Indian Journal of Experimental Biology
Vol. 58, June 2020, pp. 381-388

Development and evaluation of a novel herbal formula for tobacco cessation in nicotine addicted rat model

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Received 02 January 2019; revised 08 April 2020

Tobacco addiction is a major cause of disabilities and premature death. Numerous therapies for de-addiction are available; however, nicotine dependence and withdrawal symptoms pose problems for addicts. Here, we developed novel herbal formulations using natural plant parts and evaluated for de-addiction of nicotine. Parts of *Withania somnifera* (L.) Dunal, *Avena sativa* L., *Cinnamomum cassia* Blume, *Acacia catechu* (L.f.)Willd., *Ocimum tenuiflorum* L. and *Glycyrrhiza glabra* L. were formulated in three formulations containing alcohol extracts (AELF), aqueous extracts (WELF) and powdered herbs (PHLF). Swiss albino Wistar rats were addicted with nicotine 10 mg/kg/day for first five days and 20 mg/kg/day for next 10 days subcutaneously. Control rats were administered with 0.9% NaCl (Group VII) and addicted animals were treated with bupropion, 40 mg/kg, p.o. (Group I), rid-tobak, 200 mg/kg, p.o. (Group II), AELF, 200 mg/kg, p.o. (Group III), WELF, 200 mg/kg, p.o. (Group IV), PHLF, 200 mg/kg, p.o. (Group V), 0.5% sodium carboxymethyl cellulose, p.o. (Group VI) for 15 days. The animals were subjected to Y-maze test, swimming endurance test, behavioural studies on Day 0, 1, 6, 11 and on Day 16 after withdrawal of nicotine. On Day16, brain dopamine and serum cortisol levels were measured. Rats treated with AELF and PHLF showed significant improvement in behavioural parameters, increased brain dopamine level and decreased serum cortisol levels thus being a promising choice for tobacco cessation.

Keywords: Ashwagandha, *Avena sativa*, Chinese cassia, Cinnamon, Cutch tree, *Glycyrrhiza glabra*, Herbal formulation, Holi basil, Indian ginseng, Liquorice, *Nicotiana tabacum*, Oat, Tobacco de-addiction, Tulsi, *Withania somnifera*

Human beings commonly get addicted to pyridine alkaloids of tobacco than to any other naturally occurring plant products. Tobacco (*Nicotiana tabacum*, Fam.: Solanaceae) addicts exist in almost all classes of the society; while in most of the cases only alcohol rehabilitation campaigns in world are running[1]. In India, more than 42.4% of males, 14.2% females and 14.6% of children (<15 yr) are addicted to tobacco[2]. Addiction, according to World Health Organization, is a state of periodic or chronic intoxication characterized by first tolerance, craving and finally dependence of that substance produced by its repeated consumption[3].

Tobacco rehabilitation campaign is slow and very few de-addiction therapies[4] are available and are often expensive[5]. The nicotine replacement therapy products require long term therapy and have side effects[6]. We hypothesize that, the formulation developed for nicotine cessation, should have ability to compensate dopamine levels in brain. It should help in minimizing abstinence syndromes as well as reduce harmful effects of tobacco. It should have anti stress, anxiolytic, anti-psychotic, anti-oxidant, nervine tonic effects, etc[7].

The present study, attempts to design formulations depending upon the pharmacological effectiveness of herbs for tobacco cessation. The herbs have been selected based either on reports for smoking cessation effects[8] or those herbs which help in relaxing the affected area in brain or for their tonic and strengthening effects on the physiological system[9]. Thus, the main objective of the study is to develop a polyherbal formulation and evaluate it for tobacco cessation in nicotine addiction rat model for de-addiction properties.

Materials and Methods

Dried roots of *Withania somnifera* (L.) Dunal, *Glycyrrhiza glabra* L. stolons, *Ocimum tenuiflorum* L.

