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Ameliorative Effect of *Teucrium polium* Extract against Nicotine Induced Liver Toxicity in Swiss Albino Mice

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ABSTRACT

Background: Nicotine can be consumed in many forms, the most common of which is smoking tobacco. Tobacco smoking is a global problem that negatively affects human health. Natural plants are vital antioxidants that may antagonize the toxic effects of smoking. We designed the present study to assess the protective potential of Teucrium polium extract (TPE) on nicotine-induced liver toxicity in male mice.

Methods: Twenty-four animals were allocated into four equal groups and daily treated for 21 constitutive days. Control group one received distilled water, group two orally received 100 mg/kg TPE, group three received subcutaneous injections of 2.5 mg/kg nicotine and group four received 100 mg/kg TPE orally then 2.5 mg/kg nicotine subcutaneously after one hour.

Result: Nicotine exposure significantly elevated liver enzymes; alanine transaminase (ALT) and aspartate transaminase (AST). The levels of malondialdehyde (MDA) and nitric oxide (NO) were also elevated along with a reduction in glutathione reduced content (GSH), superoxide dismutase (SOD) and catalase (CAT) activity. Treatment with TPE considerably ameliorated the serum levels of liver enzymes, lowered MDA and NO levels, as well as elevated the antioxidant defense system. Also, TPE improved the histological structure and modulated apoptosis caused by nicotine. The current study proved the effectiveness of TPE as a natural antioxidant for the alleviation of nicotine-induced hepatic biochemical and histopathological alterations through attenuating oxidative stress, improving antioxidant systems and abrogating apoptosis.

Key words: Apoptosis, Liver, Mice, Nicotine, Oxidative stress, *Teucrium polium*.

INTRODUCTION

Cigarette smoking is a worldwide problem leading to high morbidity and mortality in over one hundred million people in the 20th century. Leaves of tobacco plants (*Nicotiana tabacum*) are dried to produce chewing tobacco, cigarettes and cigars. Around the world, one in three men smokes tobacco (Pham *et al*., 2007 and El-Sokkary *et al*., 2007). Numerous compounds can be generated during cigarette smoking including cytotoxic and carcinogenic compounds as well as free radicals and volatile aldehydes which can probably damage biomolecules (Abdul-Razaq and Ahmed, 2013). Non-smokers exposed to tobacco smoke absorb toxic chemicals in the same way as smokers (The American Cancer Society, 2015).

Nicotine, an extremely toxic organic compound present in tobacco, is a colourless volatile alkaloid that turns brown upon air exposure (Marzouk *et al*., 2022). It is the primary reason for satisfaction and pleasing sensation during cigarette smoking and sustains tobacco addiction (Benowitz *et al*., 1982 and Benowitz, 2009). During nicotine smoking, it is inhaled into the lungs and metabolized in the liver causing toxic, immunological, or carcinogenic effects (Hukkanen *et al*., 2005). The metabolism of nicotine and related alkaloids involves chemically reactive intermediates that can generate free radicals and harm macromolecules including lipids, proteins and DNA through oxidative stress (Birben *et al*., 2012). Nicotine causes liver inflammation by enhancing pro-inflammatory cytokins2 production (El-Zayadi, 2006), along with oxidative stress by reactive oxygen species (ROS) generation (Suleyman *et al*., 2002). There is a relationship between smoking and the ability of the liver to detoxify hazardous compounds (Alwar *et al*., 2013). Furthermore, smoking damages liver cells and has a genotoxic effect on the rat liver (Bandyopadhyaya *et al*., 2008).

Our bodies fight against free radicals and pro-oxidants to minimize their harmful effect with the help of antioxidants. Antioxidants are significant compounds that can defend the body against oxidative stress (Pal *et al*., 2014). Natural antioxidants are mainly derived from plants and have a

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potential therapeutic efficiency against several pathological conditions (Gupta and Prakash, 2014). Thus, there has been a lot of interest in medicinal plants and their active components as potential antiperoxidative agents (Lee and Park, 2003).

