The Effects of *Erythrina subumbrans* (Hassk.) Merr. Leaves Extract on Nicotine Withdrawal Syndrome and β2 nAChRs Expression in The Ventral Tegmental Area of Rats

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Abstract. *Erythrina subumbrans* (Hassk.) Merr. is an alkaloid plant with *dihydro-β-erythroidine* (DhβE) content which is considered to block α4β2 nAChRs subtype and, therefore, may suppress the desire to use nicotine. This study aimed to investigate these possible effects of *E.subumbrans* (Hassk.) Merr. extract on nicotine withdrawal syndrome and β2 nAChRs expression in rats' ventral tegmental area (VTA). The rats were divided into six groups, i.e., control (OO), nicotine treated (NO), nicotine, and *E. subumbrans (Hassk.) Merr*.-treated (NE 100, NE 200, NE 400), and *E. subumbrans* (Hassk.) Merr.-treated (OE 200) groups. Nicotine was given ad libitum via drinking water with a step-wise increase of dosage every four days for 30 days. Somatic and affective signs were observed during the dark cycle of 24 hours abstinent period (days 31and 46). The expression of β2 nAChRs in the VTA was examined semi-quantitatively. It has been found that the rearing behavior of the NE 100 group was fewer on day 46 than on day 31. The body scratching behavior of the NE 100 group was fewer than that of the OO group on day 46. The front paws and penile licking behaviors of the NE 100 group were fewer than those of the NO group on day 46. The open arm entries of the NO group were fewer than that of the NE 200 group on day 46. The β2nAChRs expression of the NO group was lower than that of the OO group. *E. Subumbrans* (Hassk.) Merr. at a dosage of 100mg/kg BW may decrease some somatic signs of nicotine withdrawal syndrome.

1 Introduction

Smoking causes nearly 5.4 million deaths worldwide each year [1]. However, people find it challenging to quit smoking [2]. Only 3% of smokers quit successfully, while 80% of smokers who attempt to quit on their own return to smoking within a month [3]. One of the causes why people find it difficult to stop smoking is nicotine, which is from tobacco. It acts as an agonist on nicotinic acetylcholine receptors (nAChRs) in the brain and causes dependence [4].

Nicotine upregulates nAChRs by increasing the number and altering the function of the receptors [4][5]. This upregulation of receptors contributes to nicotine dependence [6]. In addition, an increase in nAChRs' affinity to nicotine presents in basal ganglia, ventral tegmental area (VTA), and substantia nigra of nicotine users [7]. The VTA consists of neurons that play a role in drug addiction [8] and affect mood, cognition, and bodily functions by binding and activating nAChRs [4].

The VTA has a high density of nAChRs and is critically important for the pleasure effects of nicotine [5]. Exposure to nicotine increases α4β2 nAChRs number [3]. Consequently, nicotine alters these receptors' function [5] and directly stimulates the receptors [4], especially in the VTA. The decrease of the nicotine levels in the VTA results in the reduction of the number of dopamine receptors [3] and the increase of the CRF-1 (Corticotrophin releasing factor-1) level [3]. In turn, the increase in CRF-1 levels leads to withdrawal symptoms and stimulates smoking behavior.

Nicotine withdrawal symptoms include craving, irritability, depression, anxiety, restlessness, sleep disturbance, difficulty concentrating, increased appetite, and weight gain [9]. Tobacco users usually seek nicotine to avoid nicotine withdrawal syndrome [10]. In animal models, Sprague Dawley rats exhibit similar symptoms as smokers.

While withdrawal symptoms in people are characterized by craving, depression, restlessness, and sleep disturbances, those in rats are indicated by increased somatic and affective signs [11]. In rats, the signs are jumping, rearing, wet dog shaking, body scratching, front paws, and penile licking, as well as changes in body weight [12]. Affective signs, including anxiety [13], can be measured in rats by observing their behaviors using an elevated plus maze (EPM) [14]. The upregulation of α4β2 nAChRs causes this effect; therefore, the antagonist of

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nAChRs will decrease nicotine withdrawal symptoms [15]. nAChRs antagonist inhibits neurotransmitter release in the brain, and the nicotine withdrawal will diminish [15]. This reduces the desire to smoke. It is therefore hypothesized that a nAChRs antagonist will lessen the urge to smoke.

Erythrina spp. is an alkaloid plant containing *dihydro-β-erythroidine* (DhβE), *erysodine,* and *erythralin* [16]. They are antagonist competitive of nAChRs which block α4β2 nAChRs subtype [11]. Consequently, the α4β2 nAChRs level will decrease in VTA [17](Ohmura et al., 2012), and this will lower the desire to use nicotine [13]. *Erythrina subumbrans* (Hassk.) Merr. *(E. subumbrans* (Hassk.) Merr.*)* is a nAChRs antagonist subtype that can be potentially used for behavioral therapy [15]. To the best of our knowledge, there is no definitive treatment for nicotine dependence using nAChRs antagonist up to date. This study aims to investigate the effect of ethanol extract *E. subumbrans* (Hassk.) Merr. on nicotine withdrawal syndrome and β2 nAChRs expression in the VTA of female Sprague Dawley rats treated with oral nicotine.

