RESEARCH REPORT

Evidence for *in vitro* antiophidian properties of aqueous buds extract of *Eucalyptus* against *Montivipera bornmuelleri* venom

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ABSTRACT

Because snake venoms are complex mixtures of bioactive molecules, snake bites produce a large panel of symptoms which cannot be totally prevented by current antivenoms. Thus investigating plant extracts for antivenomics therapy approaches seemed relevant. Here, we evaluated the potency of the aqueous Buds extract of *Eucalyptus* (ABEE) to counteract the main enzymatic activities of *Montivipera bornmuelleri* venom. We showed that ABEE efficiently counteracts the proteolytic, Phospholipases A2 (PLA2), and L-aminoacid oxidase activities (LAAO) of *M. bornmuelleri* venom. ABEE was found to inhibit Acetylcholine esterase (AChE) and to exhibit a potent antioxidant activity. ~~While~~ In addition, *M. bornmuelleri* venom displays antibacterial properties against *Staphylococcus aureus*, which were not inhibited by ABEE. We also showed that of *M. bornmuelleri* venom lacks AChE, either anti-AChE activities. ABEE represents a promising natural source of antivenomics compounds against the deleterious effects of *M. bornmuelleri* or other Vipera species bites.

KEYWORDS: *Montivipera bornmuelleri* venom*, Antivenomic approaches, Aqueous bud extract of Eucalyptus, Natural anti-venoms.*

INTRODUCTION

Snake bite envenoming constitutes a neglected public health issue, where bites by the members of the families Viperidae and Elapidae snakes are responsible for severe cases (White, 2000).-For many decades, immunotherapy remains the exclusive treatment against snakebites envenomation. However, antivenoms induce diverse side effects, including itching, fever, hypotension or bronchospasm. In addition, antivenom accessibility represents a major difficulty particularly for people living in rural countries (Guttiérez et al, 2014). Thus, other alternative treatments of snake bite envenoming have been developed, such as the use of plants.

Historically, plants constitute a source of food and medicine since ancient times. The low cost and the accessibility of folk medicine triggered scientific investigations that demonstrated the ability of some plants to treat snakebites (Felix-Silva et al, 2014). Thus, vegetal extracts could be considered as promising natural sources of effective antivenom compounds (Ahmed et al, 2010).

In Lebanon, snake bite mostly occurs in mountains or deserted habitats. The *Eucalyptus* plant was used in traditional medicine as a source to treat colds and flu, respiratory infections, coughs, sore throats, asthma, bronchitis (Sinclair, 1996), reasons for which we explored its potential for antiophidian properties/activities. *Eucalyptus* genus gathers several species belonging to Myrtaceae, a family well-known for its richness in secondary metabolites as terpenoids and polyphenols, including flavonoids and tannins (Hardel and Laxmidhar, 2011). However, *Eucalyptus* genus can be considered as a promising source of antivenomics compounds, since they contain enzymatic inhibitors such as trypsin inhibitors (Tremacoldi and Pascholati, 2002).

The Lebanese Vipera *Montivipera bornmuelleri* is a scarce snake who lives on high attitudes between vegetations and rocks (Hraoui-Bloquet et al, 2012). Since it represents a potential danger for human, but no bites cases have been recorded up to date. In our previous studies, we have shown that the venom of this Viperidae species possesses enzymatic activities such as PLA2, LAAO, and proteolytic. *M. bornmuelleri* venom contains antifungal and antibacterial compounds and exhibits potent, lethal and deleterious effects, such as inflammation, pro-coagulant, anticoagulant effects, hemolytic activity and more recently, it has proven to have a relaxant effect on vascular contractility (Accary et al, 2014a,c; Accary et al, 2016).

Here, using *in vitro* assays, we aim to study the antiophidian activity of the ABEE against the main enzymatic activities of *M. bornmuelleri* venom and characterize some biological properties of the aqueous Buds extract of *Eucalyptus* extract.

MATERIALS AND METHODS

Chemicals and reagents

Formic acid (FA), acetonitrile (ACN), L-Leucine, trifluoroacetic acid (TFA), calcium dichloride ($CaCl_2$), methanol, sodium chloride (NaCl), 2,2-diphenyl-1-picrylhydrazyl (DPPH), acetylcholinesterase (AChE), 5,5'-dithiobis-(2-nitro benzoic acid) (DTNB), acetylcholine iodide, trypsin, were from Sigma-Aldrich (USA). Muller Hinton agar was purchased from Bio-Rad.

Snake venom

Freeze dried *Montivipera bornmuelleri* venom was obtained from the American University of Beirut (Beirut), and stored at -20°C in a dry and light free place.

