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Title: Effect of Hydro-Alcoholic Root Extract of Delphinium Denudatum Wall on Mecamylamine-Precipitated Nicotine-Withdrawal and Corticosterone Levels in Rats

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Abstract

This study evaluates the effects of hydro-alcoholic root extract of *Delphinium denudatum W*all (Jadwar, family; Ranunculaceae) for its ability to attenuate the nicotine-withdrawal in nicotine-dependent rats. The physical dependence on nicotine was induced in male adult Wistar albino rats (175-250 g) by subcutaneous implantation of Alzet mini osmotic pumps which supplied nicotine at 9.0mg/kg/day, while control rats received saline via osmotic pumps. For seven days four separate test doses of hydro-alcoholic root extract of *Delphinium denudatum Wall* (200, 400, 800, 1600 mg/kg) were given orally. On the 7th day, a mecamylamine injection (1 mg/kg, s.c.) was given to precipitate nicotine-withdrawal. The global Gellert's–Holtzman rating scale was used to rate somatic signs of withdrawal for 15 minutes, followed by a measurement of motor activity. Drug Bupropion was used as a positive control. Serum levels of nicotine (cotinine) and corticosterone were done by ELISA. In nicotine-dependent rats, oral administration of *Delphinium denudatum Wall* root extract suppressed the hyper-locomotion and decreased the corticosterone levels at all dosages. Higher doses (800 and 1600 mg/kg) of extract, significantly attenuated nicotine withdrawal whereas, lower doses (200 and 400 mg/kg) had no significant effect. These results suggest that hydro-alcoholic root extract of Delphinium denudatum Wall may prove to be a potential therapeutic agent to attenuate nicotine dependence and facilitate tobacco smoking cessation.

Keywords: Delphinium denudatum Wall, Nicotine, Hydro-alcoholic, Withdrawal, Bupropion, Mecamylamine

1. Introduction

The primary addictive component identified in tobacco is nicotine, which exerts its psychoactive effects via interaction with nicotinic acetylcholine receptors (nAChRs) in the central nervous system (Jenson and Sofuoglu 2016). Nicotine produces physical dependence and tolerance both in humans and animals (Wang and Sun, 2005). Repeated exposure to nicotine induces neural adaptations in brain reward and stress response pathways in the central nervous system, which are important for the development of dependence (Benowitz 2010; Koob and Volkow. 2010; Rohleder and Kirschbaum. 2006). This leads to nicotine's positive and negative reinforcing effects, that facilitate dependence. When a smoker undergoes nicotine withdrawal, the negative reinforcing properties of nicotine occur. In rodents (Malin et.al., 1992) and humans (Hughes et al., 1991), abstinence from chronic nicotine use leads to a withdrawal syndrome. This syndrome can be characterized by the emergence of somatic signs such as difficulty in concentration, restlessness, irritability, anxiety, impatience, insomnia, excessive hunger, drowsiness, leading to an increase in craving for nicotine. As a result, the number of binding sites located on the nicotinic cholinergic receptors in the brain increases. This could be due to nicotine-mediated desensitization of receptors, which contributes to tolerance and dependence on nicotine (Dani and De Biasi, 2001). Nicotine administration has been shown to activate the hypothalamic-pituitary-adrenal (HPA) axis, the main mediator of the neuroendocrine stress pathway, stimulating the secretion of adrenocorticotrophic hormone (ACTH) and corticosterone (Jenson and Sofuoglu 2016). Plasma levels of ACTH and corticosterone are raised in rats experiencing stress due to nicotine withdrawal (Rhodes et al., 2004; Lutfy et al., 2006; Torres et al., 2013). Rodent models of nicotine withdrawal are important tools for evaluating the mechanisms underlying nicotine dependence and are potentially useful for designing interventions to aid in smoking cessation (Jain et al., 2008). Many pharmacological therapies have been developed or are currently under evaluation for smoking cessation (Cahill et al., 2014; Prochaska and Benowitz, 2016). These interventions have had limited success and some have shown side effects. Existing pharmacotherapy for tobacco cessation including bupropion, varenicline, or nicotine replacement is effective in around one-third of patients, indicating the need for additional therapies (Fiore et al., 2008; Cahill et al., 2014). Plants and natural products are a potential source of novel and safer compounds to use for drug withdrawal management (Ward et al., 2011; Tabatabai et al., 2014).

Delphinium denudatum Wall (DD) (Jadwar) is an annual herb that belongs to the family Ranunculaceae occurring in the western Himalayan region from Kashmir to Kumaon in India. It is one of the most important drugs used in the Indian systems of medicines for different complaints and diseases, especially in Unani Medicine (Qudsia and Jafri, 2006). The roots of this plant are used in various medical formulations in Unani and Ayurveda. Its phytochemical and pharmacological properties have been evaluated in several studies (Singh and Chopra, 1962; Zafar et al., 2001). Chemically DD comprises many bioactive constituents (Singh and Chopra, 1962). Its use in the treatment of opium dependence has been mentioned in classical literature (Ghani, 1928; Shirazi, 1737 and 1744) and has potent effects on nervous disorders (Husain, 1875; Husain, 1897). Its ability to aid in de-addiction in morphine-dependent rodents has been reported (Rahman et al. 2002). These authors reported that alcoholic DD root extract significantly attenuated the scores for all parameters in morphine withdrawal syndrome through central action, suggesting that it could be effective in morphine de-addiction. The protective effect of DD root extract on the development of tolerance and dependence in mice was studied by Zafar et al. (2002). Based on these findings, it is hypothesized that hydro-alcoholic root extract of DD may attenuate nicotine withdrawal due to common neurobiological mechanisms for dependence.

The present experiments examined the effects of hydro-alcoholic root extract of DD on mecamylamine-precipitated nicotine withdrawal and corticosterone levels in rats.

2. Methodology

2.1. Animals

Adult male albino Wistar rats (175-250 g, n = 60) were used for the study (Flow chart - I). Rats were individually housed under standard laboratory conditions ($23\pm1°C$, 12–12 h light-dark cycle) and had unlimited access to food and water. Before the experiment, they were acclimatized to laboratory conditions for three days. All experimental procedures were approved by the Institutional Animal Ethics Committee.