2 Material and Methods

2.1 Animals

The experimental protocol and animal handling of the present study were approved by the Ethical Committee of the Faculty of Medicine, Public Health, and Nursing, Universitas Gadjah Mada/UGM (approval number KE/FK/116/EC). The subjects were adolescent Sprague Dawley female rats obtained from Integrated Testing and Research Laboratory (LPPT) UGM aged ± 30-50 days with body weights of 50-125 g. They were divided into six groups, i.e., OO (aquabidestilata), NO (nicotine + aquabidestilata), NE 100 (nicotine +100mg/kg BW of *E. subumbrans* (Hassk.) Merr.), NE 200 (nicotine + 200 mg/kg BW of *E. subumbrans (Hassk.) Merr*.), NE 400 (nicotine + 400 mg/kg BW of *E. subumbrans* (Hassk.) Merr.), and OE 200 (200 mg/kg BW *E. subumbrans* (Hassk.) Merr.) groups. The dosages of *E. subumbrans* (Hassk.) Merr. was referred to those of Pitchaiah et al. [18].

2.2 E.subumbrans (Hassk) Merr preparation

E. subumbrans (Hassk.) Merr. was collected from Banjarsari village, Leses, Manisrenggo, Klaten, Indonesia, during dry seasons. The determination of the *E.subumbrans (Hask) Merr.* leaves were conducted in the Faculty of Pharmacy, Universitas Gadjah Mada, prior to the extraction. The *E. subumbrans (Hask) Merr.* leaves were washed and dried. The leaves were cut into pieces and incubated at 50^{o}C in an oven for four or five days before being mashed into powder. The powder was soaked in 70% ethanol for 24 or 48 hours and filtered. The liquid filtrate was evaporated until the liquid became viscous and ready for use.

2.3 Nicotine and E.subumbrans (Hassk.) Merr treatments

Firstly, the rats acclimatized for seven days before treatments and were housed in cages under natural light/dark cycle. They had ad libitum access to food and water during the experiment. Nicotine (nicotine bitartatedyhidrate, Nacalai Tesque INC, Japan) was given via drinking water at increasing doses from 10, 20, 35, 50, 65, 80, 100, up to 125µg/mL, which were changed every 4 days for 30 days. The nicotine administration was stopped for the following 24 hours (day 30 - day 31). The rats were examined for nicotine withdrawal syndrome during the dark cycle of this abstinent period (starting from 6 pm).

On the 31st day, the nicotine administration was continued, accompanied by the oral administration of *E. subumbrans* (Hassk.) Merr. extract once a day for 14 days (day 31 - day 45). The administration of nicotine and *E. subumbrans* (Hassk.) Merr. extract was terminated on day 45. The nicotine withdrawal syndrome was again observed during the dark cycle starting from 6 pm.

2.4 Nicotine withdrawal syndrome

The signs and symptoms of the syndrome which were observed included the body weight as well as affective and somatic signs [11]. The somatic signs were jumping, rearing, wet dog shaking, body scratching, front paws licking, penile licking, and body weight change [19]. The body weights of rats were measured on days 30, 31, 45, and 46. The jumping, rearing, wet dog shaking, body scratching, front paws licking, and penile licking behaviors were examined on days 31 and 46 during the 24 hours of nicotine abstinence. Video recorded every somatic sign was observed and counted for 10 minutes [20] by video recorded. The affective sign was anxiety which was observed using an elevated plus maze (EPM) video recording. The anxiety was represented by open arm time and open arm entries in the EPM. Open arm time was the latency spent by any given rat in the open arms of EPM. Open arm entries were the frequency of any given rat entering the open arms [14]. The affective signs were examined on days 31 and 46 during the 24 hours period of nicotinic abstinence.

2.5 Immunohistochemistry

All rats were sacrificed on day 47 by transcardial perfusion under the anesthesia of 20-40 mg/kg BW of ketamine hydrochloride (PT. Guardian Pharmatama, Jakarta, Indonesia). The rats' skulls were opened, and the cerebrums were immediately removed from the heads. A coronal slice of 5 mm thick starting from lambda to the direction of bregma of each of the cerebrums was taken. This slice of the brain was the approximate location of the VTA. The slice was immersed in 10% formalin in phosphate-buffered saline (PBS) solution for 24 hours at room temperature. The slices were subsequently dehydrated using ethanol with increasing concentrations,

cleared with xylol, infiltrated with, and embedded in paraffin blocks.

The blocks containing the brain tissues with VTA were coronally sectioned at three μm thicknesses using a Reichert Germany 1980 microtome. Four to nine sections were taken from each block. The first section was taken at a distance of -5.20 mm from bregma in the direction of lambda as defined by Paxinos and Watson (1998). The second and the following sections were taken systematically, with every 43rd section sampled (sections 43, 86, 129, and so on) until the tissue containing VTA was exhaustively sectioned.