T. polium Lamiaceae is an annual flowering plant found in arid stony regions of hills and deserts in Europe, North Africa and South-Western Asia. It is an important component in different traditional medical prescriptions used for several pathological disorders such as gastrointestinal diseases, inflammation, rheumatism, diabetes and stomach ulcers (Bahramikia and Yazdanparast, 2012). It has been previously reported that *T. polium* extract is rich in phenolic compounds, which have hepatoprotective roles through inhibiting lipoperoxidation and enhancing antioxidant enzymes (Rahmouni *et al*., 2018). In Saudi Arabia, the aerial parts and leaves of *T. polium* are used to treat parasites, fever and gastrointestinal problems. Additionally, local people use their steam baths to treat colds and fevers (Mossa *et al*., 2000), while the hard parts are boiled before being used as a medication for wounds (Akin *et al*., 2010). Also, various secondary metabolites were investigated and many of which exhibited important medicinal and/or ecological importance (Alreshidi *et al*., 2020).

Therefore, the present work was designed to determine the possible protective efficacy of *T. polium* extract against oxidative stress, histopathological changes and apoptosis associated with nicotine.

MATERIALS AND METHODS

This experiment was completed in the zoology department of King Saud University's College of Sciences between October 2022 and May 2023.

T. polium leaves were collected from Al-badyah Tabuk, Saudi Arabia in May 2022. Position: 27°45′59.5″N 36°31′48.8″E .80 km south of Tabuk. The plant was identified by a specialist at the herbarium (Botany Department, Science College, King Saud University, Riyadh, Saudi Arabia). *T. polium* leaves extract was prepared following the method of Qabaha *et al*. (2021) with some adjustments. The leaves were air-dried and then ground into a powder. The obtained powder was subjected to a cold maceration extraction technique using the ethanol solvent system for 24 hours. The ethanolic extract was filtered and concentrated by a rotary evaporator under pressure and temperature of 50°C, then was collected and kept in sealed bottles at -20°C.

Nicotine was acquired from SOMATCO (Riyadh, Saudi Arabia). Other analytical-grade chemicals and reagents were purchased from commercial providers.

In the present study, twenty-four adult Swiss albino male mice of 8-10 weeks of age and 30-35 g weight were purchased from the animal house, College of Science, King Saud University. Mice were maintained in well-aerated cages under specific pathogen-free conditions at a controlled temperature (23±5°C) and 12 hrs light /12 hrs dark cycles. Animals had free access to standard pellet diets and tap water ad libitum and were acclimated for seven days before the onset of the experiment.

Mice were distributed into four equal groups with six animals per each and were treated once/day for 3 weeks as follows:

Group 1 (Control): Mice received distilled water.

Group 2 *(T. polium*): Mice were treated orally with 100 mg/kg *T. polium* extract dissolved in distilled water (Forouzandeh *et al*., 2013).

Group 3 (Nicotine): Mice were injected subcutaneously with 2.5 mg/kg nicotine dissolved in distilled water (Shakir *et al*., 2015).

Group 4 (Nicotine + *T. polium*): Mice were orally gavaged with 100 mg/kg TPE and after one-hour mice were subcutaneously injected with 2.5 mg/kg nicotine.

24 hours following the experimental period, blood samples were collected through retro-orbital bleeding from six mice per group under ketamine/xylazine anaesthesia. After clotting of blood samples at room temperature, they were centrifuged for 15 min at 3000 rpm. The serum was collected and kept frozen at -20°C as aliquots for biochemical analysis.

Mice were sacrificed and blood was then collected. The liver was extracted from each animal, washed with saline and divided into parts. One part was homogenized in cold 10% w/v phosphate-buffered saline, centrifuged for 15 minutes at 5000 rpm, then the supernatant was collected and kept at -20°C. For histological examination, another part was fixed in freshly prepared 10% neutral buffered formalin (pH 7.4) for at least 24 hours.