2.2. Preparation of hydro-alcoholic extract of *Delphinium denudatum Wall* (DD) roots

Soxhlet extractor was used to prepare hydro-alcoholic root extract of *Delphinium denudatum* (DD) (Rakhee et al., 2018; Redfern et al., 2014). The roots of DD were shade-dried and pulverized to make a fine powder. The solvent mixture containing an equal amount of alcohol and distilled water (1:1) was used for extract preparation. The powdered drug was packed in the filter paper and placed gently in the thimble. The extract was carried out by applying heat on the heating mantle at 50°C for 8 hr. Vapors of a fresh solvent was produced in a distillation flask. These vapors were passed

through the thimble containing powdered drug and were liquefied in the condenser. When the liquid reached the overflow level in the thimble, a siphon aspirated the solution, and the liquid fell back into the distillation flask, carrying the extracted drug into the bulk liquid. The distillation flask was used to separate the drug from the solvent. The extracted drug was then left in the flask, with fresh solvent vapors passing back into the solid bed of the powdered drug. The extract was filtered, collected, and dried on a water bath. The obtained semi-solid mass was weighted to determine the weight of the drug for subsequent usage.

2.3. Induction of Physical Dependence

Under Ketamine anesthesia, all rats were implanted subcutaneously (s.c.) in the scapular region with an Alzet $2ML1$ (7 days) osmotic mini-pump filled with Nicotine tartrate di-hydrate in saline (Alza Scientific Products, Palo, Alto, CA, USA). For 7 days, the nicotine concentration was adjusted for subject weight differences to deliver a continuous subcutaneous infusion at the rate of 9 mg/kg/day (equivalent to 3.15/kg/day expressed as the nicotine base). Each pump was primed in normal saline for four hours before implantation (Malin et al., 1992, 1994).

2.4. Experimental design

Five experiments were conducted to study the effects of hydro-alcoholic root extract of DD on mecamylamine-precipitated nicotine withdrawal in nicotine-dependent rats (Flow chart - I). Experiments 1, 2, 3, and 4 were designed to examine the effects of four different doses of DD (200, 400, 800, 1600 mg/kg, respectively) on nicotine withdrawal (Table 1). In total 60 rats were used in the study and were randomly assigned to each experiment containing 12 rats each. Each experiment had two groups of rats: a control group (Group 1) and an experimental group (Group 2), each with six rats. Group 1 rats received saline-filled Alzet mini-osmotic pumps, while Group 2 rats received nicotine-filled Alzet mini-osmotic pumps. Experiment 5, served as a positive control group, with 12 rats divided into two groups: a control group (Group 1) and an experimental group (Group 2), each with six rats. In both groups, bupropion (20 mg/kg) was administered intraperitoneally for 7 days to assess its effects on nicotine withdrawal and to compare it with other experimental groups who were administered hydro-alcoholic root extract of DD (Table 1).

2.5. Behavioral observation

2.5.1. Measurement of Precipitated withdrawal

Rats were weighed on day 7 of nicotine infusion (168 hours after the pump was implanted). They were given injection mecamylamine (1 mg/kg) subcutaneously after 2 hours of test drug (DD) to precipitate withdrawal. Thereafter, animals were immediately placed in an activity cage and visually observed for 15 min after receiving mecamylamine injection. They remained in the activity cage for another ten minutes (Malin, 1994).

The global Gellert–Holtzman rating scale was used to rate the individual somatic signs of withdrawal (Gellert and Holtzman, 1978; Jain et al., 2004; Raghav et al., 2018). The global GH rating scale included graded signs such as weight loss, numbers of escape attempts, abdominal constrictions, number of escape attempts, and the presence or absence of checked signs like swallowing movements, diarrhea, ptosis, facial fasciculation/teeth chattering, penile grooming/erection/ejaculation, profuse salivation, abnormal posture, chromodacryorrhea, and irritability upon handling. With the exception of weight loss, graded signs were given a weighting factor from 1 to 4 on the global Gellert–Holtzman rating scale based on how often they appeared. Depending on the withdrawal signals detected, but independent of the frequency of appearance, the checked signs receive values of 2–7 (Jain et al., 2008; Raghav et al., 2018). Except for weight loss, all somatic signs were recorded during the first 15 minutes following the injection (Jain et al. 2008). The difference between the weight measured immediately before the mecamylamine injection and the weight measured 60 minutes later was used to compute the weight reduction. During this time, the rats were not provided any food. Thus, for the first 15 minutes, the scores of precipitated withdrawal were recorded along with the measurement of gross activities (Jain et al. 2008).

2.5.2. Measurement of Motor Activity

The Activity Monitor (True Scan Photo beam Sensor-E63-22, Coulbourn Instruments, Inc. USA) was used to measure the motor activity. On days 0, 2, 4, 6, and 7, before the mecamylamine challenge, the activity was monitored for 25 minutes at 5-minute intervals. The animals were weighed each day before measuring a motor activity and again 2 hours following the mecamylamine challenge.

2.6. Measurement of serum cotinine and corticosterone levels

After behavioral experiments, rats were anesthetized by the ketamine injection. The heart was incised open and 1 ml of blood was collected in BD Vacutainer tube and was allowed to clot in an upright position for at least 30 minutes at room temperature. Thereafter, serum was separated by centrifugation for 15 minutes at 2500 RPM at 4°C and was immediately transferred to a sterile microcentrifuge tube and stored at -80°C till further analysis. ELISA Kits were used to determine the levels of serum corticosterone (EC3001-1, Assaypro, USA) and cotinine (nicotine metabolite) (BC-ER141403, Biocodon Technologies, USA) as per the manufacturer's guidelines. Samples or, standards were run in duplicate. The M200 PRO, Multimode ELISA Reader (TECAN, Austria) instrument was used to read the ELISA Plate. The standard curve was plotted using the standard concentrations on the x-axis and the corresponding mean absorbance (OD) on the y-axis. Finally, the statistical software computed the serum corticosterone and serum cotinine levels (ng/ml) based on the optical density values of the standard and the samples.