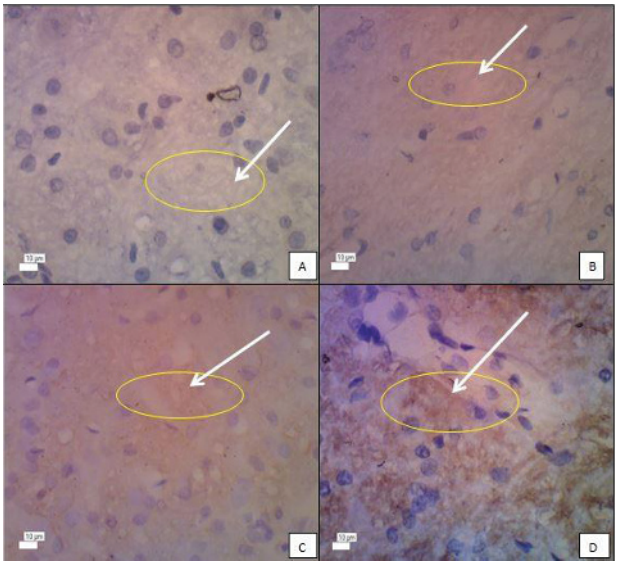

Figure 1. β2nAChRs expression in VTA. A, 0 degree; B, 1st degree; C, 2nd degree; D, 3rd degree of color intensity

Subsequently, the sections were stained using anti-CHRNB 2 polyclonal antibodies (bs-4247R, Bioss Antibody Inc, Massachusetts, USA) in order to examine the β2 nAChRs expression in the VTA. The immunopositive reaction was visualized using the Starr Trek Universal HRP detection system (STUHRP700 H, L1, Biocare Medical, CA, USA). Sections not incubated with primary antibodies served as negative controls. β2 nAChRs expression was viewed under a CX100 (Olympus, Japan) light microscope at 4xmagnification. The VTA neurons expressing β2 nAChRs were identified as those having brown cytoplasm around the blue nucleus (Figure 1B-D). The sections were photographed using a digital camera connected to a computer and Optilab software (PT Miconos, Indonesia). Two fields of view, from both right and left VTAs, were photographed from each section.

The β2 nAChRs expressions were quantified using a semi-quantitative method based on the degree of color intensity expressed by neuronal soma (Figure 1). The degree of color intensity was categorized into 4 degrees (0, 1, 2, 3). There was no brown color in 0 degrees (Figure 1A), light brown in the 1st degree (Figure 1B), and increasingly darker brown in the 2nd and 3rd degrees (Figure 1C and 1D, respectively). In order to maintain the reliability of the examination, all sections were examined by three observers. The validity test of the measurement analysis by Pearson Product Moment. There was a strong correlation between observers (p=0.001).

2.6 Statistical Analyses

The data of body weights, body scratching on day 46, front paws licking on day 46, and open arm time on day 31, were normal and homogeneous and therefore were analyzed using a one-way ANOVA procedure. A post-hoc least significant difference (LSD) was conducted where necessary. The data of rearing, wet dog shaking, body scratching on day 31, front paws licking on day 31, penile licking, open arm time on day 46, and open arm entries were not normally distributed and not homogeneous, and hence were analyzed using Kruskal-Wallis procedure. Post-hoc Mann-Whitney and Wilcoxon tests were applied where needed. The β2 nAChRs expressions were analyzed using a semi-quantitative method.

3 Results and Discussion

3.1 Somatic signs

3.1.1 Body weights

The data of the body weights of all rats are shown in Table 1. One-way ANOVA of these data showed no significant

Table 1. Mean ± SD of the body weights of the rats

Groups (n=6)	Body weights (g)				
	Day 1	Day 30	Day 31	Day 45	Day 46
OO	82.16±12.31	144.83±22.89	145.83 ± 23.23	161.33±25.46	161.16±26.92
NO	75±20.81	141.83±20.81	142.50 ±14.98	165.16±17.39	166.50±17.38
NE 100	76±14.65	145.83±14.01	146.33±13.87	165.33±15.03	171.16±15.59
NE 200	78.5±13.57	141.50±13.52	142.50±13.79	163.00±20.14	161.00±20.66
NE 400	69.66±16.39	142.00±12.84	142.67±12.79	170.33±14.36	170.00±16.26
OE 200	78.5±13.51	156.16±22.60	156.50±22.72	170.50±21.39	169.50±19.32
One-way ANOVA	df=5,30	df=5,30	df=5,30	df=5,30	df=5,30
	F = 0.447	F = 0.0663	F = 0.575	F = 0.227	F = 0.31
	p = 0.81	p = 0.64	p = 0.71	p = 0.94	p = 0.90

OO: Aquabidestilata treated group; NO: Nicotine treated group; NE 100: Nicotine + 100 mg/kg bw of *E. subumbrans* (Hassk.) Merr.; NE 200: Nicotine + 200 mg/kg bw of *E. subumbrans* (Hassk.) Merr.; NE 400: Nicotine + 400 mg/kg bw of *E. subumbrans* (Hassk.) Merr.; OE 200: 200 mg/kg bw of *E. subumbrans* (Hassk.) Merr.
ANOVA, analysis of variance; df, degree of freedom, F, F values; p, p values

main effects of groups on days 30, 31, 45, and 46. The present study revealed no significant differences between groups in body weights on all days of measurements. The group with chronic nicotine administration tended to have higher weights on day 30 than on day 1 (Table 1). There was also no difference in body weights following *E. subumbrans* (Hassk.) Merr. administration, which suggested that *E. subumbrans* (Hassk.) Merr. did not affect the body weights of rats.