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(syn. *Ocimum sanctum*) leaves, *Acacia catechu* (L.f.)Willd. extract, *Cinnamon cassia* Blume bark and *Avena sativa* L. seeds were purchased from ACS Chemicals, Ahmedabad and got authenticated at Raw Materials, Herbarium and Museum, Delhi (RHMD) CSIR-NISCAIR, New Delhi. L-Nicotine (99% of assay purity) was purchased from ACROS Organicals Ltd., India; Bupropion hydrochloride from SIGMA Organicals and Chemicals Ltd. 'Rid tobak' antismoking mouth drops was purchased from local drug store. Dopamine estimation Kit was purchased from LABOR Diagnostic Nord GmbH & Co. KG (Germany), Serum cortisol level (ADVIA Centaur Assay) estimation kit was obtained from Sigma Diagnostic Kit, Sigma-Aldrich Corp., St. Louis, MO. All the solvents used were of Analytical grade, purchased from Qualigens, Mumbai, India.

Method of preparation

Withania somnifera (8 parts), *G. glabra* (6 parts), *O. tenuiflorum* (6 parts), *A. catechu* (4 parts), *C. cassia* (3 parts) and *A. sativa* (3 parts) were selected for preparing the formulation. All the crude drugs were pulverized and passed through sieve 80 mesh. Three formulations were prepared using the alcohol extracts (AELF), water extracts (WELF) and powdered crude drug (PHLF). The extracts were prepared using soxhlation and concentrated using rotary evaporator, and then dried at room temperature (25°C) in desiccators[10]. The extracts were weighed and mixed together. The formula was prepared under the guidance of experienced doctors of Indian Medicine and as per reported literature[8].

The PHLF was prepared from powdered plants using 'rolling between palm' method with 20% hydroalcoholic gum solution[11]. Similarly, AELF and WELF were also prepared from ethanol and aqueous extracts, respectively and 'rolling between palm' method using 20% hydroalcoholic gum solution.

Animals

Female Balb/c mice weighing between 20-25 g and adult female Swiss albino rats of Wistar strain having average body weight between 160 and 200 g were housed for at least 5 days before being used in a room with controlled temperature (23±1°C), humidity (50±10%) and light (6.00 a.m. to 6.00 p.m.). Food and water were made available continuously. Animal care and use for experimental procedures were approved by the Institutional animal care and use committee. All anaesthetic and other procedures were in compliance with the guidelines established by the Animal Care Committee (Pharmacy Department, M. S. University, Baroda/404/01/1/CPCSEA). All behavioural parameters were studied during the light phase of the cycle.

Acute toxicity of formulations

Acute Toxicity study of formulations was carried out as per the OECD guidelines[12]. Female Balb/c mice weighing between 20-25 g were administered orally with single dose of 2000, 1550, 550, 175, 55 mg/kg of formulations after overnight fasting. The animals were kept under observation for 12 h for any signs of abnormal behaviour or symptoms and for seven days for any mortality or abnormality.

Effect of formulation on nicotine addicted rats

Nicotine habituation[13] was induced in Group I to VI by injecting nicotine s.c. 10 mg/kg per day for first 5 days and 20 mg/kg per day for next 10 days. Treatments were given simultaneously as; Group I (bupropion, 40 mg/kg, p.o.), Group-II (rid-tobak, 200 mg/kg, p.o.), Group-III (AELF, 200 mg/kg, p.o.), Group-IV (WELF, 200 mg/kg, p.o.), Group-V (PHLF, 200 mg/kg, p.o.), Group-VI (0.5% sodium carboxy methyl cellulose, p.o.) and Group VII (Normal), were dosed 0.9% NaCl (normal saline) (s.c.) for all 15 days. Study formulations were administered to rats from Day 1 to Day 16, one hour before nicotine administration. Behavioural tests were performed on Day 0, 1, 6, 11 and 16 (the day of nicotine withdrawal). AELF, WELF and PHLF were dissolved in 0.5% sodium carboxy methyl cellulose, p.o. in distilled water vehicle.

