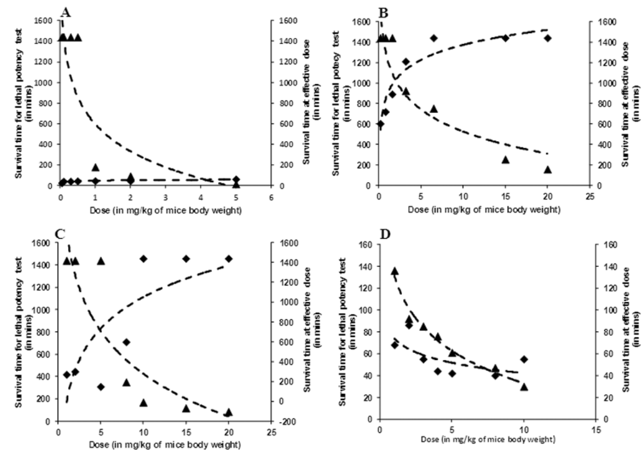

Fig. 2. Inhibition of lethality of "Big four" venoms (A. *Naja naja*; B. *Daboia russelli*; C.*Echis carinatus*; D. *Bungarus caeruleus*) by ACME: For inhibition studies, twice the lethal potencydose of Big four venoms was pre-incubated with different doses of ACME (1:0.5 - 1:100) for 1h at 37°C and injected intraperitonially. Lethal potency of respective venom. Neutralization of venom lethality by plant extract.

extract of *Anthocephalus cadamba* named as ACME showed the highest inhibitory activity. Hence ACME was tested against the "Big Four" crude venoms which inhibited the PLA2 activity in a dose-dependent manner(Figure 1). The IC_{50} of the ACME for the enzymatic activity of PLA2 enzyme was calculated using the Origin 8 software. The highest inhibitory

Table 2. Neutralization of few activities of crude venom by methanolic extract of *A. cadamba* leaves. Results are means of three experiments. The activity in absence of plant extract was considered as 100% activity. CV: crude venom; PE: methanol extract of *A. cadamba* leaf.

In-vitro snake venom activities	Crude venom	% inhibition (CV: PE in µg)				
		1:1	1:2.5	1:5	1:25	1:50
Direct hemolytic activity	*N. naja*	65±5.13	85±4.13	100±3.14	100	100
	D. russelii	100	100	100	100	100
	E. carinatus	8536±1.13	100	100	100	100
	B. caeruleus	100	100	100	100	100
Indirect hemolytic activity	*N. naja*	55±2.13	77±2.17	99±4.15	100	100
	D. russelii	Nil	Nil	Nil	Nil	Nil
	E. carinatus	Nil	Nil	Nil	Nil	Nil
	B. caeruleus	Nil	Nil	Nil	Nil	Nil
Recalcification time	*N. naja*	Nil	Nil	Nil	Nil	Nil
	D. russelii	Nil	Nil	Nil	Nil	Nil
	E. carinatus	Nil	Nil	Nil	Nil	Nil
	B. caeruleus	Nil	Nil	Nil	Nil	Nil
Proteolytic activity	*N. naja*	Nil	Nil	Nil	Nil	Nil
	D. russelii	Nil	Nil	Nil	Nil	Nil
	E. carinatus	Nil	Nil	Nil	Nil	Nil
	B. caeruleus	Nil	Nil	Nil	Nil	Nil

concentration (IC) of ACME was recorded against the crude venom of *Echis carinatus* (IC_{50}=17 µg). Whereas moderate to low PLA2 inhibition activity against the venom of *Bungarus caeruleus* (IC_{50}=13.5 µg), *Naja naja* (IC_{50}=3.54 µg) and *Daboia russelii* (IC_{50}=1.9 µg) was observed when pre-incubated with ACME (Fig. 1). The result of this study showed that 5µg of the ACME was required to achieve complete neutralization ofPLA2 activity of 1µg of crude venom of *Daboia russelii*. However, the amount of ACME extract needed to neutralize the venoms of other snakes was higher than the venom of *Daboia russelii*. The direct activities of "Big Four" crude venoms were inhibited by the ACMEand the complete inhibition was obtained in the venom-extract proportion at 1: 25 (venom: ACME) (Table 2). However, the indirect haemolytic of only *Naja naja* venom was found to be inhibited by ACME. The recalcification time and proteolytic activity of the "Big Four" venoms were not inhibited by ACME even at a ratio of 1:50 (Table 2). Hence the bioactive compounds present in this extract might be only targeting the active site of the PLA2 enzyme.

Table 3. In-vivo neutralization of lethality of "Big four" venoms by ACME, NE: not effective. Results are expressed as a mean of three observations. The ED50 value is expressed in terms of ±SD. PE: Plant extract.

Venom property	Snake species	LD50 dose of crude venom (mg/kg)	2x LD50 dose of crude venom (in µg)	Fold neutralization by PE	ED50 Value (in µg)
Lethal action	*Naja naja*	0.6	2.4	0.01	NE
	Bungarus caeruleus	1.76	3.6	0.24	NE
	Echis carinatus	2.21	4.42	3.404	144.16± 0.5
	Daboia russelli	2.36	4.72	2.9	164.04± 1.2

Neutralization of lethality of crude venoms

The lethal doses of big four venoms were calculated according to the mathematical calculations of Meier and Theakston (Meier and Theakston, 1986). To determine the anti-venom potential of ACME, twice the lethal doses of big four venoms was incubated with varying doses of ACME (1: 0.5 w/w to 1:100 w/w) for 1hr and injected through intra-peritoneal route (Table 3). ACME failed to neutralize toxicity as it did not increase the survival time of experimental animals injected with *Naja naja* and *Bungarus caeruleus* venoms at tested doses (Fig. 2A & 2D). Intriguingly, ACME was effective in neutralizing the toxicity and increased the survival time of experimental animals treated with *Daboia russelii* and *Echis carinatus* venoms (Fig. 2B & 2C).

