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Teucrium polium Extract Ameliorates Neurobehavioral, Neurochemical Induced by Nicotine in Brain of Mice

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ABSTRACT

Background: Smoking tobacco is a serious global health problem that is associated with psychiatric disorders and increased mortality rates. Nicotine, the main compound consumed during smoking, causes damage to different organs, especially the brain. The current study assessed the modulatory impact of *Teucrium polium* extract (TPE) on nicotine-induced behavioral and biochemical, alterations in mice brains.

Methods: Twenty-four mice were divided into four groups and treated for three weeks. Group one was control, group two was subcutaneously injected with 2.5 mg/kg nicotine, group three received 100 mg/kg TPE orally, and group four was given 2.5 mg/kg of nicotine subcutaneously after an hour of oral administration of 100 mg/kg TPE.

Result: The HPLC results of the plant extract showed the presence of 11 bioactive compounds. Nicotine administration increased anxiety and decreased locomotor activity and forelimb grip strength. Dopamine, serotonin, and acetylcholinesterase activity showed a significant decline in the nicotine-induced group. Treatment with TPE showed anxiolytic effects, modulated muscle strength and locomotor activity. Also, there was an increase in neurotransmitters compared with the nicotine group. In conclusion, TPE protected against nicotine-induced neurotoxicity through modulating behavior and neurotransmitter levels in mice.

Key words: Behavior, Brain, Neurotransmitter, Nicotine, *Teucrium polium*.

INTRODUCTION

Nicotine, a toxic alkaloid in tobacco, is consumed worldwide by millions of people during cigarette smoking. There are several ways to consume nicotine including inhalational products, gums, patches and electronic cigarettes (Fagerström, 2005). Nicotine is the main reason for addiction in tobacco smokers. About 1.27 billion people use to-bacco worldwide, which causes 5.4 million deaths each year and might result in one billion fatalities in this century if current usage continues (Chaturvedi *et al*., 2015). Chronic administration of nicotine leads to nicotine dependence mainly through stimulation of the mesolimbic dopamine neurons (Balfour, 2009). Nicotine negatively affects many organs, including the brain (Yuan *et al*., 2015). It is rapidly transported from the blood to the brain and can cross the blood-brain barrier (Yang *et al*., 2019). Moreover, smoking has been linked to a higher risk of Alzheimer's disease (Toda and Okamura, 2016) and dementia (Zhong *et al*., 2015) and lower cognitive flexibility and memory (Bashir *et al*., 2017). In addition, nicotine has been shown to have anxiolytic (Szyndler *et al*., 2001) and anxiogenic (Biala and Kruk, 2009) effects in experimental animals.

The World Health Organization indicated that about 80% of the worldwide population depends on plant extracts and their active constituents to avoid or treat health problems (Khan *et al*., 2022). *T. polium*, a perennial shrub of the Lamiaceae family, is mainly found in the dry stony hills and deserts of North Africa, southwestern Asia and the Mediterranean region (Bahramikia *et al*., 2022). Owing to its remarkable pharmaco-logical properties, it has been considered a widely used herbaceous plant in folk medicine for over 2000 years. It has anti-inflammatory, antibacterial, antitumor, hepatoprotective and hypoglycemic activities (Rahmouni *et al*., 2021), in addition to diuretic, tonic, antirheumatic, antifungal, antispasmodic and antipyretic properties. Its main constituents include asparagine, ditryne, β-caryophyllene, diterpenoids, flavonoids, saponins, terpenoids and sterol (Khazaei *et al*., 2018).

Although *T. polium* has been reported to improve mental performance (Hasanein and Shahidi, 2012) there have been scarce reports of *in vivo* neurobehavioral modulation effects in nicotine-induced animals. Therefore, the main objective of the current study was to assess behavioral changes,

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motor performance and functional deficits in the brains of mice exposed to nicotine for three weeks. In addition, we evaluated the protective efficacy of *T. polium* in terminating these effects through modulating neurotransmitter levels.

MATERIALS AND METHODS

Pure liquid nicotine was purchased from SOMATCO in Riyadh (Saudi Arabia). Other chemicals and kits were supplied from commercial sources.