Existing literature on the effects of nicotine on body weight and appetite have been conflicting. It has been reported that nicotine decreases appetite and leads to losing body weight. Nicotine also increases energy expenditure and metabolic rate. Studies showed that mean body mass index (BMI) tended to be lower among smokers than among non-smokers in many populations [20]. However, while chronic exposure to nicotine decreases body weight, withdrawal or abstinent period leads to weight gain [20]. An increasing body weight occurs after withdrawal [21].

Heavy smokers usually experience higher weight gain than weaker ones, despite the unclear relationship between obesity and smoking. Nicotine seems to cause an accumulation of visceral adipose tissue, thus causing weight gain [22]. It has also been reported that female rats decreased appetite in the first five days after oral nicotine administration through pellets. Afterward, the rats experienced weight loss for the first nine days before the appetite level became normal until day 21 [21].

The ethanolic extract of *E. subumbrans* (Hassk.) Merr. has an effect of increasing dopamine levels. Increased dopamine levels can cause weight loss and alter eating behavior [23]. In the case of smoking cessation, the dopamine level decreases, and thus it might lead to an increase in CRF-1 level and appetite. Therefore, smoking cessation may lead to weight gain. It has been thought that the ethanolic extract of *E. subumbrans* (Hassk.) Merr. may reduce nicotine withdrawal syndrome by preventing body weight gain. However, such effect was not clearly evident in the present study since there was no difference between groups in the body weight.

3.1.2 Jumping, rearing, wet dog shaking, body scratching, front paws licking, and penile licking

This study shows no significant differences between groups in somatic signs following 30 days of oral nicotine administration (Table 2). Jumping behavior was not observed in this study. The data on rearing behavior is shown in Table 2. Kruskal-Wallis test of these data revealed no significant differences between groups on days 31 and 46. However, the Wilcoxon test showed that the rearing behavior of NE 100 on day 46 was fewer than that on day 31 ($p=0.02$). The data of wet dog shaking behavior are demonstrated in Table 2. Kruskal-Wallis test of these data showed no significant differences between groups on days 31 and 46.

The data of body scratching behavior are shown in Table 2. Kruskal-Wallis test of these data showed no significant differences between groups on day 31. Similarly, the one-way ANOVA procedure also revealed no significant main effects of groups on day 46. Nevertheless, the post-hoc LSD test of these data showed no significant differences between groups on day 31. Similarly, the one-way ANOVA procedure also revealed no significant main effects of groups on day 46. Nevertheless, the post-hoc LSD test of these data showed that the body scratching behavior of the NE 100 group was fewer than that of the OO group on day 46 ($p=0.04$).

The data of front paw licking behavior are shown in Table 2. Kruskal-Wallis test of these data showed no significant differences between groups on day 31. One-way ANOVA of these data also showed no significant main effects of groups on day 46. The post-hoc LSD test, however, revealed that the front paws licking behavior of the NE 100 group were fewer than that of the NO group on day 46 ($p=0.03$).

Table 2. Mean ± SD of somatic signs of rats (n = 6) on days 31 and 46

Somatic signs	Day	Frequency in 10 minutes (times)						P values
		OO	NO	NE 100	NE 200	NE 400	OE 200	
Jumping	31	0	0	0	0	0	0	0
	46	0	0	0	0	0	0	0
Rearing	31	3.66±3.50	2.33±2.58	4.16±2.40	2.16±2.48	3.33±2.33	2.83±2.32	0.70
	46	1.33±2.06	1.66±2.58	0.33±0.51	0.66±0.81	0.66±0.81	0.16±0.40	0.85
Wet dog shaking	31	0.16±0.41	0.33±0.82	0.00±0.00	0.33±0.52	0.00±0.00	0.00±0.00	0.38
	46	0.16±0.40	0.00±0.00	0.33±0.51	0.50±0.54	0.16±0.40	1.00±0.89	0.09
Body scratching	31	4.83±3.76	4.00±2.76	3.50±1.76	7.16±4.83	3.00±2.28	3.66±2.34	0.63
	46	5.50±2.50	6.66±3.77	3.33±3.01	4.66±3.01	4.16±2.40	5.83±1.72	0.37
Front paws licking	31	5.00±3.52	4.16±2.56	3.50±1.76	7.00±4.93	2.83±2.31	3.50±2.34	0.60
	46	5.50±2.50	6.66±3.93	3.00±2.44	5.00±3.03	4.00±2.75	4.83±2.22	0.35
Penile licking	31	0.83±0.98	0.67±1.21	0.83±0.75	3.67±3.72	1.00±1.55	2.00±2.76	0.13
	46	3.16±2.78	3.50±2.58	0.33±0.51	3.33±3.01	1.83±1.94	2.50±2.58	0.13