Behavioural studies

All behavioural parameters were studied in dark and silent room. Details of tests performed are given below.

Y-maze test

Y-maze test was performed 15 min after acute administration of nicotine or saline to the rats[14]. The light of 10 watts was used to enlighten the light arm, Exposure time for test was 5 min. Each rat was placed at the centre of a Y-shaped wooden runway (Y maze, YM; 65×45× 35 cm) with one of the three arms closed. The percentage cumulative time spent by the rat in the open arm, the latency period when the animal remains immobile and the number of visits in each arm was recorded for 5 min[15].

Tail immersion test

The test was performed on the same rats after Y-maze test. The time of flicking of rat tail was measured in seconds with a cut off latency of 10 s, to prevent tissue damage[16].

Swimming endurance test

This test was performed after 35 min of nicotine administration or withdrawal[17]. The dimensions of test apparatus were 55 cm diameter and 60 cm depth[18]. A weight of 7% of rat body weight was tied on rat's tail and made to swim until they start sinking[19]. The swimming time was recorded in min. The water temperature was maintained at 50±0.5°C.

Somatic signs

Nicotine abstinence syndromes were observed and measured on Day 16 of treatment[20]. Signs like sniffing, rearing, teeth chattering, backing, nibbling, noisy respiration, climbing, scratching and head shaking were measured in occurrence per min[21].

Preparation of brain homogenate

On day 16, the rats were decapitated after 4 hours of withdrawal, brains removed, washed in pre-chilled normal saline and homogenized in an isotonic pre-chilled Tris HCl buffer (7.4 pH) with a glass homogenizer at 1000 rpm. The homogenized brain was used for dopamine estimation[22].

Estimation of brain dopamine in rat

Dopamine was extracted using a cis-diol-specific affinity gel and then acylated to N-acyldopamine and after this converted enzymatically during the detection procedure into N-acyl-3-methoxytyramine[23]. The competitive EIA kit was based on the microtiter plate format[24]. About 100 µL of the extracted standards and samples were transferred to the appropriate wells and incubated for 30 min at room temperature on a shaker set at 400-500 rpm. About 50 µL of dopamine antiserum was added into all wells and incubated for 2 h at room temperature on an orbital shaker (400-500 rpm). The contents of the wells were aspirated, discarded and washed thoroughly with 300 µL diluted wash buffer. This process was done twice. It was blotted dry by inverting plate on absorbent material. About 100 µL of the enzyme conjugate was transferred into all wells and incubated for 30 min at room temperature on an orbital shaker (400-500 rpm)[25]. Again, the aspirates were collected and rest of the samples were discarded and washed thrice. About 100 µL of substrate was transferred into wells and incubated for 20-30 min at room temperature on an orbital shaker (400-500 rpm). Then, 100 µL of the stop solution was added to each well and the micro titre plate was shaken to ensure a homogeneous distribution of the solution. The absorbance of the solution in the wells was observed within 10 min, using a micro plate reader set to a reference wavelength between 450 and 630 nm. The amount of antibody bound to the solid phase dopamine is inversely proportional to the dopamine concentration of the sample[26].

Estimation of serum cortisol in rat

Female albino rats were incised by aseptic surgical blade at the neck aorta portion and then bled. The blood was collected in a polypropylene container. Serum was isolated by centrifugation at 8000 rpm. About 20 µL of sample was dispensed in to a cuvette followed by 50 µL of Lite Reagent and 250 µL of solid phase and incubated for 5 min at 37°C. The aspirates were separated and the cuvettes washed with reagent water. Later, 300 µL each of acid reagent and base reagent were added to initiate the chemiluminescent reaction, and the readings were taken[27].

Statistical analysis

The mean ± SEM values were calculated for each group. For determining significance of intergroup difference, each parameter was analyzed separately by using one-way ANOVA. Student t test was also applied to detect 'p' value of $P < 0.05$ was considered to be the level of significance[28].