Between October 2022 and May 2023, this experiment was finished in the zoology department of King Saud University's College of Sciences. In May 2022, *T. polium* leaves were collected from Saudi Arabia (Al-badyah, 80 km south of Tabuk) and examined by a specialist in the Botany and Microbiology Department, College of Science, University of King Saud (Riyadh, Saudi Arabia). For preparing *T. polium* leaves extract, we followed the protocol described by Qabaha *et al*. (2021) with few adjustments. The leaves were dried in air and then converted into a powder which was subjected, for about to 24 hours, to a cold maceration extraction technique using the ethanolic solvent system. The prepared extract was subjected to filtering and concentration in a rotary evaporator under low pressure at a temperature of 50°C. Finally, *T. polium* extract was collected and kept in closed bottles at -20°C.

We utilized an Alliance chromatographic system from Waters Instruments, Inc., which was equipped with dual wavelength absorbance detectors, to conduct our chromatographic analyses. For reverse phase analyses, we employed a Restek™ Pinnacle™ II C18 column with dimensions of 4.6 × 250 mm and a particle size of 5 µm. The mobile phase consisted of two solutions: Solution A, composed of water with 1% formic acid and Solution B, which was a mixture of methanol and acetonitrile in a 60:40 ratio. To achieve our desired gradient flow rate of 1 ml/min, we followed this gradient profile: from 0 to 7 minutes, Solution B increased from 5% to 30% from 7 to 15 minutes, Solution B increased from 30% to 55% from 15 to 30 minutes, Solution B increased from 55% to 65% from 30 to 47 minutes, Solution B increased from 65% to 90% and from 47 to 55 minutes, it was maintained at 90%. Before injecting into the system, we filtered our samples with a 0.45 µm membrane filter from Millipore, using a volume of 20 µL. The identification and detection of our substances were performed at a wavelength of 280 nm, by comparing their retention times with those of the corresponding commercial standards.

Twenty-four adult male Swiss albino mice weighing 30-35 g and aged 8-10 weeks were utilized in the present study. Mice were provided from the animal house of Zoology Department (College of Science, University of King Saud) and were housed in plastic cages under optimal hygienic circumstances; a temperature of 23±5°C and a 12 h light/12 h dark cycle. Mice were provided with a commercial pellet diet and tap water ad libitum. Four groups (six mice/each) were allocated and daily treated as follows:

Group 1: Mice were orally administered saline for three weeks (negative control).
Group 2: Mice were subcutaneously injected with 2.5 mg/kg nicotine, dissolved in distilled water, for three weeks (Shakir *et al*., 2015).
Group 3: Mice were orally treated with 100 mg/kg *T. polium* extract for 3 weeks (Forouzandeh *et al*., 2013).
Group 4: Mice were subcutaneously injected with 2.5 mg/kg nicotine and were orally treated with 100 mg/kg *T. polium* extract for three weeks.

Mice were subjected to behavioral tests after the dosing period of the experiment before sacrifice. Behavioral changes were examined using the plus-maze (Ajarem *et al*., 2017), grip strength (Allam *et al*., 2016) and activity cage (Kraeuter *et al*., 2019) tests.

According to Abu-Taweel *et al*. (2013), anxiety was tested in male mice using the elevated perpendicular plus maze.

Mice's spontaneous coordinate activity and variations in this activity over time, such as horizontal or vertical movements, were recorded using the Ugo Basile 47420-Activity Cage.

The mice's forelimb grip strength is automatically measured by the Ugo Basile 47200 Grip-Strength Meter. The goal is to measure the muscle strength in the forelimbs. Three tests were performed on each animal and the greatest force exerted by each mouse was observed. According to Allam *et al*. (2016), Retro-orbital bleeding was applied to obtain blood samples from six mice in each group after they had received injections of ketamine and xylazine to induce anesthesia. After blood clotting at room temperature, samples were centrifuged for 15 minutes at 3000 rpm. The clear serum was collected and stored at -20°C until biochemical analysis.