2.7. Drugs

The roots of DD were purchased from Dawakhana Tibbiya College, Aligarh Muslim University (AMU), Aligarh, India and, were air-dried. Thereafter, it was identified at the National Institute of Science Communication and Information Resources (NISCAIR) (Ref. no. NISCAIR/RHMD/2017/3130-79) New Delhi, India, under the supervision of faculty of Unani Medicine, Jamia Hamdard, Delhi. The Hydro-alcoholic (1:1) extract of the air-dried powdered roots was prepared in Soxhlet's apparatus. Nicotine tartrate dihydrate salt and Bupropion hydrochloride were procured from Tokyo Chemical Industry (TCI), Japan. Mecamylamine was procured from Cayman Chemical Company, USA, and Ketamine hydrochloride of Claris Injectables Limited, India. Nicotine and mecamylamine were administered subcutaneously while ketamine (50mg/Kg) was given intraperitoneally. Hydro-alcoholic extract of DD roots dissolved in normal saline and given by oral route with help of flexible cannula, as this is the route of choice of drug administration in Unani System of Medicine. Drug mecamylamine was given at the volume of 1 mg/kg and Bupropion at 20mg/kg body weight expressed with the free base.

2.8. Data Analysis

The software SPSS 22 was used to conduct all of the analyses. For comparing motor activity within the groups, repeated measure ANOVA (two-way ANOVA) was applied followed by multiple comparisons using the Bonferroni test. The motor activity between the groups was compared using a one-way ANOVA with the Bonferroni test for multiple comparisons. The individual behavioral signals were analysed using the Chi-square test. The significance level was set at 0.05 percent.

3. Results

3.1. Comparison of motor activity in five experiments: Repeated measure ANOVA with post-hoc test revealed that nicotine resulted in a significant increase in motor activity in the nicotine-treated rats compared to saline-treated rats from day 0 to 4 ($F4/20 = 3.223$, $p < 0.01$). As indicated in figure 1A-D, there was a non-significant decrease in motor activity on day 6. Furthermore, after the mecamylamine challenge on day 7, nicotine-dependent rats showed a significant increase in motor activity ($p < 0.001$), whereas saline and bupropion-treated rats showed no change after the mecamylamine challenge. On the other hand, no significant change was observed over the period of 7 days in motor activity of sal ($F_{4/20} = 0.333$, $p > 0.05$), nic + bup ($F_{4/20} = 0.663$, $p > 0.05$) and nic + DD (200 DD mg/kg: $F_{4/20} = 1.698$, $p > 0.05$; 400 DD mg/kg: $F_{4/20} = 1.698$, $p > 0.05$; 800 DD mg/kg: $F_{4/20} = 0.547$, $p > 0.05$; 1600 DD mg/kg: $F_{4/20} = 0.792$, $p > 0.05$)-treated rats as compared to nicotine-treated rats challenged with mecamylamine (Figure 1A-D). Comparison of motor activity between the groups during withdrawal was done by one way ANOVA followed by Bonferroni test. On day 7 nicotine-dependent rats after mecamylamine injection showed significantly higher motor activity than in DD-treated rats at all doses (200 DD mg/kg: $F4/25 = 18.881$, $p < 0.001$; 400 DD mg.kg: $F4/25 = 18.976$, $p < 0.001$; 800 DD mg/kg: $F4/25 = 10.738$, $p < 0.0001$; 1600 DD mg/kg: $F4/25 = 13.120$, $p < 0.001$). Hence, all doses of DD (200, 400, 800, 1600 mg/kg) appeared to be effective in attenuating the hyperlocomotion in nicotine-dependent rats (Figure 1A-D).

3.2. Comparison of GH-sore in five experiments:

DD (200 mg/kg): One-way ANOVA revealed a significant difference in GH-Score during withdrawal in mecamylamine-precipitated nicotine-dependent rats. During nicotine withdrawal, the posthoc test confirmed the significant increase in GH-Score in nicotine-dependent rats ($F_{4/25} = 14.014$, $p < 0.001$) as compared with saline, nic + bup, and sal + DD. The downward trends of

GH-score were observed in rats treated with 200 mg/kg dose of DD e but not up to the significant level ($p > 0.05$) (Figure 2A).

DD (400 mg/kg): A similar downward trends of GH-score was found in 400 mg/kg DD-treated rats as observed in 200 mg/kg DD-treated rats (Figure 2B). Post-hoc test confirms that there was no significant difference in GH-Score of mecamylamine challenged 400 mg/kg DD and nicotine-treated rats ($F_{4/25} = 15.585$, $p > 0.05$). Whereas, GH-Score was found to be significantly lower in sal ($p < 0.001$), nic + bup ($p < 0.001$) and sal + nic ($p < 0.001$) (Figure 2B).

DD (800 mg/kg): The GH-score was found to be significantly lower in rats treated with 800 mg/kg DD dose than in the mecamylamine-precipitated nicotine-dependent rats ($F_{4/25} = 11.500$, $p < 0.05$). No significant difference was found in GH-score between the sal, nic + bup, and DD-treated rats by one-way ANOVA (Figure 2C).

DD (1600 mg/kg): The rats treated with an oral dose of 1600 mg/kg DD was found effective and significantly decreased the GH-Score as compared with mecamylamine challenged nicotine-dependent rats ($F_{4/25} = 15.990$, $p < 0.0001$). No significant difference was observed in GH-score between the rats treated with DD dose and control rats (Figure 2D).

in GH-Score of mecamylamine challenged 400 mg/kg DD and nicotine-treated rats

Individual behavioral signs (Graded and checked signs): The graded and checked signs were analyzed by using Fisher-exact test (Table 2). Escape attempts, ptosis, abnormal posture, ejaculation and irritability were highly prominent in mecamylamine-precipitated nicotine-dependent rats. The oral administration of DD significantly attenuated the graded sign: escape attempts at all doses (200 mg/kg: $\chi = 11.387$, $p < 0.05$; 400 mg/kg: $\chi = 15.572$, $p < 0.01$; 800 mg/kg: $\chi = 13.846$, $p < 0.01$; 1600 mg/kg: $\chi = 13.708$, $p < 0.01$). The higher doses of DD (800 and 1600 mg/kg) significantly reduced the checked signs like abnormal posture (800 mg/kg: $\chi = 7.177$, $p < 0.05$; 1600 mg/kg: $\chi = 9.196$, $p < 0.05$) and ptosis (800 mg/kg: $\chi = 7.177$, $p < 0.05$; 1600 mg/kg: $\chi = 7.164$, $p < 0.05$), whereas, lower doses of DD (200 and 400 mg/kg) showed reduction in abnormal posture (200 mg/kg: $\chi = 2.570$, $p > 0.05$; 400 mg/kg: $\chi = 5.673$, $p > 0.05$) and ptosis (200 mg/kg: $\chi = 1.363$, $p > 0.05$; 400 mg/kg: $\chi = 2.197$, $p > 0.05$), but was found to be non-significant. The suppression was also observed in checked signs of irritability and genital grooming in rats treated with all doses of DD but was statistically non-significant. The facial fasciculation was

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observed equally in all rats and no change was observed after oral administration of DD at all does (Table 2).