Results

Acute toxicity studies

In the acute toxicity study, all three formulations up to the dose level of 2000 mg/kg of body wt. did not exhibit any lethality or toxic symptoms. As per the OECD guidelines, these formulations were found to be safe and well tolerated without any behavioural changes during study.

Behavioural studies

Y-maze study

Latency period in each arm was noted, it is the time span in which an animal remains in immobile state. Less latency time indicates anxious state of brain. The histogram (Fig. 1) of Day 1 indicates that rats remained more immobile in dark arm. But during addiction i.e. at day 6 latency in dark arm reduces but on Day 16 (the day of nicotine withdrawal) the negative control group has highest latency in the dark arm. The treatment groups like market formulation and PHLF treated groups significantly reduced the latency compared to negative control i.e. the formulations proved to be good in reducing anxiety at the time of withdrawal. The studies were performed to assess effect of treatment on latency effect of nicotine.

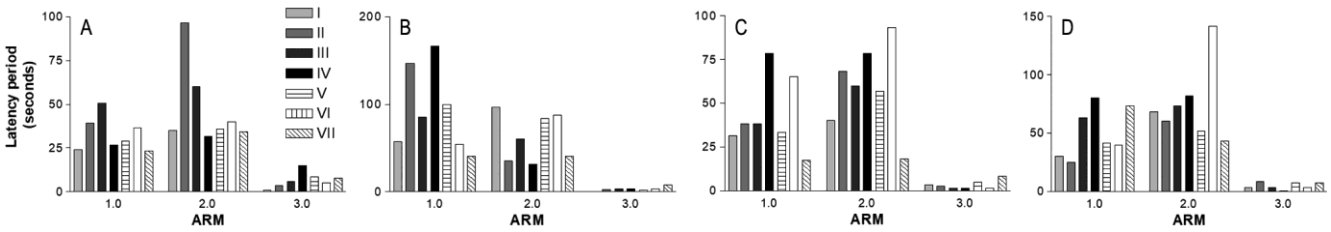

Fig. 1 — Latency period (A-D: Days 1, 6, 11 & 16, respectively) in each arm. [1.0, Open arm; 2.0, Dark arm; 3.0, Light arm. Groups: I, bupropion @ 40 mg/kg, p.o.; II, rid-tobak @ 200 mg/kg, p.o.; III, AELF, @ 200 mg/kg, p.o.; IV, WELF @ 200 mg/kg, p.o.; V, PHLF @ 200 mg/kg, p.o.; VI, 0.5% sodium carboxy methyl cellulose, p.o.; and VII, normal saline (0.9% NaCl)]