After blood collection, mice were killed blood was then collection. The brain was dissected and samples were homogenized as 0.5 g tissue in 5 ml buffered phosphate saline. After centrifugation for 10 min at 3000 rpm, the clear supernatant of the homogenate was collected and stored at -20°C until measuring oxidative stress parameters. The cerebral cortex of the fore-brain was isolated, frozen in liquid nitrogen and stored at -80°C for determination.

The monoamines, dopamine (DA) and 5-hydroxytryptamine or serotonin (5-HT), were determined following the technique of Patrick *et al*. (1991) with a brief modification.

In a Teflon-glass-homogenizer, the brain homogenate was prepared in ice-cold phosphate buffer (0.067 M, pH 7.2) at 4±1°C. The cell debris was removed by centrifuging the homogenate for 15 minutes at 5000 rpm to obtain supernatant, which was then used for the enzyme assay. Using acetylcholine chloride as the substrate, AChE activity was measured and expressed as µmoles of hydrolyzed acetylcholine chloride/g of wet tissue weight per hour at 37±1°C (Hestrin, 1949).

Data were presented as means±standard error of the mean (SEM). Statistical analysis was performed by SPSS for Windows version 28.0. One-way ANOVA tests were used to compare groups and Tukey's test post hoc analysis was performed afterward. P-values of less than 0.05 were deemed statistically significant.

RESULTS AND DISCUSSION

T. polium extract was thoroughly investigated using HPLC-UV, the goal was to identify specific phenolic compounds present in the extracts. The analysis involved comparing the retention parameters of each assay with standard controls and evaluating peak purity using UV-visible spectral reference data (Table 1). A reversed-phase gradient system was employed to qualitatively determine 11 bioactive compounds (gallic acid, chlorogenic acid, catechin, caffeic acid, ellagic acid, rutin, luteolin 7-o-glucoside, myricetin, quercetin, apigenin and naringenin) in ethanolic extract. These compounds showed a high UV absorption at 280 nm therefore this wavelength was used for their determination. The results of the analysis revealed the presence of additional compounds, alongside, in smaller quantities (Fig 1).

Therefore, we assessed the impact of *T. polium* extract (TPE) supplementation in improving anxiety, motor activity and muscle strength in nicotine-exposed animals as well as its role in ameliorating the neurotoxicity induced by nicotine. It is obvious from our results that TPE alone is not toxic and did not exert any behavioral and neurotoxicity deficits in mice brains.

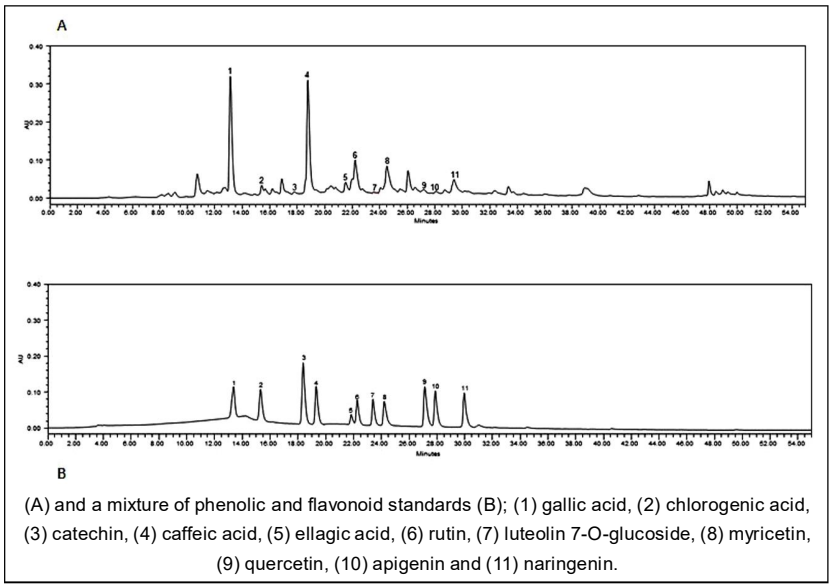
The number of entries of nicotine-exposed mice in open arms was significantly ($p<0.001$) declined compared to the number of entries of control mice, while the number of entries in close arms was significantly ($p<0.05$) enhanced in the nicotine-administered group demonstrating high anxiety as compared to controls (Fig 2). Similarly, the time spent by nicotine-administered mice in open arms was significantly ($p<0.001$) decreased as compared to the control mice, whereas the time spent in close arms was significantly ($p<0.001$) in-creased as shown in Fig 3. *T. polium* extract treated mice showed no significant change in any of the above parameters as compared to the control mice (Fig 2 and 3).