3.3. Serum Corticosterone levels of DD-treated rats: One-way ANOVA revealed significant difference in corticosterone levels in mecamylamine-precipitated nicotine-dependent rats during withdrawal (Figure 3). The post-hoc test confirmed the significant increase in serum corticosterone levels ($F_{2/15}$ =22.708, $p < 0.0001$) during withdrawal in nic + mec-treated rats as compared with sal and nic + bup + mec-treated rats as shown in Figure 3. The serum corticosterone levels were found to be significantly lower in DD-treated rats at all four doses (DD 200 mg/kg: $F_{4/25}$ =11.011, $p < 0.0001$; DD 400 mg/kg: $F_{4/25}$ =12.308, $p < 0.0001$; 800 DD mg/kg: $F_{4/25}$ =17.832, $p < 0.0001$; 1600 DD mg/kg: $F_{4/25}$ =16.528, $p < 0.0001$) than in the mecamylamine-precipitated nicotine-treated as shown in figure 3.

3.4. Serum cotinine levels DD-treated rats: To examine the differences in serum cotinine levels between the groups, one-way ANOVA followed by Bonferroni test (Post-hoc) was applied. The rats implanted with nicotine filled Alzet mini-osmotic pumps showed significantly higher cotinine levels as compared with rats implanted with saline filled Alzet mini-osmotic pumps (DD 200 mg/kg: $F_{4/25}$ =25.237, $p < 0.0001$; DD 400 mg/kg: $F_{4/25}$ = 37.779, $p < 0.0001$; 800 DD mg/kg: $F_{4/25}$ =42.427, $p < 0.0001$; 1600 DD mg/kg: $F_{4/25}$ =39.197, $p < 0.0001$) (Figure 4). There was no significant difference in cotinine levels in nicotine-dependent rats after treatment with bupropion and DD at all four doses ($F_{5/30}$ = 0.387, $p < 0.05$) as shown in figure 4.

4. Discussion

The study examined the ability of hydro-alcoholic root extract of DD to attenuate nicotine dependence in nicotine-dependent rats. Nicotine was administered subcutaneously through an osmotic mini-pump followed by a mecamylamine challenge (Malin et al., 1993, 1994; Jain et al., 2008). The present study applied the global Gellert–Holtzman (GH) rating scale, for assessing the mecamylamine-precipitated nicotine withdrawal signs. This scale has been previously validated for measuring nicotine withdrawal signs (Jain et al., 2008). The global GH rating scale assesses the total index of withdrawal intensity. The present study indicated a significantly higher withdrawal intensity in nicotine-dependent rats after the mecamylamine challenge as compared to control rats (Fig. 2 and Table 2). Graded signs like escape attempts were highly prominent in

nicotine-dependent rats(Table 2). Checked signs, viz. ptosis, irritability, ejaculation or genital grooming, and abnormal posture, were also found to be highly intense in nicotine-dependent rats, whereas, teeth chattering or facial fasciculation were equally observed in all rats. The nicotine delivered by the mini- osmotic pump in 24 hours was in the same dose range as that used in earlier studies of nicotine withdrawal in rats and approximated to the daily dose of nicotine ingested by a heavy smoker (Jain et al., 2008; Malin et al., 1992, 1993, 1994).

The results from the present study demonstrated that higher doses of hydro-alcoholic DD root extract (800, 1600 mg/kg) significantly suppressed the mecamylamine-precipitated withdrawal in rats dependent on nicotine when compared to control rats, whereas, lower doses (200, 400 mg/kg) were found to be non-significant. There was a significant reduction in GH-Score after oral administration of higher doses of DD root extract (Fig 2A-D). The escape attempts were found to be suppressed at higher doses of DD root extract while lower doses were not found to be effective in suppressing the escape attempts. Similarly, abnormal posture and ptosis were significantly reduced when DD was administered at higher doses of (800, 1600 mg/kg), whereas, no significant decrease was observed at lower doses (200 and 400 mg/kg). Also, no changes were seen in some checked signs like facial fasciculation, genital grooming, and irritability after all administered oral doses of DD (Fig 2A-D). Behavioral signs like abdominal constriction, wet dog shakes, diarrhea, weight loss, grasps, tremors, and chews were not seen in our study. Some previous studies showed that ethanolic extract of DD has a significant effect in reducing the withdrawal signs like wet-dog shake, jumping, head shake, writhing, lacrimation, yawning, rearing, eye twitching, digging in morphine-dependent rats (Zafar et al., 2001, 2002; Rahman et al.,2002; Zaheer et al., 2016). The effects of the control drug, bupropion were highly significant in our study. Acute and chronic bupropion exposure alleviates the expression of somatic signs and precipitated withdrawal (Cryan et al., 2003; Malin et al., 2006). The findings of this study are in line with the earlier research studies, which also report an increase in motor activity with nicotine exposure (Ksir et al., 1987; Di Franza and Wellman 2007; Li et al., 2008; Jain et al. 2008). Th could be related to qualitative changes in nicotine receptor agonism's motor effects during the development of behavioral sensitization, a vital role in the development of dependence (Kayir et al., 2009). Thus sensitization may be a suitable target for developing therapies for treatment or prevention of addiction. The results also showed that motor activity significantly increased further in nicotine-dependent rats after the mecamylamine challenge when compared to saline-dependent rats ($P < 0.001$) (Fig 1A-

D). All four doses of hydro-alcoholic DD root extract are equipotent in attenuating the locomotor sensitization in nicotine-dependent rats.