Plant material

The buds of *Eucalyptus genus* plant were collected from Deir Ammar town in the north governorate (Lebanon). The plant part was dried at room temperature, crushed to powder and stored in a sealed container until needed.

Preparation of the aqueous extract

Eucalyptus plant crushed buds were dissolved in PBS buffer/deionized water and left at room temperature to soak properly. The plant suspension was centrifuged 10 minutes (full speed) and the

plant essence found in the supernatant constitutes the aqueous buds extract of *Eucalyptus* (ABEE) used for all experiments.

Proteolytic activity assay

Protease activity was determined using milk agar plates. 100µg venom was preincubated with 100µg of ABEE for 1hr at 37°C. Briefly, the preincubated sample was loaded onto 6 mm diameter wells of milk agar plates and incubated overnight at 37°C. The protease inhibition was evaluated by measuring the zone of clearance. Trypsin effect served as a positive control.

Phospholipase A2 activity assay

PLA2 activity assay was evaluated using egg yolk as a substrate in agar plates according to the method described by Habermann and Hardt (Habermann and Hardt, 1972). Dried snake venom was dissolved in PBS buffer and preincubated for 1hr at 37°C with ABEE. Then this mixture-were loaded onto 6mm egg yolk agar plate containing egg yolk and 0.01mM $CaCl_2$ followed by overnight incubation at 37°C. The PLA2 activity of venom served as control.

Antioxidant activity evaluation

DPPH assay provides an easy and rapid way to evaluate the antioxidant potential of a product (Brand-Williams et al, 1995). DPPH free radical method is an antioxidant assay based on electron-transfer that produces a violet solution in methanol. This free radical, stable at room temperature (RT) is reduced in the presence of an antioxidant molecule, giving rise to a yellow solution. The free radical scavenging activity of the aqueous extract of *Eucalyptus* plant was measured in terms of hydrogen donating or radical scavenging ability using this method. DPPH solution was prepared by dissolving 0.4mg in 5ml methanol. In a glass tube, 1ml of ABEE (correspondent to 500µg suspended in 1ml deionized (DI)

water) was added to 1ml DPPH solution. 2ml DPPH solution serves as negative control. The absorbance changes were monitored at 517nm at 0min and after 30min, using a UV-VIS spectrophotometer. The higher percentage of the index of scavenging activity, the more powerful is the antioxidant capacity. The equation used to determine the %RSA (radical scavenging index): [(Acontrol – Asample)/Acontrol]*100.

L-Amino Acid Oxidase activity measurement

L-Amino Acid Oxidase (LAAO) activity measurement was determined by a spectrophotometric method using L-leucine as substrate (Shuqing et al, 2011). The reaction was performed in a 96 well plate, containing L-leucine substrate (0.023µg/µl), *M. bornmuelleri* venom (140µg), and with or without ABEE (1mg of dried ABEE). The absorbance was monitored at 450nm and the plate was incubated at 37°C for 2hr. The reaction was then stopped by the addition of 100µl of trifluoroacetic acid (TFA 27%) and the absorbance was measured once again. LAAO activity is associated with an increase in absorbance at 450nm.

Measurement of acetylcholinesterase activity

AChE activity was determined by a spectrophotometric method according to Ellmane et al (1961). The enzyme activity was measured by following the increase of yellow colour produced from thiocholine when it reacts with dithiobisnitrobenzoate (DTNB) ion. The reaction was performed at room temperature (RT) for 5 minutes in a final volume of 3ml, containing 0.2M phosphate buffer (pH = 8), DTNB (0.01M), acetylthiocholine iodide substrate (0.07 M). The absorbance was followed at 412nm for 5 minutes. To assay AChE inhibitory effects of ABEE, standard AChE (0.25u) was added to the reaction mixture and incubated for 1hr at RT before measurement. Standard AChE enzyme was used as a positive control.

RESULTS AND DISCUSSION

Since the *M. bornmuelleri* venom has been shown to exhibit proteolytic, PLA2, LAAO and also antibacterial activities (data not showed), we challenged the effects of ABEE on these four properties. First, we assayed ABEE on each of those activities as well on the standard AChE activity. A protein contains analysis was conducted by LC-ESI-MS to have an overall idea on the composition of the extract (data not showed).