Treatment of nicotine-administered mice with *T. polium* extract attenuated the induced anxiety and behavioral abnormalities as indicated by a significant ($p<0.01$) increase in the number of entries and spent time in open arms and a significant ($p<0.01$) decrease in the number of entries and spent time in close arms (Fig 2 and 3).

Administration of nicotine to mice significantly affected the vertical ($p<0.05$) and the horizontal ($p<0.01$) motor activity as compared to the control mice (Fig 4). Treatment of nicotine-induced mice with *T. polium* extract significantly ($P<0.05$) modulated the vertical and horizontal motor impairment (Fig 4). The forelimb muscles of nicotine-induced mice recorded a significantly ($P<0.01$) smaller beaks in the

Table 1: Retention times of the compounds under study.

Compound	Retention time (min)
Gallic acid	13.33
Chlorogenic acid	15.42
Catechin	18.36
Caffeic acid	19.29
Ellagic acid	21.86
Rutin	22.27
Luteolin 7-o-glucoside	23.46
Myricetin	24.22
Quercetin	27.38
Apigenin	28.23
Naringenin	29.91

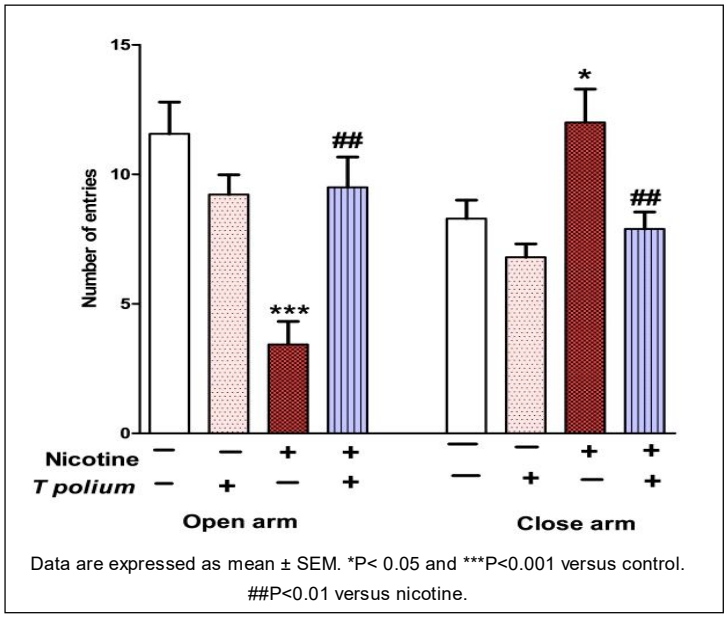


(A) and a mixture of phenolic and flavonoid standards (B); (1) gallic acid, (2) chlorogenic acid, (3) catechin, (4) caffeic acid, (5) ellagic acid, (6) rutin, (7) luteolin 7-O-glucoside, (8) myricetin, (9) quercetin, (10) apigenin and (11) naringenin.

Fig 1: Representative reversed-phase HPLC-UV chromatograms (280 nm) of *T. polium* extract.