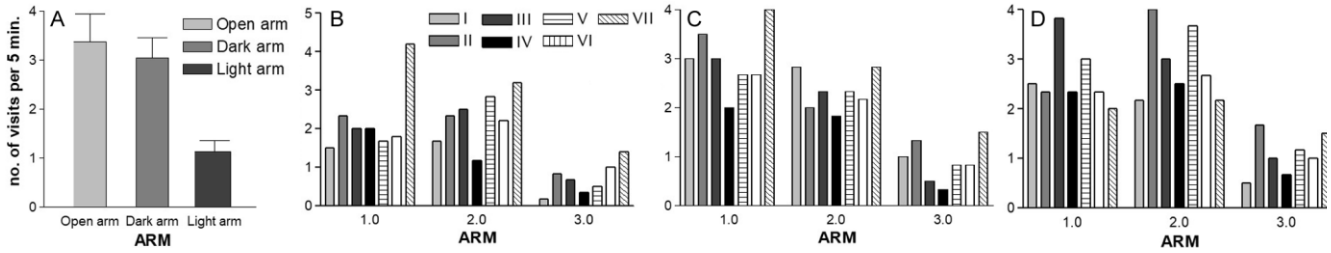

Fig. 2 — Number of visits on (A-D: Days 1, 6, 11 & 16, respectively) in each arm. [1.0, Open arm; 2.0, Dark arm; 3.0, Light arm. Groups: I, bupropion @ 40 mg/kg, p.o.; II, rid-tobak @ 200 mg/kg, p.o.; III, AELF, @ 200 mg/kg, p.o.; IV, WELF @ 200 mg/kg, p.o.; V, PHLF @ 200 mg/kg, p.o.; VI, 0.5% sodium carboxy methyl cellulose, p.o.; and VII, normal saline (0.9% NaCl)]

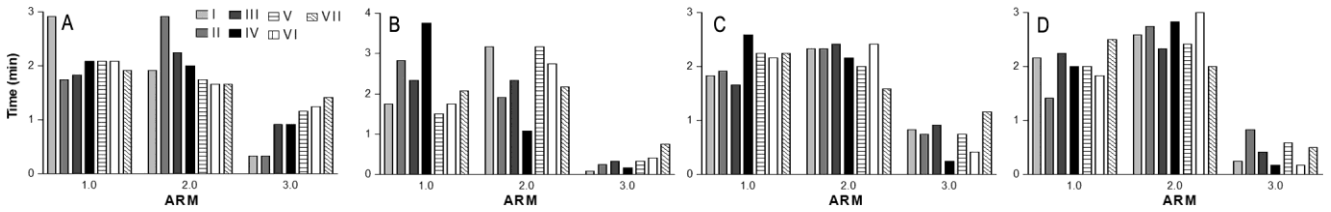

Fig. 3 — Total time spent on day 1, 11, 16 & 16 (A-D) in each arm. [1.0, Open arm; 2.0, Dark arm; 3.0, Light arm. Groups: I, bupropion @ 40 mg/kg, p.o.; II, rid-tobak @ 200 mg/kg, p.o.; III, AELF, @ 200 mg/kg, p.o.; IV, WELF @ 200 mg/kg, p.o.; V, PHLF @ 200 mg/kg, p.o.; VI, 0.5% sodium carboxy methyl cellulose, p.o.; and VII, normal saline (0.9% NaCl)]

More the visits in dark arm and less or no visit in lighted arm indicate the depressed state of brain. More the visits in every arm indicate the anxious state of mind. Fig. 2 (6th day) depicts decrease in total visits of all groups in all arms compared to normal Group VII. However, all groups except positive control and AELF treated group showed increased visits on 16th day of nicotine withdrawal (Fig 2) than the normal group.

On day 6 after nicotine treatment, time spent in light arm had decreased (Fig. 3) compared to day 1 (without nicotine treatment). At Day 11, AELF treated and bupropion treated group have significantly increased time spent in light arm. PHLF treatment has decreased time spent in dark arm (Fig. 3).

Tail immersion study

Nicotine produces analgesia within 5 min of subcutaneous administration in rats. During nicotine withdrawal period, the potentiating effect of nicotine

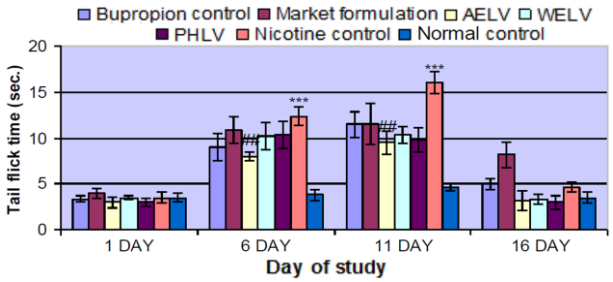

Fig. 4 — Tail flick time observed on day 1, 6, 11 and 16 of tail immersion study. [* compared to control, and # nicotine treated group]

induced analgesia would be diminished. Rats of all groups showed significant increase in tail flick time compared to normal group after nicotine treatment depicting the effect of nicotine as an analgesic. On 6 and 11 days of AELF treatment significantly reduced the analgesic effect of nicotine than nicotine control group (Fig. 4). There was no effect of nicotine withdrawal on the tail flick time when compared with 1st day.