The aqueous buds extract of *Eucalyptus* inhibits proteolytic activity of *M. bornmuelleri* venom

Agar well diffusion method revealed that of *M. bornmuelleri* venom produced a lysis zone equal to 0.9 ± 0.06cm (n=3), indicating a high caseinolytic activity of the venom. 100µg of *M. bornmuelleri* venom-induced 35.5 ± 2.3% (n=3) of protease activity normalized with trypsin. In the presence of 100µg of ABEE, the lysis zone disappeared, showing the complete inhibition of the proteolytic activity of *M. bornmuelleri* venom (Figure 1A). A strong protease inhibitory effect of the extract of another *Eucalyptus* species (*Eucalyptus Urophylla*) has been already reported, but the component was not characterized (Tremacoldi and Pascholati, 2002). Indeed, the presence of trypsin inhibitors has been observed in a seeds extracts of *Eucalyptus- Urophylla* and the authors hypothesized that secondary metabolites, such as phenolic compounds and others (asterpenoids, alkaloids, or steroids) were responsible for the inhibition metalloproteases (Tremacoldi and Pascholati, 2002). In addition, four Taiwanese plants (*D. discolor*, *E. deflex*, *M. japonica* var. *kusanoi*, and *P. taiwanensis*) have been shown to have the highest phenolic content and the ability to strongly inhibit metalloproteases activity (Lee et al, 2009). The idea that plants are natural sources of metalloprotease inhibitors are also

supported by the study of *Euphoria longana* (Panyathep et al, 2013) and *Macrocystis pyrifera, Camellia sinensis, Eucommia ulmoides* (Załuski and Smolarz, 2009).

The aqueous buds extract of *Eucalyptus* inhibits PLA2 activity of *M. bornmuelleri* venom

The PLA2 activity of *M. bornmuelleri* venom was assayed using egg yolk agar well diffusion plate. When present in the reaction mixture, PLA2 clears suspensions of egg yolk, even when the substrate is loaded into pores of agar gels (Habermann and Hardt, 1972). *M. bornmuelleri* venom produced a lysis zone equal to 1.9cm. With a quantity of 88µg, *M. bornmuelleri* venom exerts a strong PLA2 activity. We observed a strong inhibition of the PLA2 activity of *M. bornmuelleri* venom by adding 100µg of the aqueous extract of *Eucalyptus* plant to the mixture (Figure 1B). The results are correlated with the previous studies revealing the presence of a mass of 13664.7Da, matching the molecular weight of PLA2 enzyme in *M. bornmuelleri* venom (Accary et al, 2014a). That led afterwards to an isolation and purification of the PLA2 enzyme from *M. bronmuelleri* venom (Accary et al, 2014b). PLA2 is the most abundant enzyme that belongs to group II in Viperidae (Fatima and Fatah, 2014; Accary et al, 2014b). It catalyzes the Ca^{2+} dependent hydrolysis of the 2-acyl ester bond, thereby producing free fatty acids and lysophospholipids (Vivek et al, 2013). The inhibition of PLA2 by ABEE is also in agreement with other works, showing that the ethylacetate extract of *Azima tetracantha* Lam. plant leaves inhibit PLA2 of *Bungarus caeruleus* and *Vipera russelli* venoms. The opportunity to inhibit PLA2 of snake venoms opens new perspectives in the management of snakebite poisonings since PLA2 plays a key role in neurotoxicity, myotoxicity, hemolytic activity, anti-bacterial, anti-coagulant, anti-platelet effect, antitumor and anti-angiogenic activities (Zouari-Kessentini et al, 2013).

Antioxidant activity of *Eucalyptus* plant extract

Here, we intended to assess the antioxidant activity of ABEE, in order to validate our obtained results, concerning the investigation of the capacity of the aqueous extract of *Eucalyptus* plant to inhibit PLA2 activity. The data showed some high antioxidant activity, where the percentage of scavenging was 97% after 30 minutes (Table 1). The presence of a 'significant' statistical correlation between the extracts of Colombian plant with antioxidant activity and the inhibitory activity of PLA2 snake venom had been reported by Pereañez et al, (Pereañez et al, 2010). These results are in a good argument with previous reports which demonstrated that antioxidant compounds are group II PLA2 inhibitors; and that the active extracts previously studied against PLA2 activity revealed the presence of higher concentrations of phenolic compounds, suggesting that these metabolites could be responsible for the inhibition of the toxic effects of PLA2s. Also, vitamin E (α-tocopherol, an antioxidant molecule) was able to decrease both the enzymatic and the inflammatory activities of an isolated PLA2 (Pereañez et al, 2010). In this direction, we suggest that the inhibitory potential of the *Eucalyptus* extract toward PLA2 is due to the presence of phenolic compounds such as flavonoids.