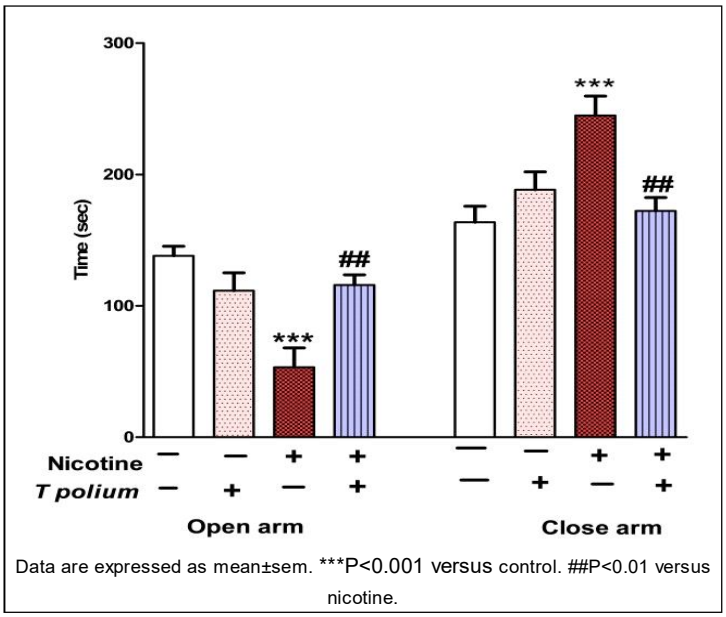
grip strength examination scores as compared to the control mice. T. extract treated group showed a significant (P<0.01) improvement in the grip strength scores and recorded a stronger beak than the nicotine-administered group (Fig 5).

Increasing evidence indicated that oxidative stress triggered anxious behavior in rodents (Rammal *et al*., 2008). Smoking was reported to increase anxiety in young adults (Pedersen and von Soest, 2009). The present results showed that nicotine administration exerted an anxiogenic effect in mice in accordance with the results of Zarrindast *et al*. (2000) and Zarrindast *et al*. (2010). For evaluating anxiety-like behavior, the elevated plus maze test is commonly used. The test relies on the innate aversion of mice to open and elevated spaces and to their tendency to explore new environments (Crawley, 2007), thus the reduced number of entries and the time spent in open arms is thought to be the result of higher fear levels induction (Rodgers and Dalvi, 1997). Consistent with our results, adult rats subcutaneously injected with nicotine showed a reduction in the percentage of open-arm entries (Elliott *et al*., 2004). The anxiogenic effect of nicotine may result from altering the normal neurodevelopment (Dwyer *et al*., 2008)



Data are expressed as mean ± SEM. *P< 0.05 and ***P<0.001 versus control. ##P<0.01 versus nicotine.

Fig 2: Protective effect of *T. polium* extract against nicotine-induced anxiety in mice (number of entries in open and close arms).



Data are expressed as mean±sem. ***P<0.001 versus control. ##P<0.01 versus nicotine.

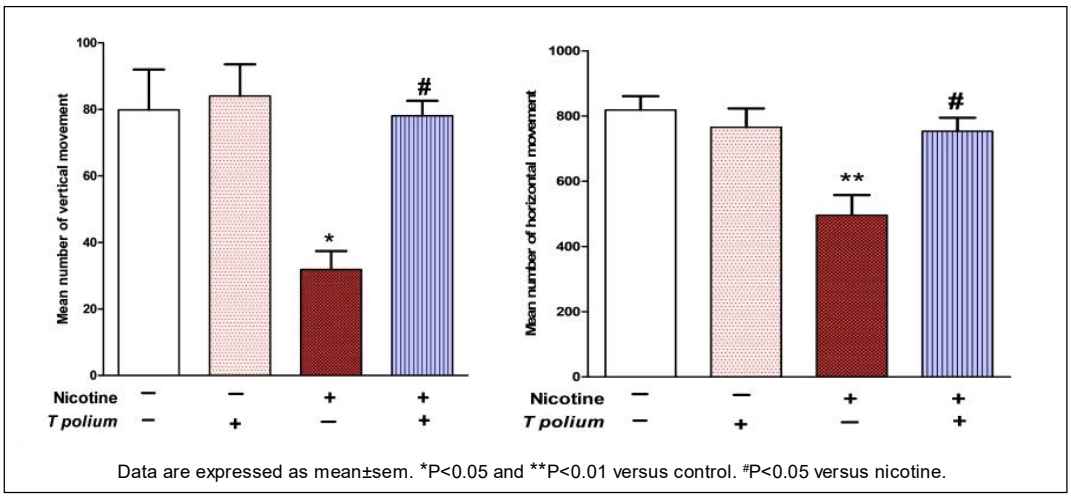
Fig 3: Protective effect of *T. polium* extract against nicotine-induced anxiety in mice (time spent in open and close arms).

or altering the neurotransmitter release in the brain. For instance, increasing glutamate after nicotine treatment has been linked to enhanced anxiety-like behaviors (Bergink *et al*., 2004).