OO : Aquabidestilata treated group; NO : Nicotine treated group; NE 100 : Nicotine + 100 mg/kg bw of *E. subumbrans* (Hassk.) Merr.; NE 200 : Nicotine + 200 mg/kg bw of *E. subumbrans* (Hassk.) Merr.; NE 400 : Nicotine + 400 mg/kg bw of *E. subumbrans* (Hassk.) Merr.; OE 200 : 200 mg/kg bw of *E. subumbrans* (Hassk.) Merr.
ANOVA, analysis of variance; df, degree of freedom, F, F values; p, p values

Table 3. Mean ± SD open arm time

Groups (n=6)	Before therapy (day 31)	After therapy (day 46)
OO	20.66±13.77 %	45.82±33.01 %
NO	26.88±18.46 %	47.04±49.04 %
NE 100	23.16±16.44 %	45.16±43.68 %
NE 200	38.00±31.70 %	22.88±8.85 %
NE 400	42.11±27.07 %	49.38±34.22 %
OE 200	32.38±25.31 %	36.16±27.77 %
	one-way ANOVA	Kruskall-Wallis
	df= 5,30	
	F = 0.64	df = 5,30
	p = 0.64	p = 0.86

OO : Aquabidestilata treated group; NO : Nicotine treated group; NE 100 : Nicotine + 100 mg/kg bw of *E. subumbrans* (Hassk.) Merr.; NE 200 : Nicotine + 200 mg/kg bw of *E. subumbrans* (Hassk.) Merr.; NE 400 : Nicotine + 400 mg/kg bw of *E. subumbrans* (Hassk.) Merr.; OE 200 : 200 mg/kg bw of *E. subumbrans* (Hassk.) Merr.
ANOVA, analysis of variance; df, degree of freedom, F, F values; p, p values

The data of penile licking behavior are shown in Table 2. Kruskal-Walis test of these data shows no significant differences between groups on days 31 and 46. However, the Mann-Whitney test revealed that the penile licking behavior of the NE 100 group was fewer than that of the NO group on day 46 (p=0.01).

There were several factors that might have affected these results. The present study used adolescent rats with nicotine withdrawal syndrome usually expressed less than their adult counterparts [20]. It needs a long time to make adolescent rats dependent on nicotine [24]. Adolescent rats have not shown decreasing addiction signs in the brain when there is a nicotine withdrawal syndrome [20]. Adolescent rats showed little or no nicotine withdrawal symptoms, while a similar plasma nicotine profile occurred in both adolescent and adult rats [22]. Adolescent rats are less sensitive to nicotine withdrawal due to the reduced function of nAChRs. Since the cholinergic system of adolescent rats is still immature, it will lower the nAChRs functions than normal [20].

Another factor that might play a role in the absence of nicotine withdrawal syndrome was that this study administered nicotine via drinking water. Nicotine through drinking water will not be immediately absorbed [25]. Orally, but not intravenously, administered nicotine will enter the first-pass metabolism through the liver [25]. Consequently, the amount of nicotine that reaches the brain will be low and variable [26]. Nicotine is a lipophilic substance and can directly influence neurons without affecting nAChRs [27]. However, the behavioral effects of orally administered nicotine are still unclear [28].

Significant differences between OO versus NE 100 groups for body scratching, front paws licking, and penile licking occurred on day 46. This study also shows the significant differences between NE 100 (day 31) and NE 100 (day 46) (Table 2). These results indicate that *E. subumbrans* (Hassk.) Merr. at the dosage of 100 mg/ kg may exert beneficial effects via its components, such as *erysodine, erysothrine, eryhtravine*, and *11- α-hydroxy-erythravine*, which have been reported to have anxiolytic [29], as well as *β erythroidine* that showed mostly sedative activity [30]. The anxiolytic activity of *Erythrina spp.* increases the number of a receptor complex of GABA and serotonin [31]. Hence, *E. Subumbrans* (Hassk.) Merr. may act as an anxiolytic and sedative agent that decreases the nicotine withdrawal syndrome for rearing, body scratching, front paws licking, and penile licking at a dosage of 100 mg/kg.

3.2 Affective signs

The data of open arm time are shown in Table 3. ANOVA test of these data showed no significant main effects of groups on day 31. Kruskal-Wallis tests also revealed no significant differences between groups on day 46. The data of open arm entries are shown in Table 4. Kruskal-Wallis test of these data showed no significant differences between groups on days 31 and 46. However, the Mann-Whitney test showed that the arm entries of the NO group were fewer than that of the NE 200 group on day 46 (p= 0.03).