The serum activities of alanine transaminase (ALT) and aspartate transaminase (AST) were estimated spectrophotometrically at 340 nm using kits from BioSystems, Spain (REF 11533 and11531 respectively) and following new IFCC reference procedures (Schumann and Klauke, 2003).

Malondialdehyde (MDA) level, an indicator of lipid peroxidation, was assessed spectrophotometrically in the liver homogenate by Biodiagnostic kits (Cat. No: MD 25 29; at 535 nm) according to the method of Ohkawa *et al*., 1979. Glutathione reduced (GSH) (Biodiagnostic kits, Cat. No: GR 25 11; at 412 nm), superoxide dismutase (SOD) (Biodiagnostic kits, Cat. No: SD 25 21; at 560 nm) and catalase (CAT) (Biodiagnostic kits, Cat. No: CA 25 14; at 510 nm) were determined following the protocols of Beutler *et al*. (1963); Nishikimi *et al*. (1972) and Aebi, (1984) respectively.

NO level in the liver homogenate was measured spectrophotometrically by reagent kits supplied by Biodiagnostic (Cat. No: NO 25 33; at 540 nm), based on the technique described by Archer (1993).

Formalin-fixed specimens were washed in distilled water, sliced, dehydrated in ascending ethyl alcohol series and embedded into paraffin wax. The wax blocks were sliced into 4 µm sections. Hematoxylin and eosin (H&E) were used to stain the sections (Adam and Caihak, 1964). A light microscope (Leica, Wetzlar, Germany) was used to image stained sections.

Paraffin blocks were subjected to immunohistochemical analysis. Terminal deoxynucleotidyl transferase-dUTP nick end labeling (TUNEL) assay was utilized for apoptosis identification. TUNEL assay was analyzed using a diagnostic kit obtained from Abcam (ab206386) and the provided protocol was strictly followed. It detects the DNA breaks formed when DNA was fragmented in the last stage of apoptosis. The method depends on the enzyme terminal deoxynucleotide transferase (TdT), which attaches deoxynucleotides to the 3'- hydroxyl end of DNA breaks. TdT is expressed in certain immune cells and acts during V(D)J recombination - the process that generates antibody diversity. ABC detection kits depend on the affinity of avidin (or streptavidin) for biotin (Alhusayan *et al*., 2020). The endpoint of apoptosis which is indicative of positive staining can be represented by a dark brown signal. Lighter shades of brown and/or blue-green to green-brown shades indicate a negative cell.

Results were expressed as means ± standard error of the mean (SEM) of different groups. Differences between the values of mean were calculated by one-way ANOVA followed by Scheffe post-hoc test using version 28 of SPSS software (SPSS Inc., Chicago, IL, USA). The results were judged statistically significant when p<0.05.ne-induced hepatotoxicity in male mice.

RESULTS AND DISCUSSION

Smoking cigarettes and-other tobacco products is one of the most common perilous addictions and is considered a major factor of high mortality and morbidity throughout the world (Abdel-Aziz, 2010). Nicotine as the main toxic compound of cigarette smoke exerts a major action in the progress of several diseases and disorders even in organs that don't have direct contact with the smoke itself such as the liver (Azab and Albasha, 2015). Since the liver is a vital organ for metabolism and excretion and controls the flow and safety of substances before entering the circulatory system (Allen, 2002), it is crucial to estimate the toxicological impact of nicotine smoking on the liver and to assess the hepato-protective potential of proposed natural products. Understanding the biological/therapeutic action of natural products and their structural diversity has increased attention to their applications in drug discovery. Also, plant whole extracts are reported to be more effective than separated compounds (Rahmouni *et al*., 2022).