Actually, nicotine was reported to exert both anxiogenic and anxiolytic effects depending upon the dosing regimen and duration, animal strain and experimental model utilized (Zarrindast *et al*., 2012). Low nicotine doses were reported to have anxiolytic properties by elevated plus maze test (Cheeta *et al*., 2001), while higher doses produced anxiogenic effects (Balerio *et al*., 2006).

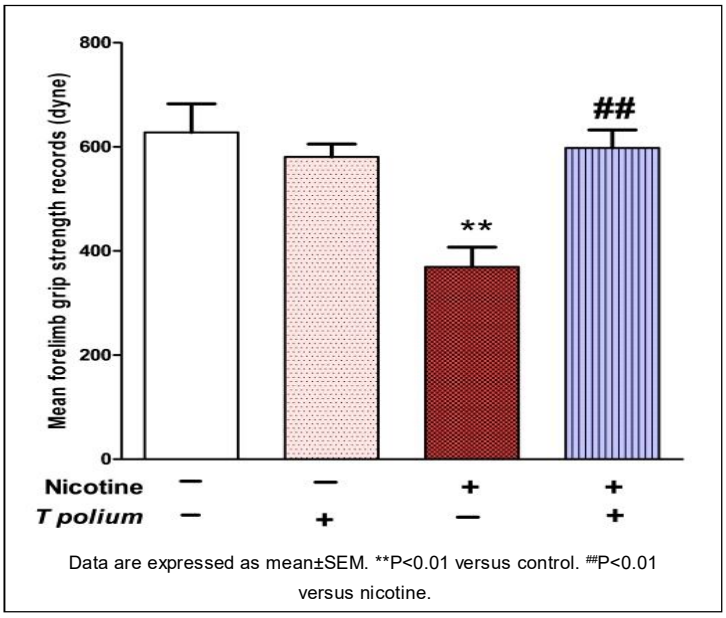
Plants belonging to Lamiaceae family have been previously reported to exert anxiolytic effects under stressful conditions (Hamed *et al*., 2021). In our study, the positive effect of *T. polium* extract supplementation on nicotine-induced anxiety was reported. Our result is supported by the fact that the extract is used in traditional medicine in Africa for relaxation, decreasing stress and management of sleep (Rahmouni *et al*., 2021). We concluded that the anxiolytic effect of *T. polium* extract relies on its antioxidant properties. This is parallel with the results of Dhingra *et al*. (2012) and Lee *et al*. (2020) who indicated the anxiolytic effects of phenolic acids and flavonoids, the main active antioxidant compounds in the plant extract, using the elevated plus maze test.

The antioxidant impact of TPE could also have a role in the modulation of motor activity and muscular strength in the forelimbs of mice exposed to nicotine. Rinaldi (2011) showed that mice subjected to smoking are more susceptible to muscle fatigue than nonsmokers due to neuromuscular transmission failure. The general fatigue sensation in smokers was accomplished via the increased oxidative



Data are expressed as mean±sem. *P<0.05 and **P<0.01 versus control. #P<0.05 versus nicotine.

Fig 4: Protective effect of *T. polium* extract on nicotine-induced locomotor impairment (vertical and horizontal movement) in mice.



Data are expressed as mean±SEM. **P<0.01 versus control. ##P<0.01 versus nicotine.

Fig 5: *T. polium* extract improved forelimb grip strength in nicotine-induced mice.

stress in the muscles, the declined activity of mitochondrial enzymes and the depleted oxygen level delivered to the muscles following nicotine smoking (Wüst *et al*., 2008). Thus, the administration of efficient antioxidants could modulate grip strength through by diminishing the potentially damaging effect of reactive oxygen species on the musculoskeletal system (Cooper *et al*., 2002).