Discussion

Many plant species have been reported from Assam and its adjoining areas with medicinal properties against various diseases including snakebite (Bhattacharya *et al.*, 1991). Recently plants used as folk medicine by various communities of this region have also been reported (Namsa *et al.*, 2009; Namsa *et al.*, 2011; Shankar *et al.*, 2012; Tarak *et al.*, 2011). The systemic evaluation of these plants in future would be source for drug development including treatment of snakebite cases (Heinrich, 2000; Heinrich and Bremner, 2006; Kumar *et al.*, 2013; Lawal *et al.*, 2012). The medicinal plants used by the traditional healers of Morigaon district of Assam reveals roots, leaves, tuber, and fruits are the parts of the plant used for preparation of the medicine used against various diseases including snakebite (Kalita *et al.*, 2014). Due to ease in collection and presence of compounds like ellagic acid, tannins, polyphenols, flavanoids and other alkaloids, leaf has been found to be commonly used for treating snake bite patients (Biondo *et al.*, 2003; Cavalcante *et al.*, 2007; Mukherjee *et al.*, 2008; Shirwaikar *et al.*, 2004; Vishwanath *et al.*, 1987).

PLA2 enzyme is one of the most toxic proteins of snake venom and is present ubiquitously in all snake venoms. In addition to its enzymatic activity it exhibits various other pharmacological properties such as myotoxicity, anticoagulant, cardiotoxicity, neurotoxicity, edema inducing, platelet aggregation etc which are either dependent or independent

on its enzymatic activity (Kini, 1997; Kini, 2005). ACME showed inhibition of enzymatic activity of PLA2 activity of crude venom as well as the direct and indirect haemolytic activity. Direct haemolytic activity of PLA2 enzyme is due to hydrolysis of the RBC membrane which is dependent on its enzymatic activity (Doley *et al.*, 2004; Kini and Evans, 1989; Mukherjee *et al.*, 1998). Therefore loss of direct haemolytic activity in presence of ACME is due to inhibition of enzymatic activity of PLA2 enzymes. Whereas indirect haemolytic activity is due lysophospholipid and free fatty acids which are hydrolysis products phospholipid by PLA2 enzyme (Condrea *et al.*, 1964). Inhibition of the indirect haemolytic activity indicates that the plant compounds might be inhibiting the enzymatic activity of the PLA2 but not the pharmacological sites. Inability to inhibit the indirect haemolytic activity of the *Echis carinatus*, *Bungarus caeruleus* and *Daboia russelii* might be because of the high rate of enzymatic which generates lysophospholipid and free fatty acids and causes the hemolysis. The anticoagulant activity of the crude venom is mostly exhibited by proteins belonging to proteases, C-type lectin, three-finger toxins kunitz-type serine proteases and PLA2 families. The inhibitory compound present in the ACME might not be inhibiting the activity of these protein families except PLA2 family hence the anticoagulant activity of the crude venom is not neutralized. Most of the anti-snake venom compounds have been reported from the methanol extract of the medicinal plants (Alam and Gomes, 2003; Chatterjee *et al.*, 2006; Mahadeswaraswamy *et al.*, 2008; Mukherjee *et al.*, 2008) and our results also indicated the presence of anti-PLA2 compounds in the methanolic extract. The lower amount of antagonist required for neutralization of PLA2 activity of crude *Daboia russelii* venom might be due to presence of catalytically inactive PLA2 whereas in elapids the PLA2 enzymes are catalytically active. Isolation and structural characterization of this compound would be helpful in designing anti-PLA2 drugs that could be used to treat snakebite patients. However, absence of anti-PLA2 compounds in other plants of the present study does not rule out the possibility of using as antidote to snake bites as it might be neutralizing the toxic effect of other components of snake venom proteins.

Lethality of *Daboia russelii* and *Echis carinatus* venom was found to neutralize up to 2.9 and 3.404 fold, respectively. The extract could not protect the mice when challenged with *Naja naja* and *Bungarus caeruleus* venom. The components present in ACME might be effective in neutralizing the toxins that act locally causing myotoxicity, edema and haemorrhage. However ACME might be ineffective in neutralizing the toxic effects of low molecular weight toxins which are abundantly present in *Naja naja* and *Bungarus caeruleus* venoms. Several active molecules from medicinal plants possessing anti-PLA2 activity has been purified and characterized. For example sesquiterpenes and flavonoids from *Santolinacham aecyparissus* have been reported to inhibit the pharmacological activities of PLA2 (Sala *et al.*, 2000). Oleanolic acid, a triterpenoid commonly present in several medicinal plants, has been reported to form irreversible complex with PLA2, thus inhibiting its activity (Dharmappa *et al.*, 2009). It has been reported that the isoquinoline alkaloid isolated from *Cardiospermum halicacabum* binds competitively to the active site of the PLA2 enzyme (Chandra *et al.*, 2011). The inhibition of enzymatic activity of crude venom PLA2 and lethality by ACME suggest the presence of bioactive compound which might be interacting with other toxic proteins present in the venom. We are working on the isolation and characterization of bioactive molecule present in the leaf extract of *A. cadamba* to understand the molecular mechanism of interaction with snake venom PLA2 enzymes. The future study on this compound and its mechanism of inhibition might enable us to synthesize anti-PLA2 molecule for therapeutic application.

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