Table 4. Mean ± SD open arm entries

Groups (n=6)	Before therapy (day 31)	After therapy (day 46)
OO	41.17±20.68 %	47.86±7.44 %
NO	47.55±2.94 %	23.08±26.23 %
NE 100	40.00±21.00 %	36.46±18.94 %
NE 200	41.11±20.20 %	47.14±4.49 %
NE 400	48.71±2.07 %	46.03±6.96 %
OE 200	49.43±7.28 %	52.22±7.28 %
	df = 5,30	df = 5,30
	p = 0.83	p = 0.09

OO : Aquabidestilata treated group; NO : Nicotine treated group; NE 100 : Nicotine + 100 mg/kg bw of *E. subumbrans* (Hassk.) Merr.; NE 200 : Nicotine + 200 mg/kg bw of *E. subumbrans* (Hassk.) Merr.; NE 400 : Nicotine + 400 mg/kg bw of *E. subumbrans* (Hassk.) Merr.; OE 200 : 200 mg/kg bw of *E. subumbrans* (Hassk.) Merr.
ANOVA, analysis of variance; df, degree of freedom, F, F values; p, p values

Table 5. The median, mode, and frequency distribution of the degree of color intensity of β2 nAChRs expression in VTA of rats

	Group	Median*)	Mode*)	Frequency			
				0*)	1*)	2*)	3*)
Left VTA	OO	2	3	1	7	6	10
	NO	1	1	5	15	3	1
	NE 100	2	2	2	9	10	3
	NE 200	1	1,2	4	9	9	2
	NE 400	1	1	13	11	0	0
	OE 200	2	2	1	9	12	2
Right VTA	OO	2	1	0	10	5	9
	NO	1	1	6	12	6	0
	NE 100	1.5	2	2	10	11	1
	NE 200	1	2	5	8	9	2
	NE 400	1	1	1	13	10	0
	OE 200	2	2	0	9	13	2

*) **0** : no brown cytoplasm; **1** : light brown cytoplasm; **2** : dark brown cytoplasm; **3** : very dark brown cytoplasm

It has been argued that nicotine withdrawal syndrome manifests in anxiety and depression-like behaviors [9]. Our study, however, did not find any difference between groups in the open arm time and open arm entries. Our present study supports the previous investigation, which found no effect on affective signs following the oral administration of nicotine.

Nicotine shows different effects on behavior. This depends on the species, strain, and sex of experimental animals used [32]. Tizabi et al. [33] reported that Wistar-Kyoto (WKY) female rats showed depression-like behavior when nicotine entered the plasma. However, during the withdrawal period, the rats showed antidepressant-like behavior [33]. This may explain why we could not find any differences between groups in the anxiety parameters after oral nicotine administration cessation. It remains uncertain whether nAChRs modulation as an anti-depressant takes a form in nAChRs activation, inhibition, or desensitization [34].

Age may play a role in manifesting affective signs of nicotine withdrawal syndrome. The present study demonstrated that the nicotine exposure to rats during adolescence did not yield any depression-like behavior and, therefore, any possible anti-depressant effect of *E. Subumbrans* (Hassk.) Merr.could not be detected (Table 3 and 4).

3.3 β2 nAChRs expression

Immunopositive staining was detected in VTA (Figure 1). β2 nAChR was expressed in both left and right VTAs. Based on the color intensity, the expression of β2 nAChRs was lower in the NO group than in the OO group (Table 5). The NO group had the lowest intensity compared to any other group. The score of β2 nAChR immunostaining in the NE groups tended to be higher than in the NO group but lower than in the OO group. The highest dose of NE did not result in the highest score of β2 nAChR immunostaining.

Nicotine induces both the activation and inactivation of nAChRs [35]. In response to nicotine entrance to the brain, nAChRs will be inactive. The inactivation of nAChRs is followed by the decrease of the nicotine level [3]. Afterward, this condition makes the nAChRs responsive and crave nicotine [3]. In this study, the rats were terminated at a supposed-to-be withdrawal syndrome condition and during the responsive phase of nAChRs on day 46. Another study demonstrated that low β2 nAChRs expression was found in rats with low nicotine craving behavior [36], which may explain the lack of withdrawal syndrome in our study. The precise reason for this finding remains unknown at present. This study used DhβE as a β2 nAChRs antagonist for nicotine, preventing β2 nAChRs from the nicotine binding [37] and decreasing the nicotine craving [13]. This study found that the β2 nAChRs expression tended to be higher following NE administration. However, this semi-quantitative observation needs to be confirmed by quantification methods.

4 Conclusions

The present study did not find any somatic or affective signs or symptoms of nicotine withdrawal syndrome. There was, however, a decreased expression of β2 nAChRs in the ventral tegmental area of nicotine-treated rats. In addition, *E. subumbrans* (Hassk.) Merr. may affect some somatic signs, including rearing, body scratching, front paws licking, and penile licking at a dosage of 100 mg/kg. Future studies may be directed towards finding appropriate dosages of the oral administration of nicotine to cause nicotine withdrawal syndrome. Possible benefits of *E. Subumbrans* (Hassk.) Merr. in overcoming nicotine addiction or anxiety is warranted to be explored.

Authors' contributions

NR roles in the design of the experiment, data acquisition, data analyses, and manuscript preparation, RS has a task in the design of the experiment, statistical analyses, and manuscript preparation, ENR was responsible for the design of the experiment, data acquisition, statistical analyses, and manuscript review, GP was responsible in the design of the experiment, data analyses, manuscript preparation, and review.

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Ethical clearance statement

The experimental protocol and animal handling of the present study were approved by the Ethical Committee of the Faculty of Medicine, Public Health, and Nursing, Universitas Gadjah Mada/UGM (approval number KE/FK/116/EC).