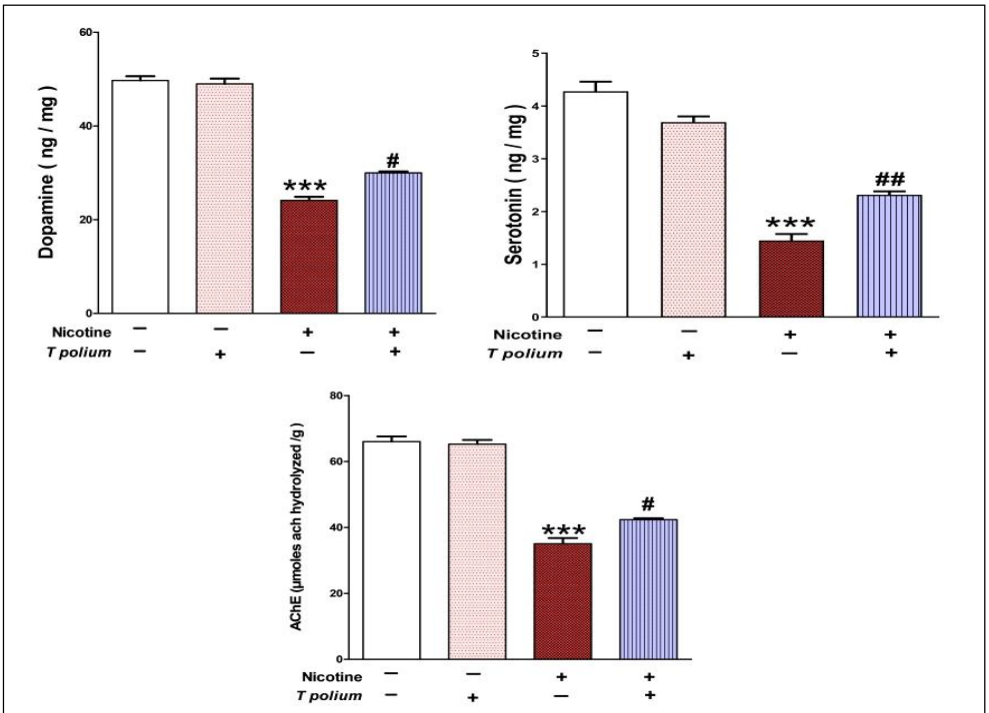
Also, antioxidant supplementation positively influenced the spontaneous locomotor activity (Nasuti *et al*., 2008). The locomotor activity is assessed by the activity cage test which is one of the methods evaluating the behavior or psychology of animals (Ambrogi *et al*., 1987). In our research, nicotine produced a decrease in the motor activity of adult mice in parallel with the results of Macphail *et al*. (2005). Other studies indicated an age-dependent influence of nicotine on locomotor activity (Belluzzi *et al*., 2004), as well as a dose-dependent decrease in motor activity in different mice strains (Marks *et al*., 1983). However, Ankarberg *et al*. (2001) reported that nicotine caused a dose-dependent increase or decrease in motor activity in adult mice following early postnatal exposure.

The behavioral alterations caused by nicotine were associated with a significant decrease in the levels of the neurotransmitters (dopamine and serotonin) in the fore-brain tissue of mice. Nicotine was actually reported to affect brain biochemistry, behavior and consequent responses to pharmacological investigations (Levin and Slotkin, 1998). It was previously noticed that prenatal nicotine exposure could alter the dopamine release (Zhu *et al*., 2012; Alkam *et al*., 2017), thus regardless of decreased or increased dopamine levels, the nervous system might have an in ordinary development during nicotine smoking. In addition, smokers showed lower concentrations of monoamine oxidase, an enzyme involved in the metabolism of dopamine, in their brains compared to nonsmokers (Hogg, 2015).

A significant ($p<0.001$) decrease in dopamine and serotonin (Fig 6) concentration was recorded in the forebrain of nicotine-induced mice compared with the control mice. Concomitant treatment of nicotine-induced mice with *T. polium* extract significantly modulated the depletion and increased dopamine ($p<0.05$) and serotonin ($p<0.01$) levels as illustrated in Fig 6.