Swimming endurance test

Nicotine reduces swimming time which is visible in nicotine control group at 11th day (Fig. 5). All groups except normal group have shown decrease in swimming time after nicotine injection. There is no effect of nicotine withdrawal on swimming performance. AELF treatment has increased the swimming time than nicotine control significantly at 6th day. All treatments did not have significant effect on swimming performance after nicotine treatment.

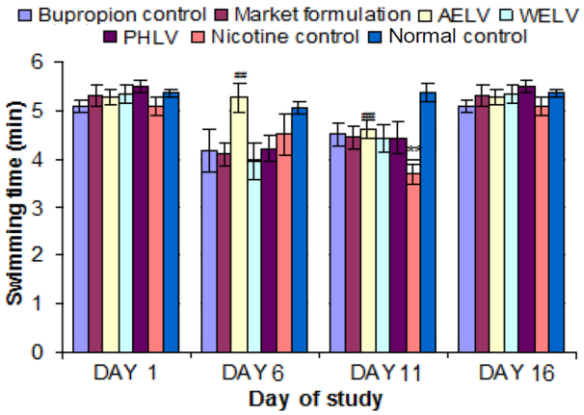

Fig. 5 — Swimming time (min) observed on day 1st, 6th, 11th and 16th day of the swimming endurance study. [* compared to control, and # nicotine treated group]

Nicotine abstinence study

In the nicotine control group, the withdrawal signs observed were exaggerated significantly as compared to the normal group on the 16th day (Table 1, & Fig. 6). Significant reduction in the nicotine withdrawal syndromes was observed in AELF treated rats when compared with the significantly from nicotine control group.

Effect on brain dopamine level

Dopamine measurement was performed after 24 h of nicotine withdrawal there was reduction in dopamine release when nicotine is suddenly withdrawn. From the graph nicotine control group rats were showing less dopamine content than normal group rats. PHLF, AELF and Market formulation treatment have significantly maintained the dopamine compared to nicotine control group (Fig. 7).

Effect on serum cortisol

Bupropion, market formulation and PHLF treatment significantly reduced the cortisol level compared to

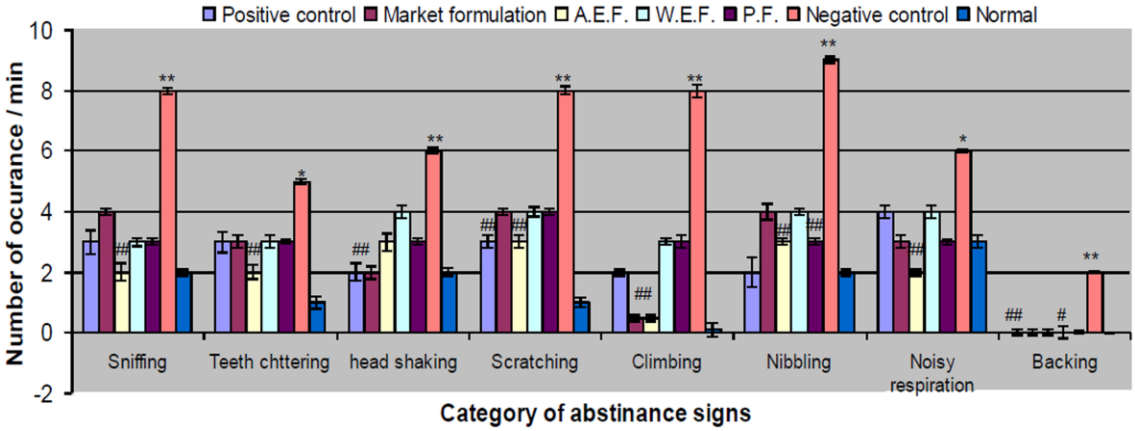

Fig.6 — Nicotine withdrawal syndrome on day 16th of study. [* compared to normal control, and # nicotine treated group]