The effects of DD root extract on corticosterone and cotinine levels in mecamylamine precipitated nicotine-dependent rats were also examined and the corticosterone levels were significantly increased in these rats. Many studies have previously shown that when rats experience nicotine withdrawal their plasma levels of corticosterone and ACTH are elevated (Rhodes et al., 2004; Semba et al., 2004; Lutfy et al., 2006). In line with other published reports, our results also demonstrate that nicotine withdrawal activates the HPA axis and stimulates the secretion of corticosterone in rodents. The rats treated with oral administration of DD root extract, at all doses, significantly decreased the corticosterone levels in mecamylamine-challenged nicotine-dependent rats. This indicated that hydro-alcoholic DD root extract also has attenuating effect on the stress pathway. The bupropion-treated rats also showed a significant reduction in corticosterone levels. Cotinine levels showed no significant changes after oral administration of DD root extract at all doses and after bupropion treatment. Earlier studies have described the mechanism involved in the prevention of morphine withdrawal by DD. This could also explain the preventive role of DD on nicotine withdrawal due to common neurobiological mechanisms for dependence (Zaheer et al., 2016; Rahman et al., 2002). Alkaloids commonly found in DD are derivatives of the norditerpenoid lycoctonine and they have been reported to inhibit acetylcholine and whole-cell currents induced by anatoxin in hippocampal neurons of cultured fetal rats (Alkondon et al., 1992). Moreover, MLA, which is an active ingredient of DD binds to the α-bungarotoxin site in the brain and suppresses the κ- bungarotoxin sensitive nicotinic receptor in ciliary ganglion (Yum et al., 1996). The suppression of motor activity decreased GH-score, and corticosterone levels, which are manifested during nicotine withdrawal could be due to the blockade of α7 nAChR receptors by the DD in experimentally treated rats. The exact mechanism of action of DD is still unclear and needs further evaluation. In the light of the above findings, hydro-alcoholic root extract of DD may prove to be an alternative treatment for nicotine cessation**.**

5. Conclusion

The hydro-alcoholic root extract of *Delphinium denudatum Wall* was effective in attenuating hyper-locomotion, mecamylamine-precipitated nicotine withdrawal, and corticosterone levels in nicotine-dependent rats. Scientific studies showed promising results on the use of *Delphinium denudatum Wall* in dependence and tolerance. Further studies are needed to elucidate the active principle and its mechanism of action. The present test drug used in the traditional system of medicine may prove to be a potential therapeutic agent to attenuate the symptoms of nicotine withdrawal and facilitate tobacco smoking cessation.

Declaration of Competing Interest

The authors declare no conflict of interest.
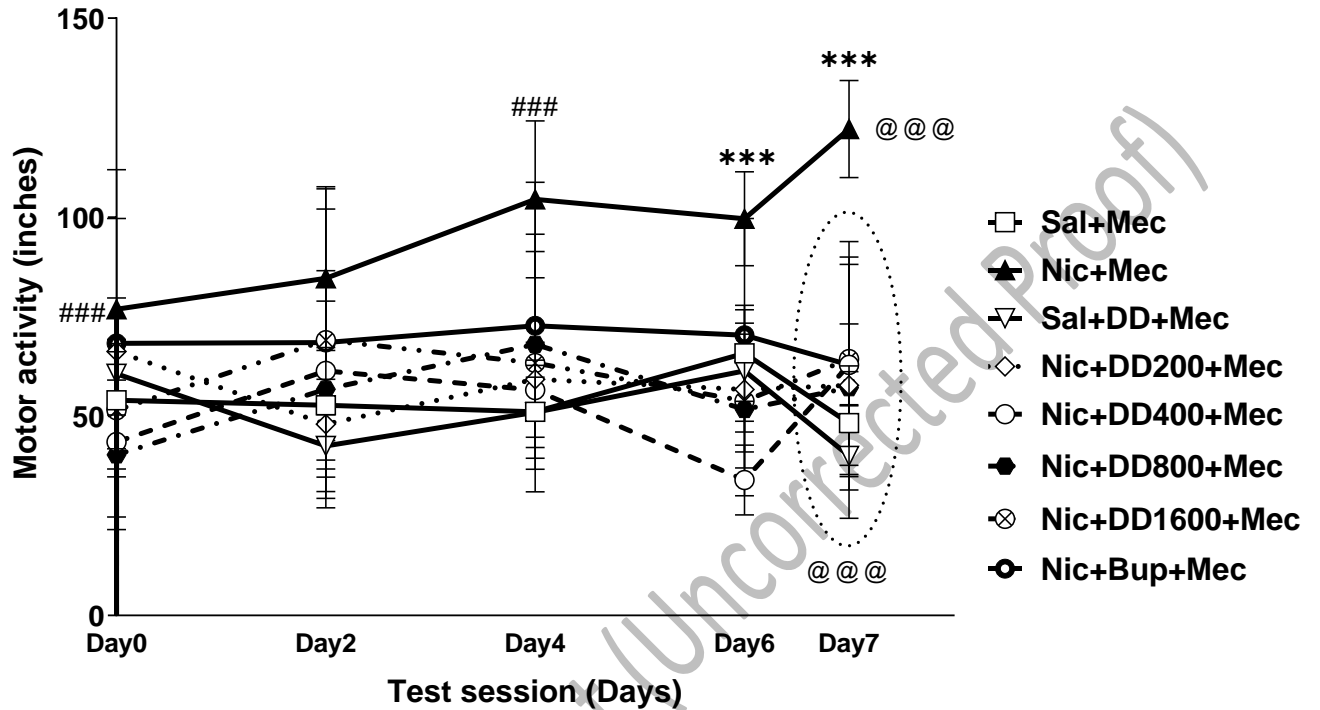
Acknowledgments