The aqueous buds extract of *Eucalyptus* inhibits LAAO activity of *M. bornmuelleri* venom

The absorbance at 450nm is 4.75 fold increased ($p<0.05$, non-parametric Wilcoxon paired t-test) in the presence of *M. bornmuelleri* venom, demonstrated that this venom possesses a strong LAAO activity (Table 2). This is agreement with our previous data (Rima et al, 2013). In the presence of ABEE, this LAAO activity is totally abolished (Table 2). The addition of ABEE in the reaction mixture led to an increase of the absorbance at t_0 (control value) was very high (t_0=0.108) compared to the venom alone (t_0=0.004), see table 2. This increase can be explained by non-specific interactions. At t_2 (after 2 hours incubation of venom with ABEE), no increase in absorbance was observed (t_2 = 0.111 minutes *vs* t_0 = 0.108 minutes), suggesting a potential inhibition of LAAO exerted within the *M. bornmuelleri* venom.

Indeed, LAAOs are the most abundant enzymes in snake venoms and they are responsible for various deleterious effects after envenomation, such as apoptotic response, cytotoxicity, platelets aggregation inhibition, hemorrhagic and hemolytic effects... (Costa et al, 2014). Thus, they constitute a relevant target for the development of snake venom antagonists. The inhibition of snake venom LAAO have been also reported with seven extracts of *Azima tetracantha* Lam. leaves was able to inhibit LAAO in both *Bungarus caeruleus and Vipera russelli* (Janardhan et al, 2014).

The aqueous buds extract of *Eucalyptus* inhibits AChE

Here, we addressed whether *M. bornmuelleri* venom and ABEE contain AChE enzyme/AChE inhibitors. In our assay, a very low O.D (Figure 1C) was observed with *M. bornmuelleri* venom (400µg) revealing the absence of AChE in the venom. These results are in agreement with previous studies demonstrating the absence of AChE in venoms of snakes belonging to the Viperidae and Crotalidae families (Accary et al, 2014a). Also, *M. bornmuelleri* venom was also examined for the presence of AChE inhibitors based on the previous citations that had indicated the presence of anti-AChE in snake venoms (Liesener et al, 2007). In fact, AChE inhibitors were found in *Bothrops moojeni* venom using an electrospray ionization/mass spectrometry (ESI/MS)-based assay (Liesener et al, 2007). Our results indicated no significant difference of between the evolution of the absorbance at 412nm during the time of the reaction with AChE alone and AChE with 400µg of *M. bornmuelleri* venom (Figure 1C). Thus, this indicates the absence of AChE inhibitors in *M. bornmuelleri* venom. Interestingly, ABEE (400µg) showed a potent AChE inhibitory activity, where it imposed almost complete inhibition after 5 minutes (Figure 1C). In conclusion, ABEE likely contains AChE inhibitor(s). AChE inhibitory activity has been already reported for *Eucalyptus* genus, but with an essential oil of *Eucalyptus camaldulensis* (Kiendrebeogo et al, 2011).

CONCLUSIONS

The venom of the viper, *M. bornmuelleri* represents a promising source of bioactive compounds such as proteases, PLA2 and antibacterial compounds. Here, we investigated the antiophidian properties of an aqueous extract of *Eucalyptus* plant using *in vitro* assays. We demonstrated that ABEE exhibits the antagonist effects against the main deleterious enzymatic activities of *M. bornmuelleri* venom. Moreover, whilst *M. bornmuelleri* venom is devoid of anti-AChE activity, we showed that ABEE is able to inhibit AChE. Our data suggest a potent antiophidic activity of ABEE against *M. bornmuelleri* venom and likely other Viperidae venoms. Moreover, these discoveries open a broad range of future perspectives concerning the isolation of the bioactive molecules in ABEE to develop new enzymatic inhibitors, such as anti-proteases, anti-PLA2, anti-LAAO and anti-AChE.

COMPETING INTERESTS

None declared.