References

1. WHO, World Health Organization. Report on the Global Tobacco Epidemic, 2008: The MPOWER Package, *Who*, pp. 1–342, 2008, [Online]. Available: http://www.who.int/tobacco/mpower/gtcr_download/en/. Acessado 23.05.2017.
2. A. L. Smith, S. M. Carter, S. M. Dunlop, B. Freeman, and S. Chapman, The views and experiences of smokers who quit smoking unassisted. A systematic review of the qualitative evidence, *PLoS One*, vol. 10, no. 5, 2015, doi: 10.1371/journal.pone.0127144.
3. N. L. Benowitz, Nicotine Addiction, *N. Engl. J. Med.*, vol. 362, no. 24, pp. 2295–2303, Jun. 2010, doi: 10.1056/NEJMra0809890.
4. A. Markou, Neurobiology of nicotine dependence, *Philos. Trans. R. Soc. B Biol. Sci.*, vol. 363, no. 1507, pp. 3159–3168, 2008, doi: 10.1098/rstb.2008.0095.
5. H. D. Mansvelder and D. S. McGehee, Cellular and synaptic mechanisms of nicotine addiction, *J. Neurobiol.*, vol. 53, no. 4, pp. 606–617, 2002, doi: 10.1002/neu.10148.
6. M. W. Quick and R. A. J. Lester, Desensitization of neuronal nicotinic receptors, *J. Neurobiol.*, vol. 53, no. 4, pp. 457–478, Dec. 2002, doi: 10.1002/neu.10109.
7. E. X. Albuquerque, E. F. R. Pereira, M. Alkondon, and S. W. Rogers, Mammalian Nicotinic Acetylcholine Receptors: From Structure to Function, *Physiol. Rev.*, vol. 89, no. 1, pp. 73–120, Jan. 2009, doi: 10.1152/physrev.00015.2008.
8. J. D. Berke and S. E. Hyman, Addiction, dopamine, and the molecular mechanisms of memory, *Neuron*, vol. 25, no. 3, pp. 515–532, 2000, doi: 10.1016/S0896-6273(00)81056-9.
9. S. Shiffman, R. West, and D. Gilbert, Recommendation for the assessment of tobacco craving and withdrawal in smoking cessation trials, *Nicotine Tob. Res.*, vol. 6, no. 4, pp. 599–614, Aug. 2004, doi: 10.1080/14622200410001734067.
10. P. J. Kenny and A. Markou, Neurobiology of the nicotine withdrawal syndrome, *Pharmacol. Biochem. Behav.*, vol. 70, no. 4, pp. 531–549, 2001, doi: 10.1016/S0091-3057(01)00651-7.
11. J. W. Daly, Nicotinic agonists, antagonists, and modulators from natural sources, *Cell. Mol. Neurobiol.*, vol. 25, no. 3–4, pp. 513–552, 2005, doi: 10.1007/s10571-005-3968-4.
12. S. S. Watkins, George F. Koob, Athina, Neural mechanisms underlying nicotine addiction: acute positive reinforcement and withdrawal, *Nicotine Tob. Res.*, vol. 2, no. 1, pp. 19–37, Feb. 2000, doi: 10.1080/14622200050011277.
13. A. J. Grottick *et al.*, Evidence that nicotinic α7 receptors are not involved in the hyperlocomotor and rewarding effects of nicotine, *J. Pharmacol. Exp. Ther.*, vol. 294, no. 3, pp. 1112–1119, 2000.
14. A. A. Walf and C. A. Frye, The use of the elevated plus maze as an assay of anxiety-related behavior in rodents, *Nat. Protoc.*, vol. 2, no. 2, pp. 322–328, 2007, doi: 10.1038/nprot.2007.44.
15. L. P. Dwoskin and P. A. Crooks, Competitive neuronal nicotinic receptor antagonists: A new direction for drug discovery, *J. Pharmacol. Exp. Ther.*, vol. 298, no. 2, pp. 395–402, 2001.
16. T. Herlina, U. Supratman, U. M. S. Soedjanaatmadja, A. Subarnas, S. Sutardjo, and H. Hayashi, Biologically active natural products from Indonesian Erythrina plants, vol. 2008, no. October, pp. 204–207, 2008.
17. Y. Ohmura, I. Tsutsui-Kimura, and M. Yoshioka, Impulsive behavior and nicotinic acetylcholine receptors, *J. Pharmacol. Sci.*, vol. 118, no. 4, pp. 413–422, 2012, doi: 10.1254/jphs.11R06CR.
18. G. Pitchaiah, G. L. Viswanatha, R. Srinath, K. Nandakumar, D. Dayabaran, and E. J. Florance, Anxiolytic and anticonvulsant activity of aqueous extract of stem bark of Erythrina variegata in rodents, *Int. J. PharmTech Res.*, vol. 2, no. 1, pp. 40–48, 2010.
19. A. T. Rafsanjani *et al.*, The effect of nicotine administration on physical and psychological signs of withdrawal syndrome induced by single or frequent doses of morphine in rats, *Basic Clin. Neurosci.*, vol. 3, no. 3, pp. 49–57, 2012.
20. L. E. O'DELL, A. W. BRUIJNZEEL, S. GHOZLAND, A. MARKOU, and G. F. KOOB, Nicotine Withdrawal in Adolescent and Adult Rats, *Ann. N. Y. Acad. Sci.*, vol. 1021, no. 1, pp. 167–174, Jun. 2004, doi: 10.1196/annals.1308.022.
21. E. D. Levin, F. J. McClernon, and A. H. Rezvani, Nicotinic effects on cognitive function: Behavioral characterization, pharmacological specification, and anatomic localization, *Psychopharmacology (Berl).*, vol. 184, no. 3–4, pp. 523–539, 2006, doi: 10.1007/s00213-005-0164-7.
22. A. Chiolero, D. Faeh, F. Paccaud, and J. Cornuz, Consequences of smoking for body weight, body fat