Figure.1: Motor activity of rats treated with nic, sal, bup, and different doses of DD (200, 400, 800, 1600 mg/kg). Motor activity is presented as an average distance traveled (inches) after receiving nic, sal, bup, and DD dosages over a 7-day test session. Each point represents the average distance mean ± SEM of 6 rats. Based on repeated measure ANOVA (Two way ANOVA) within groups, ### shows a significant difference (p < 0.001) in motor activity over 4 days with nicotine exposure, and *** indicates a significant difference (p < 0.001) in motor activity after mecamylamine injection in nic + mec-treated rats. @@@ indicates a significant difference (p < 0.001) in motor activity after mecamylamine injection on day 7 between the nic + mec, sal, nic + bup + mec, and DD-treated groups by One-way ANOVA. Abbreviations: nic = nicotine, sal = saline, mec = mecamylamine, Bup = Bupropion, DD = *Delphinium denudatum Wall*
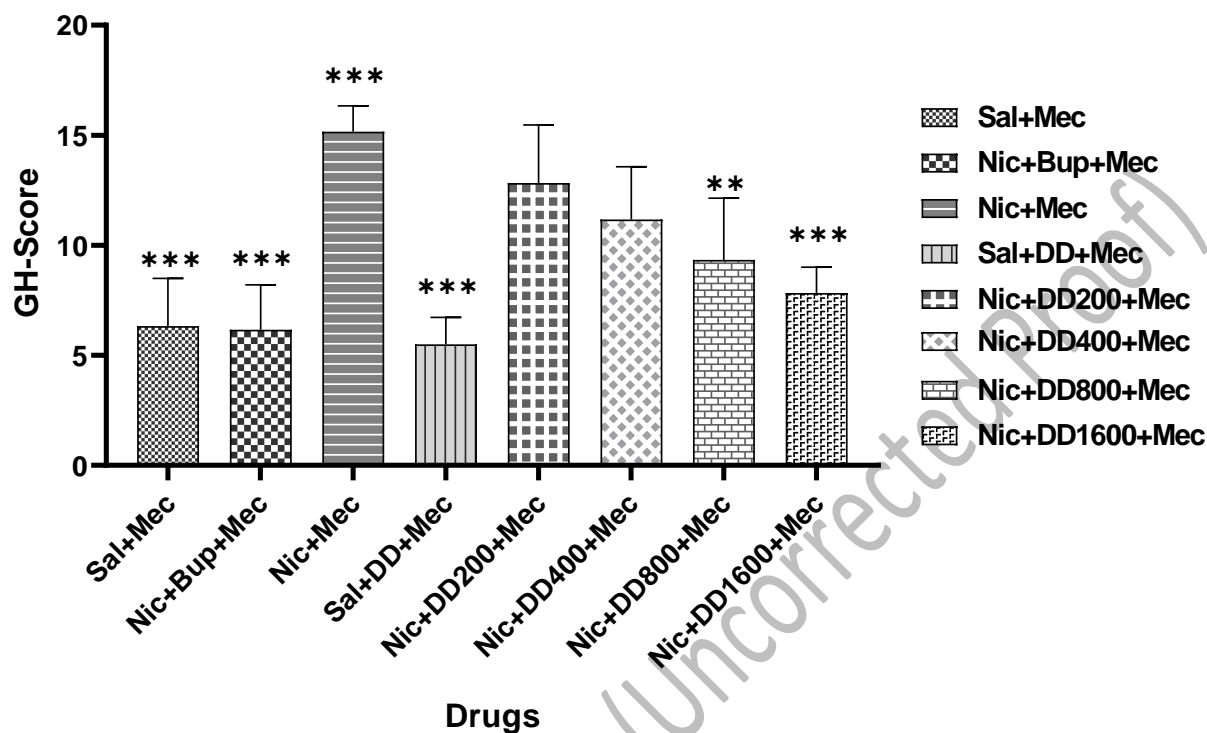
Figure 2: GH-score of rats given sal, nic, nic + bup and different does of DD (200, 400, 800, 1600 mg/kg). Each vertical bar represents the mean ± SEM GH-score of 6 rats. Based on One-way ANOVA between the groups, *** ($p < 0.001$) and ** ($p < 0.01$) indicate a significant difference in GH-score of mecamylamine-challenged nicotine-dependent rats with respect to rats treated with sal, nic + bup + mec and different doses of DD. Abbreviations: nic = nicotine, sal = saline, mec = mecamylamine, Bup = Bupropion, DD = *Delphinium denudatum Wall*
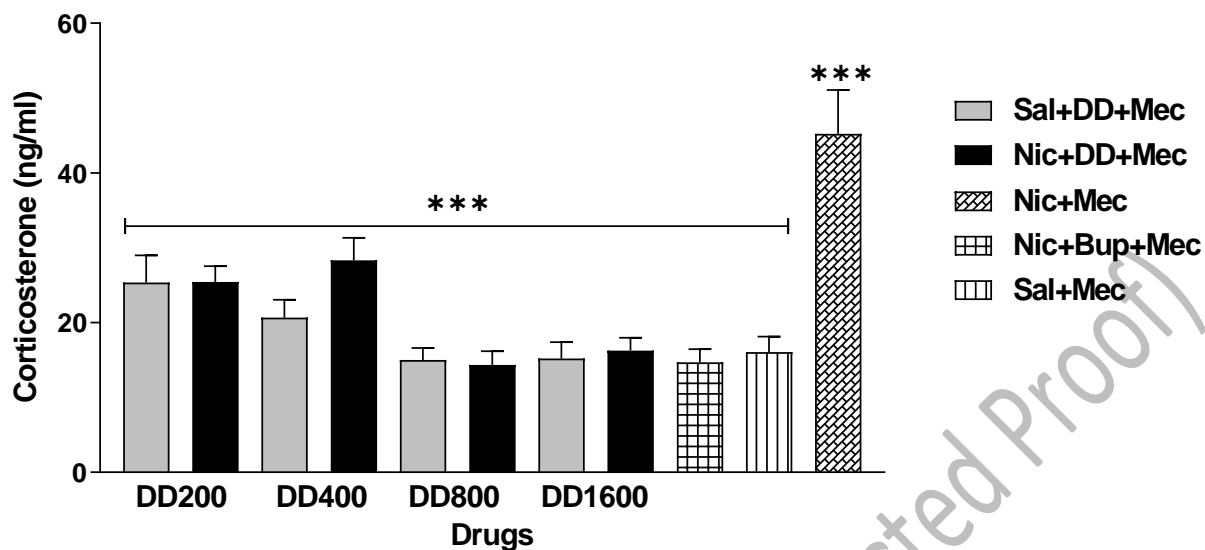
Figure 3: Corticosterone levels of rats treated with sal, nic, nic + bup, and different doses of DD (200, 400, 800, 1600 mg/kg). Each vertical bar represents mean ± SEM corticosterone levels of 6 rats. Based on one-way ANOVA between the groups, *** indicates a significant difference in corticosterone levels (p < 0.001) in mecamylamine challenged nic + mec treated rats as compared to rats treated with sal, nic + mec, nic + bup + mec, and different doses DD. Abbreviations: nic = nicotine, sal = saline, mec = mecamylamine, Bup = Bupropion, DD = *Delphinium denudatum Wall*
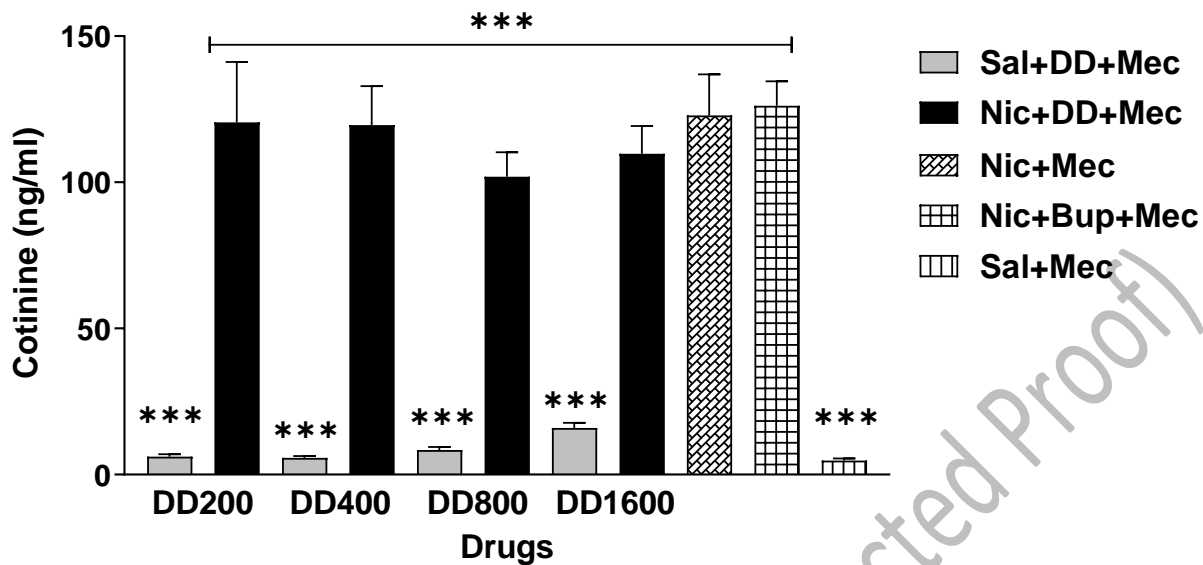
Figure 4: Cotinine levels of rats treated with sal, nic, bup, and different doses of DD (200, 400, 800, 1600 mg/kg). Each vertical bar represents mean ± SEM cotinine levels of 6 rats. Based on one-way ANOVA between the groups, *** indicates a significant difference in cotinine levels (p < 0.001) in rats implanted with nicotine-filled Alzet mini-osmotic pumps as compared to rats implanted with saline-filled Alzet mini-osmotic pumps. Abbreviations: nic = nicotine, sal = saline, mec = mecamylamine, Bup = Bupropion, DD = *Delphinium denudatum Wall*