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ABBREVIATIONS

AChE: Acetylcholine esterase

ABEE: Aqueous buds extract of *Eucalyptus*

ACN: Acetonitrile

$CaCl_2$: Calcium dichloride

Cv: Concentration of the venom

Da: Dalton

DI: Deionized water

DPPH: 2,2-diphenyl-1-picrylhydrazyl

ESI-MS: Electrospray ionization mass spectrometry

FA: Formic acid

LAAO: L-aminoacid oxidase

Mb. V: *M. bormuelleri* venom

NaCl: Sodium chloride

O.D: Optic density

PBS: Phosphate buffer saline

PLA2: Phospholipases A2

RP-HPLC: Reverse phase high-performance liquid chromatography

RSA: Radical scavenging activity

SEM: Standard error of the mean

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FIGURES

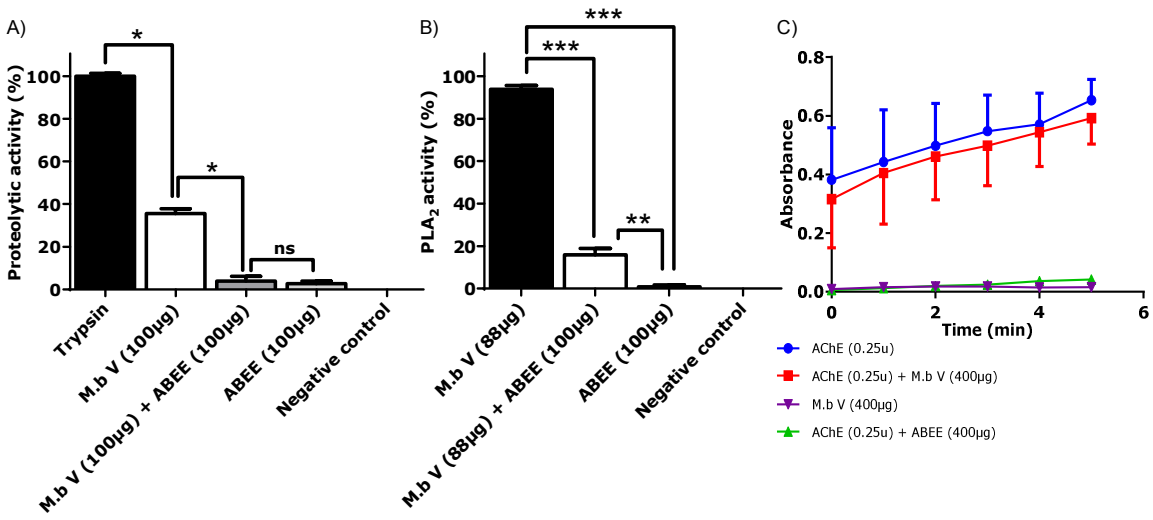

Figure 1. **A)** The *Eucalyptus* aqueous extract inhibits the proteolytic activity of *M. bornmuelleri* venom. The Inhibition of protease activity of *M. bornmuelleri* venom (M.b V) by the aqueous extract of *Eucalyptus* plant (ABEE) was investigated using plate method. 100µg of venom were preincubated with 100µg of the plant extract for 1hr at 37°C. The proteolytic activity of the venom and the aqueous plant extract was also separately measured. 10µg/µl of Trypsin was used as positive control and PBS as a negative control. Data are expressed as mean ± SEM (n=3). $p<0.005$ One way ANOVA test followed by a Tukey post-hoc test. **B)** Effects of *Eucalyptus* aqueous extract on phospholipase A2 activity of M.b V. An egg-yolk agar diffusion assay was used to measure the PLA2 activity of *M. bornmuelleri* venom. 88 µg of M.b V was pre-incubated with the aqueous bud extract of *Eucalyptus* for 1hr at 37°C. The PLA2 activity of the venom and the aqueous plant extract was also separately measured. Water was used as negative control. Values expressed as mean ± SEM with n=3. $p<0.001$ by Tukey's test (ANOVA). **C)** Inhibition of standard AChE activity by the aqueous extract *Eucalyptus* plant. This activity was assayed following the absorbance of AChE (0.25u) preincubated with either 400µg of venom (AChE+M.b V) or with plant supernatant (AChE+ABEE). 0.25u of AChE was used as positive

control. Data are expressed as mean ± SEM (n=3). $p<0.001$ One way ANOVA test followed by a Tukey post-hoc test.

TABLES

Table 1. Antioxidant activity of the aqueous buds extract of *Eucalyptus*. The radical scavenging index is shown 0 minute and 30 minutes. Data are expressed as mean ± SEM (n=3).

	Radical scavenging index (%)	
Samples	*t = 0 minutes (control)*	*t = 30 minutes*
ABEE (500µg)	97.2 ±0.141	97.85 ±0.21

Table 2. Effects of the aqueous buds extract of *Eucalyptus* on the LAAO activity of *M. bornmuelleri* venom. Data are expressed as mean ± SEM. Statistical analyses were performed using a non-parametric Wilcoxon paired t-test (* $p<0.05$). V, venom; ABEE, aqueous buds extract of *Eucalyptus*.

	Absorbance at 450nm (a.u.)	
Samples	*t = 0hr (control)*	*t = 2hr*
M. bornmuelleri V (140µg)	0.004 ± 0.0007	0.0190 ± 0.0007*
M. bornmuelleri V (140µg) + ABEE (1mg)	0.108 ± 0.008	0.111 ± 0.016 (n.s)