Erigeron annuus flower absolute prolongs botulinum neurotoxin A-induced muscle paralysis and inhibits neurotransmitter release-linked responses

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ABSTRACT
This study aimed to determine the effects of Erigeron annuus (L.) Pers. (EAP) flower absolute (EAPFAb) on neurotransmitter release-blocking events and muscle paralysis induced by botulinum neurotoxin type A (BoNT/A). For this study, EAPFAb was extracted from EAP flowers by solvent extraction and its composition was determined by GC/MS. Neurotransmitter release and SNARE protein expression were examined in PC12 cells by ELISA and immunoblotting. Rat hind limbs were tested for muscle paralysis. EAPFAb contained 23 components and prolonged the duration of BoNT/A-induced rat hind limb muscle paralysis. EAPFAb reduced neurotransmitter release induced by elevated extracellular potassium levels and attenuated SNARE protein expression in PC12 cells. These findings demonstrate that EAPFAb prolongs BoNT/A-induced muscle paralysis action, probably by inhibiting releases of neurotransmitters that are regulated by SNARE proteins in neural cells. EAPFAb may be a promising material for prolonging BoNT/A action.

ARTICLE HISTORY
Received 26 December 2021
Accepted 20 January 2022

KEYWORDS
Erigeron annuus (L.) Pers; absolute; neurotransmitter; botulinum neurotoxin type A; muscle paralysis; SNARE proteins

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Supplemental data for this article can be accessed online at https://doi.org/10.1080/14786419.2022.2036147.
1. Introduction

Natural extracts or natural compounds obtained from a wide range of natural sources are widely used for the development of natural-based therapies and medicines for disorders in various organs and have minimal risk of side effects (Alesci, Fumia et al. 2021; Alesci, Miller et al. 2021; Alesci, Pergolizzi et al. 2021; Fumia et al. 2021). Natural compounds or extracts from various plants have numerous biological activities including anti-inflammatory, anti-cancer, antioxidant, antibacterial, antiviral, immunomodulant and neuroprotective effects (Alesci, Aragona et al. 2021; Alesci, Fumia et al. 2021; Alesci, Miller et al. 2021; Fumia et al. 2021). *Erigeron annuus* (L.) Per. (EAP) (Family: Asteraceae) (known also as the daisy fleabane) is native of North America and an introduced species widely distributed in Korea (Bennington and Stratton 1998; Oh et al. 2002). EAP has been traditionally used to treat disorders such as enteritis, indigestion, hematuria and epidemic hepatitis (Yusuf 2021), and EAP and its extracts have been reported to possess multiple biological activities including anti-inflammatory, antimicrobial and antioxidant effects (Sung et al. 2011; Kumar et al. 2017; Yusuf 2021). Plant extracts showed to induce the paralysis of rat hemidiaphragms *ex vivo* and botulinum neurotoxin (BoNT)-like muscle paralysis in mice (Jung et al. 2012). However, the effect of EAP extracts on BoNT-induced muscle paralysis has not been previously investigated.

In the present study, we extracted absolute from EAP flowers (EAPFs) and examined the effect of EAPF absolute (EAPFAb) on type A BoNT (BoNT/A)-induced muscle paralysis. Based on the results obtained, we further explored the effects of EAPFAb on neurotransmission-linked responses, especially on neuronal SNARE (soluble N-ethylmaleimide-sensitive factor-attachment protein receptor) protein events in neuronal PC12 cells.

2. Results and discussion

To determine the effect of EAPFAb on the action duration of BoNT/A-induced muscle paralysis *in vivo*, we injected into rat left hind-limb muscles with BoNT/A alone or BoNT/A plus EAPFAb and assessed weekly using the Digit Abduction Score (DAS) assay, which has been reported to be reliable and sensitive for assessing muscle weakness (Broide et al. 2013). Rats in the control (PBS) and vehicle (0.01% HC-60/PBS) groups showed normal toe spreading responses (Supplementary material, Figure S1). A single injection with 5 U of BoNT/A increased DASs, but this BoNT/A-induced increase time-dependently reduced from 5 weeks after BoNT/A injection and recovered to almost baseline levels over 10 to 12 weeks (Supplementary material, Figure S1). On the other hand, this BoNT/A-induced DAS reduction was inhibited by administering EAPFAb (50 μg/ml weekly) (Supplementary material, Figure S1). These results indicate that EAPFAb lengthens the time of BoNT/A-induced hind limb muscle paralysis in rats.