Tables

**Table 1: Mode of Drug Administration**

Day	Experiment1 (Treated with 200 mg/kg, DD, orally) (n=12) Group1(n=6) Group2 (n=6)	Experiment 2 (Treated with 400 mg/kg, DD, orally) (n=12) Group1(n=6) Group2 (n=6)	Experiment 3 (Treated with 800 mg/kg, DD, orally) (n=12) Group1(n=6) Group2 (n=6)	Experiment 4 (Treated with 1600 mg/kg, DD, orally) (n=12) Group1(n=6) Group2 (n=6)	Experiment 5 (Treated with 20 mg/kg, Bupropion, i.p.) (n=12) Group1(n=6) Group2 (n=6)
	3 Days of Acclimatization				
Day 0 (behavior)	(No drug given)	(No drug given)	(No drug given)	(No drug given)	(No drug given)
Day 1	Sal+DD200 Nic+DD 200	Sal+DD400 Nic+DD400	Sal+DD800 Nic+DD800	Sal+DD1600 Nic+DD1600	Sal+Bup Nic+Bup.
Day 2 (behavior)	Sal+DD200 Nic+DD 200	Sal+DD400 Nic+DD400	Sal+DD800 Nic+DD800	Sal+DD1600 Nic+DD1600	Sal+Bup Nic+Bup.
Day 3	Sal+DD200 Nic+DD200	Sal+DD400 Nic+DD400	Sal+DD800 Nic+DD800	Sal+DD1600 Nic+DD1600	Sal+Bup Nic+Bup.
Day 4 (behavior)	Sal+DD200 Nic+DD 200	Sal+DD400 Nic+DD400	Sal+DD800 Nic+DD800	Sal+DD1600 Nic+DD1600	Sal+Bup Nic+Bup.
Day 5	Sal+DD200 Nic+DD 200	Sal+DD400 Nic+DD400	Sal+DD800 Nic+DD800	Sal+DD1600 Nic+DD1600	Sal+Bup Nic+Bup.
Day 6 (behavior)	Sal+DD200 Nic+DD 200	Sal+DD400 Nic+DD400	Sal+DD800 Nic+DD800	Sal+DD1600 Nic+DD1600	Sal+Bup Nic+Bup.

Day 7 (behavior)	Sal+DD200+ Mec Nic+DD 200+Mec	Sal+DD400+ MecNic+DD400+ Mec	Sal+DD800+ Mec Nic+DD800+ Mec	Sal+DD1600+ Mec Nic+DD1600+Mec	Sal+Bp+Mec Nic+Bup+Mec