distribution, and insulin resistance, *Am. J. Clin. Nutr.*, vol. 87, no. 4, pp. 801–809, 2008, doi: 10.1093/ajcn/87.4.801.

23. J. Reinholz, O. Skopp, C. Breitenstein, I. Bohr, H. Winterhoff, and S. Knecht, "Compensatory weight gain due to dopaminergic hypofunction: New evidence and own incidental observations," *Nutr. Metab.*, vol. 5, no. 1, pp. 1–4, 2008, doi: 10.1186/1743-7075-5-35.
24. S. Pogun and A. C Collins, Oral Nicotine Self-Administration in Rodents, *J. Addict. Res. Ther.*, vol. 01, no. S2, 2012, doi: 10.4172/2155-6105.s2-004.
25. T. Nesil, L. Kanit, A. C. Collins, and S. Pogun, Individual differences in oral nicotine intake in rats, *Neuropharmacology*, vol. 61, no. 1–2, pp. 189–201, Jul. 2011, doi: 10.1016/j.neuropharm.2011.03.027.
26. S. G. Matta *et al.*, Guidelines on nicotine dose selection for in vivo research, *Psychopharmacology (Berl).*, vol. 190, no. 3, pp. 269–319, 2007, doi: 10.1007/s00213-006-0441-0.
27. S. Ferrea and G. Winterer, Neuroprotective and Neurotoxic Effects of Nicotine, *Pharmacopsychiatry*, vol. 42, no. 06, pp. 255–265, Nov. 2009, doi: 10.1055/s-0029-1224138.
28. J. Le Houezec, C. Martin, C. Cohen, and R. Molimard, Failure of behavioral dependence induction and oral nicotine bioavailability in rats, *Physiol. Behav.*, vol. 45, no. 1, pp. 103–108, 1989, doi: 10.1016/0031-9384(89)90171-6.
29. M. A. R. Serrano *et al.*, Anxiolytic-like effects of erythrinian alkaloids from erythrina suberosa, *Quim. Nova*, vol. 34, no. 5, pp. 808–811, 2011.
30. R. Marcos, R. Garcia-Mateos, R. San, G. Kite, M. Martinez-Vazquez, and A. C., Erythrina, a Potential Source of Chemicals from the Neotropics, in *Bioactive Compounds in Phytomedicine*, InTech, 2012.
31. R. Marcos, R. Garcia-Mateos, R. San, G. Kite, M. Martinez-Vazquez, and A. C., Erythrina, a Potential Source of Chemicals from the Neotropics, *Bioact. Compd. Phytomedicine*, 2012, doi: 10.5772/26188.
32. Y. Tizabi *et al.*, Antidepressant effects of nicotine in an animal model of depression, *Psychopharmacology (Berl).*, vol. 142, no. 2, pp. 193–199, Feb. 1999, doi: 10.1007/s002130050879.
33. Y. Tizabi *et al.*, Effects of nicotine on depressive-like behavior and hippocampal volume of female WKY rats, *Prog. Neuro-Psychopharmacology Biol. Psychiatry*, vol. 34, no. 1, pp. 62–69, Feb. 2010, doi: 10.1016/j.pnpbp.2009.09.024.
34. J. T. Andreasen, G. M. Olsen, O. Wiborg, and J. P. Redrobe, Antidepressant-like effects of nicotinic acetylcholine receptor antagonists, but not agonists, in the mouse forced swim and mouse tail suspension tests, *J. Psychopharmacol.*, vol. 23, no. 7, pp. 797–804, 2009, doi: 10.1177/0269881108091587.
35. S. M. Anderson and D. H. Brunzell, Low Dose Nicotine and Antagonism of β2 Subunit Containing Nicotinic Acetylcholine Receptors Have Similar Effects on Affective Behavior in Mice, *PLoS One*, vol. 7, no. 11, pp. 1–11, 2012, doi: 10.1371/journal.pone.0048665.
36. M. R. Picciotto *et al.*, Acetylcholine receptors containing the β2 subunit are involved in the reinforcing properties of nicotine, *Nature*, vol. 391, no. 6663, pp. 173–177, 1998, doi: 10.1038/34413.
37. R. Exley and S. J. Cragg, Presynaptic nicotinic receptors: A dynamic and diverse cholinergic filter of striatal dopamine neurotransmission, *Br. J. Pharmacol.*, vol. 153, no. SUPPL. 1, pp. 283–297, 2008, doi: 10.1038/sj.bjp.0707510.