Table 1 — Nicotine abstinence syndrome on Day 16 (the day of withdrawal)

	Nicotine Abstinence Signs							
Group	Sniffing	Teeth chattering	Head shaking	Scratching	Climbing	Nibbling	Noisy respiration	Backing
I	3.0±0.4	3.0±0.33	2.0±0.3$^{\#\#}$	3.0±0.2$^{\#\#}$	2.0±0.1	2.0±0.5	4.0±0.2	0.0±0.1$^{\#\#}$
II	4.0±0.1	3.0±0.2	2.0±0.2	4.0±0.1	0.5±0.1$^{\#\#}$	4.0±0.25	3.0±0.2	0.0±0.1
III	2.0±0.3$^{\#}$	2.0±0.24$^{\#}$	3.0±0.3	3.0±0.2$^{\#\#}$	0.5±0.1$^{\#\#}$	3.0±0.1$^{\#\#}$	2.0±0.1$^{\#\#}$	0.0±0.1
IV	3.0±0.13	3.0±0.2	4.0±0.2	4.0±0.15	3.0±0.1	4.0±0.1	4.0±0.2	0.0±0.2$^{\#}$
V	3.0±0.1	3.0±0.05	3.0±0.11	4.0±0.1	3.0±0.21	3.0±0.1$^{\#\#}$	3.0±0.08	0.0±0.05
VI	8.0±0.1**	5.0±0.08*	6.0±0.1**	8.0±0.14**	8.0±0.2**	9.0±0.1**	6.0±0.05*	2.0±0.02**
VII	2.0±0.1	1.0±0.2	2.0±0.12	1.0±0.15	0.1±0.25	2.0±0.1	3.0±0.2	0.0±0.02

[Each column represents the mean ± S.E.M. (n=6). $^{\#}$ P <0.05, $^{\#\#}$ P <0.01, $^{\#\#\#}$ P <0.001 as compared to nicotine treated group. * P <0.05, ** P <0.01, *** P <0.001 as compared to normal group]

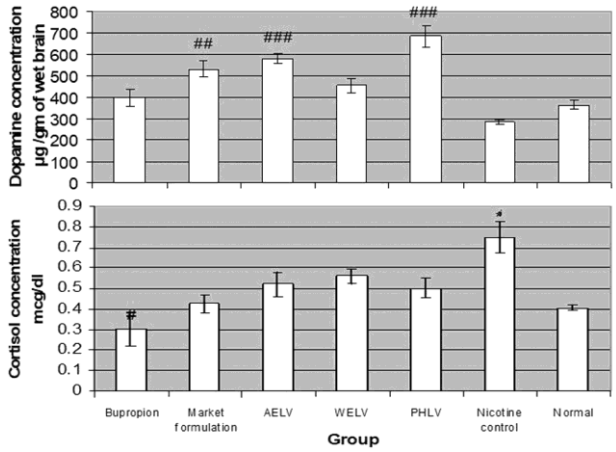

Fig. 7 — (A) Brain dopamine levels (µg/g); and (B) serum cortisol level (µg/dL) in rat brain on day 16. [* compared to normal, and # nicotine treated group]

nicotine control group during nicotine withdrawal as shown in Fig. 7B.

Discussion

Nicotine dependence was induced successfully in mice and on 16th day, significant withdrawal symptoms were observed in comparison to the control group. Nicotine binds to the neurons in certain areas of the brain, on consumption of tobacco, which quickly secrete some dopamine, which acts on the "reward" areas of the brain and is perceived as very pleasurable. But the receptors on these cells lose their sensitivity to nicotine in a short time, mere seconds to a few min, and the cells stop secreting dopamine. As it turns out, this same type of receptor for nicotine also occurs on pre-synaptic terminals in this same area of the brain which are activated by nicotine. This leads to the release of a signal molecule, glutamine, which acts on certain dopaminergic neurons to stimulate them to release more dopamine. This activation results in continued release of dopamine for hours, a case of long-term potentiation of excitatory input. The whole mechanism is similar to events seen during learning and memory and may be an important early step in the development of addiction[2].