Nic= Nicotine, Sal= Saline Bup= Bupropion (20mg/kg IP), Mec= Mecamylamine (1mg/kg SC), DD= Delphinium denudatum (200, 400, 800, 1600 mg/kg body wt.). Group 1 rats (n = 6) in each experiment were administered saline and Group 2 rats (n = 6) in each experiment were administered nicotine.
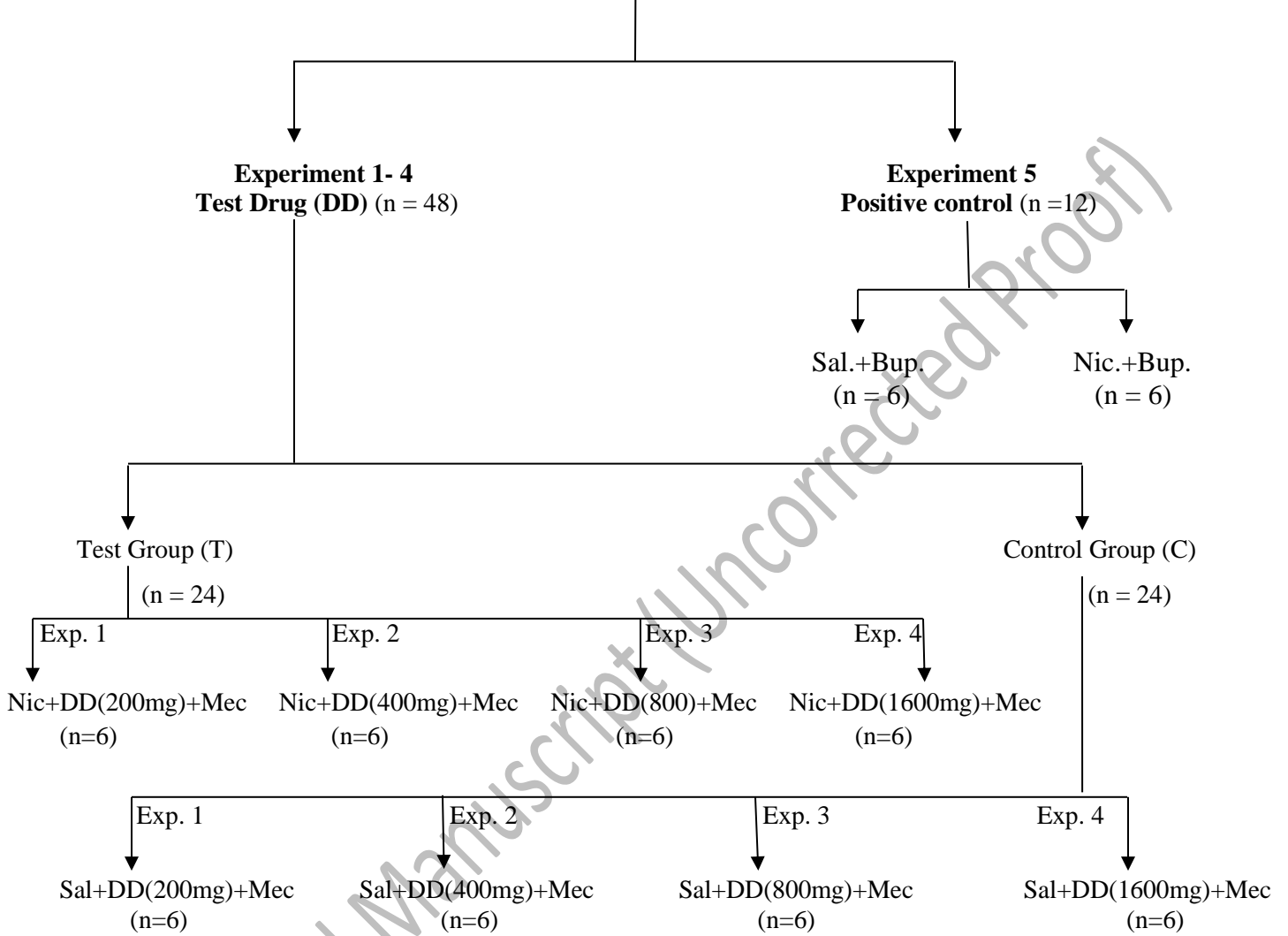
Table 2: Behavioral signs of rats at different doses of DD in nicotine administered rats

Behavioral signs	Nic.	Bup+Nic	Nic+Dd200	Nic+Dd400	Nic+Dd800	Nic+Dd1600
GH-Score	**15.166***	**6.166***	**12.833**	**12**	**9.33***	**7.833***
Graded Signs						
No. of Esc Attempts						
Two-Four	**0 (0%)**	**3 (50%)**	**4 (66.6%)**	**4 (66.6%)**	**6 (100%)**	**5 (83.3%)**
Five –Nine	**2 (33.3%)***	**0 (0%)***	**1 (0%)**	**1 (0%)**	**0 (0%)***	**0 (0%)***
Ten or more	**4 (66.6%)***	**0 (0%)***	**0 (0%)**	**0 (0%)**	**0 (0%)***	**0 (0%)***
Checked Signs						
Facial Fasciculation	**6 (100%)**	**6 (100%)**	**6 (100%)**	**6 (100%)**	**6 (100%)**	**6 (100%)**
Ptosis	**6 (100%)***	**2 (33.3%)**	**5 (83.3%)**	**5 (83.3%)**	**2 (33.3%)***	**2 (33.3%)***
Abnormal Posture	**6 (100%)***	**3 (50%)***	**4 (66.6%)**	**4 (66.6%)**	**2 (33.3%)***	**2 (33.3%)***
Ejaculation	**6 (100%)***	**2 (33.3%)**	**5 (83.3%)**	**4 (66.6%)**	**4 (66.6%)**	**4 (66.6%)**
Irritability	**6 (100%)***	**4 (66.6%)**	**6 (100%)**	**5 (83.3%)**	**4 (66.6%)**	**4 (66.6%)**

a. The minimum criterion for statistical significance was when the probability level was less than 0.05 (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). Data are expressed as mean values for rats in each group (n = 6).

b. Data is presented as the percentage of subjects that showed individual signs during a 15-minute test in each group.

Flow Chart I

Male adult Albino Wistar Rats
(n = 60)

Experiment 1- 4
Test Drug (DD) (n = 48)

Experiment 5
Positive control (n =12)

Sal.+Bup.
(n = 6)

Nic.+Bup.
(n = 6)

Test Group (T)

(n = 24)

Control Group (C)

(n = 24)

Exp. 1

Exp. 2

Exp. 3

Exp. 4

Nic+DD(200mg)+Mec
(n=6)

Nic+DD(400mg)+Mec
(n=6)

Nic+DD(800)+Mec
(n=6)

Nic+DD(1600mg)+Mec
(n=6)

Exp. 1

Exp. 2

Exp. 3

Exp. 4

Sal+DD(200mg)+Mec
(n=6)

Sal+DD(400mg)+Mec
(n=6)

Sal+DD(800mg)+Mec
(n=6)

Sal+DD(1600mg)+Mec
(n=6)

Abbreviations: Nic = Nicotine; Sal = Saline; Bup = Bupropion; Mec = Mecamylamine, DD = Delphinium;

N = Numbers; Exp = Experiment

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