Administration of nicotine resulted in a significantly ($p<0.001$) decreased activity of acetylcholinesterase in the fore-brain of mice as compared with the control mice. Treatment of nicotine-exposed mice with *T. polium* extract for 3 weeks markedly enhanced ($p<0.05$) the enzyme activity (Fig 6).

Nicotine exposure showed a significant reduction in serotonin level in the brain of mice. Serotonin is a neurotransmitter that has been linked to anxiety (Gordon and Hen, 2004). Therefore, nicotine-exposed mice in our investigation showed a decreased serotonin level associated with a tendency towards increasing anxiety. A previous study documented that smoking is associated with a decrease in serotonin levels in the humans2 brain (Benwell *et al*., 1990).



Data are expressed as mean±sem. ***$p<0.001$ versus control. #$p<0.05$ and ##$p<0.01$ versus nicotine.

Fig 6: *T. polium* extract increased neurotransmitters in the brain of nicotine-exposed mice.

These conflicting results of serotonin levels during smoking were explained by Awtry and Werling (2003). Nicotine reaches the cerebrum in a few seconds stimulating the release of neurotransmitters, including serotonin, but smoking continuation causes a physical change in the mind and inhibits serotonin formation. Bombardi *et al*. (2020) reported that intraperitoneal administration of acute and chronic nicotine has a biphasic effect on the serotonin expression in the brain.Similarly, nicotine exposure was recorded to decline the activity of acetylcholinesterase (AChE) in consistent with the results of Jain and Flora (2012).

The available studies presuppose that long-term nicotine exposure changes the homeostasis of several neurotransmitters in the brain, which is regarded to be the main contributor to nicotine dependence (Alasmari *et al*., 2019). Both acetylcholine (Ach) and acute nicotine exposure activate the nAChR function in terms of functional consequences. There are numerous fail-safe mechanisms in place to ensure that nAChRs are not continually exposed to ACh and that nAChRs are activated by pulses of ACh release rather than continuously.

The current study findings showed the *T.polium* plus Nicotine group had an increase in Acetylcholineesterase level compared to the Nicotine group. The findings of this study accord with Ashare *et al*. (2017) observed that repeated administration of an acetylcholinesterase inhibitor attenuates nicotine taking in rats and smoking behavior in human smokers. Another study also in agreement with the present results demonstrated that Nicotine administration in male mice inhibits acetylcholinesterase (Hasan *et al*., 2018).

Neurotransmitters and acetylcholinesterase activity were modulated after *T. polium* extract treatment. Since it was reported that a decline in the level of the neurotransmitter acetylcholine and a rise in free radicals production play a significant role in the development of neurodegenerative diseases (Hassan *et al*., 2014). We herein explained the modulatory role of *T. polium* extract against nicotine-induced neurotoxicity through its antioxidant properties. *T. polium* showed high neurotransmitter activity and elicited neurodegenerative changes in ovariectomized rats (Simonyan and Chavushyan, 2016). The detected neuroprotective effect was supported by the results of Han *et al*. (2012) who showed the antioxidant and neuroprotective efficacy of apigenin, a major flavonoid in the plant extract. Also, Havsteen (2002) clarified the role of flavonoids in the stimulation of neurotransmitters and scavenging free radicals.

CONCLUSION

In conclusion, the present study intensified the hazardous consequences of smoking habit and revealed that adult male mice exposed to nicotine had numerous behavioral impairments, altered neurotransmitter levels and neuro-degenerative changes in the brain tissues. While treatment with *T. polium* extract protected against the neurotoxicity of nicotine. It showed an anxiolytic effect, better muscular activity and protection against neurotransmitter dysfunction in the brain. Therefore, we encourage smokers to consume *T. polium* extract as a dietary supplement to protect against nicotine-induced health problems. We plan to perform additional studies in the future to elucidate the protective effect of each single component in the extract.

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Institutional review board statement

"The animal study protocol was approved by the Institutional Review Board (or Ethics Committee) of Kingdom of Saudi Arabia (Ethics Committee, King Saud University, Ethics Agreement ID: KSU-SE- 23-79)".

Conflict of interest

The authors declare that there are no conflicts of interest.

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