BoNT-induced muscle paralysis may be associated with acetylcholine release inhibition (Baldwin and Barbieri 2009). PC12 cells can release acetylcholine by stimulating with high-concentrated potassium (high K⁺) solution and are widely used as model of neuronal function such as the synthesis and secretion of neurotransmitters including acetylcholine (Amino et al. 2002; Mandela and Ordway 2006). We thus determined
whether EAPFAb influences neurotransmitter secretion using PC12 cells. Treatment with 50 mM high K⁺ solution increased acetylcholine release from PC12 cells (309.68 ± 1.28% of untreated control) and this was reduced in the presence of EAPFAb at 25 or 50 μg/mL to 245.37 ± 7.86% and 246.55 ± 8.73% of untreated controls, respectively (Supplementary material, Figure S2a). In addition, EAPFAb at concentrations of 10 to 50 μg/mL did not reduce PC12 cell viability (Supplementary material, Figure S2b). BoNT/A decreased acetylcholine release in high K⁺ stimulated PC12 cells (Ray 1993). Therefore, our results indicate that EAPFAb, like BoNT/A, may inhibit acetylcholine release in nerve cells.

The release of acetylcholine from synaptic vesicles is induced by the fusion of synaptic vesicles with the presynaptic plasma membrane mediated by SNARE proteins (Jankovic 2004; Breidenbach and Brunger 2005). SNARE proteins, such as vesicle-associated membrane protein (VAMP)2 and presynaptic plasma membrane proteins, syntaxin 1a and synaptosome-associated protein (SNAP) 25 have been shown to participate in the fusion events by forming complexes (Baldwin and Barbieri 2009; Archana 2016). Thus, we investigated whether EAPFAb affects the expressions of SNAP25, syntaxin 1a and VAMP2 proteins in PC12 cells by Western blotting. EAPFAb (10–50 μg/mL) downregulated the expressions of all three proteins in a concentration-dependent manner (Supplementary material, Figure S3). Treatment with EAPFAb at 50 μg/mL maximally reduced SNAP25 (16.74 ± 6.28% of untreated control; Supplementary material, Figure S3a and b), syntaxin 1a (9.99 ± 5.96% of untreated control; Supplementary material, Figure S3a and c), and VAMP2 expressions (21.58 ± 6.81% of untreated control; Supplementary material, Figure S3a and d). These results indicate that EAPFAb suppresses the expressions of all three SNARE proteins. BoNTs prevent acetylcholine release from presynaptic vesicles by blocking complex formation or by down-regulating of SNARE proteins, which induce muscle paralysis (Mandela and Ordway 2006; Archana 2016). Thus, it appears EAPFAb may inhibit neurotransmission-related events associated with SNARE proteins, probably leading to a prolonged duration of hind limb muscle paralysis induced by BoNT/A.

To identify the components of EAPFAb, we performed GC-MS analysis. The yield of EAPFAb was 0.057% (v/w fresh material) and EAPFAb contained 23 compounds (Supplementary material, Table S1, Figure S4). A search of the literature revealed that none of these compounds and EAPF absolute has been associated with muscle paralysis-associated responses. Accordingly, we report for the first time that EAPFAb extends the action duration of BoNT/A-induced muscle paralysis in rats.

3. Conclusions

The present study shows that EAPFAb contains 23 compounds and prolongs BoNT/A-induced hind limb muscle paralysis in rats. Furthermore, EAPFAb inhibited high K⁺-induced releases of neurotransmitter acetylcholine and the expressions of SNARE proteins in PC12 cells, which indicates EAPFAb may act to extend BoNT/A-induced muscle paralysis by inhibiting SNARE protein-induced releases of neurotransmitters in neural cells. Accordingly, we suggest that EAPFAb may be useful for prolonging the action duration of BoNT/A. However, further study will be needed to identify the major
bioactive components of EAPFAb and to elucidate the mechanism responsible for the inhibition of neurotransmission by EAPFAb.

**Disclosure statement**

The authors declare no potential conflicts of interests.

**Funding**

This study was supported by the R&D Program for Forestry Technology (Project no. 2020190B10-2122-BA01) of the Korean Forest Service.

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