Prepared novel tobacco cessation formulations showed their efficacy in many ways in comparison with marketed herbal formulation and allopathic molecule i.e. bupropion treatments. AELF and PHLF treatment results have proved to be effective to reduce the nicotine craving by maintaining the dopamine level in brain and reducing cortisol level in serum. Therefore, AELF and PHLF were found to be potent for nicotine cessation[29]. The herbs incorporated in the formulations, for example, *W. somnifera* contain some major active constituents such as withanolide A, withanoside IV and withanoside VI[30]. These compounds have GABA mimetic and acetyl cholinesterase activity. The aqueous and alcoholic extracts of this plant have been proved to be effective in alcohol withdrawal induced anxiety and convulsions in mice. Studies have reported that 1.0 µM doses of each compound produces significant neurite outgrowth in dopaminergic SH-SY5Y cells. They have also been used traditionally to protect health of mind and body as an adaptogenic, rejuvenative, anti-inflammatory and antistress[31]. Studies have proved the effect of *Withania somnifera* in conditions of stress, fatigue, mood swings and weakness due to disease or drug withdrawal[32]. It helps to restore mental and physical vitality and adaptability[33]. *Ocimum tenuiflorum* has been reported to show antistress activity in despair swim test model of mice[34]. It is also reported to treat the respiratory tract problems and thus the issues related to nicotine withdrawal like coughing and sore throat can be eliminated[35].

Chronic constipation and intestinal cramps are the major withdrawal symptoms showing physical dependence and *A. catechu* has been used traditionally for treatment of digestive problems like constipation[36]. *Cinnamomum zeylanicum* has been reported to make the taste of tobacco unpalatable thus reducing the craving for nicotine[37]. *Avena sativa* has been reported to reduce the pressor response produced by nicotine in anesthetized rat[38]. It is also reported to be a nervine tonic. *Glycyrrhiza glabra* has been well studied for its effect on norepinephrine and dopamine leading to an anti-depressant activity[39,40]. Bupropion, a nor-epinephrine-dopamine re-uptake inhibitor has been used extensively as one of the drugs in smoking cessation[41]. However, it has been reported to have numerous side effects like epileptic seizures, nausea, headache, etc.[42].

Conclusion

In this study, we made an attempt to develop a polyherbal formulation using parts of Ashwagandha or Indian ginseng (*Withania somnifera*), Oat (*Avena sativa*), Cinnamon (*Cinnamomum cassia*), Cutch tree (*Acacia catechu*), Tulsi (*Ocimum tenuiflorum*) and Liquorice (*Glycyrrhiza glabra*) for tobacco addictive patients. The results of behavioural studies and biochemical markers showed that the formulation

AELF (alcoholic extract) and PHLF (powdered crude drug) have significantly reduced the nicotine abstinence symptoms, stress and cortisol levels and managed to maintain the brain dopamine levels in rats. The present study performed in rats induced with nicotine dependence, proposes the option of using AELF and PHLF formulations for nicotine de-addiction as well as for treatment of mental and physical withdrawal symptoms. The two formulae, AELF and PHLF should be studied further to determine the dose dependent efficacy followed by clinical studies.

Acknowledgement

The authors are thankful to Dr. Swami, Ex-dean, Manibhai Civil Ayurvedic Hospital, Ahmedabad for designing novel formulation. We are also thankful to Dr. Manubhai Agravat, Ayurvedacharya, Rajkot for helping in formulation development and validation.

Conflict of interest

Authors declare no conflict of interests.

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