Nicotine administered mice showed a significant elevation (P<0.01) in ALT (Fig 1) and AST (Fig 2) serum levels when compared to the control mice. On the contrary, treatment of nicotine administered mice with TPE effectively ameliorated ALT (P<0.05) and AST (P<0.01) serum levels as shown in Fig 1 and 2 respectively. ALT and AST were not significantly changed in control TPE compared with the negative control mice.

In the present study, nicotine administered mice showed an elevation in the hepatic enzymes; ALT and AST. Transaminases are normally found in the cytoplasm of the hepatic cells but can be released into the blood circulation after the rupture of the plasma membrane (Ramaiah, 2007). Thus, high serum levels of these enzymes reveal damaged structural integrity of the liver. Indeed, the ALT level denotes a disease progression and the risk increases if ALT is steadily elevated (Sherman, 2009). Fahim *et al*. (2014) stated an increase in ALT and AST serum levels in mice after intraperitoneal injection of nicotine for 3 weeks. Further studies of Al Anany *et al*., (2015) and Salahshoor *et al*., (2016) are consistent with our results in rats and mice respectively.

The hepatoprotective effect of *T. polium* against nicotine- induced liver damage was supported by the decreased ALT and AST circulating levels in the serum. Previous studies showed the hepatoprotective potential of

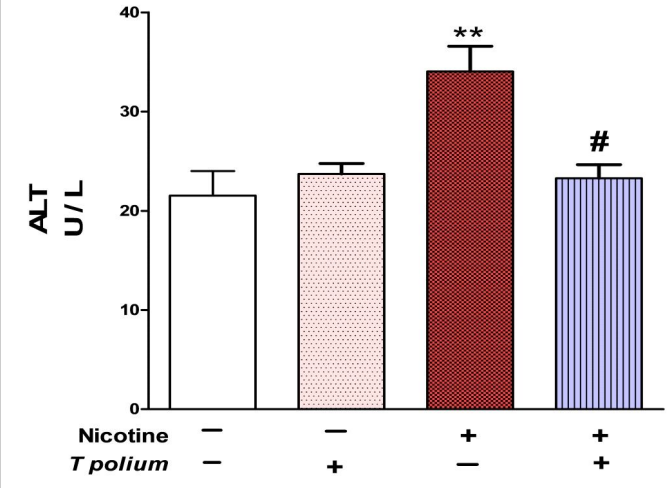


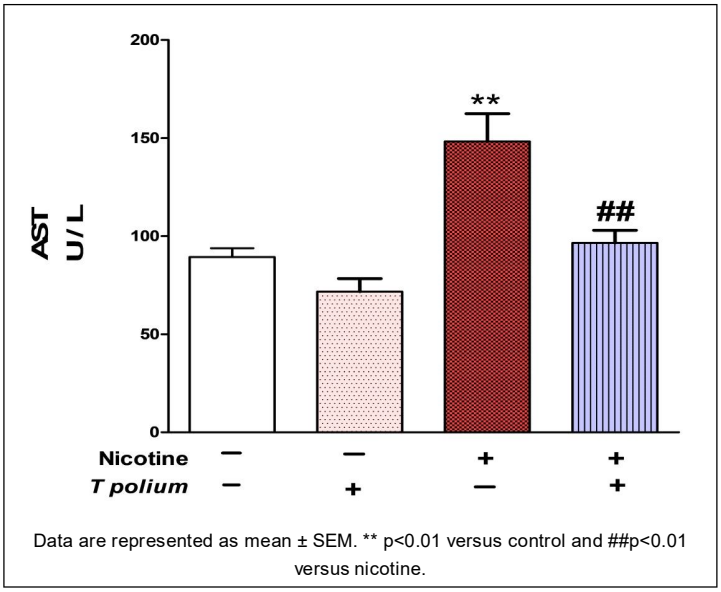
Fig 1: *T. polium* ameliorated serum alanine aminotransferase (ALT) in nicotine-administered mice.

T. polium against different toxic agents through its role in ameliorating liver function biomarkers (Rahmouni *et al*., 2022 and Forouzandeh *et al*., 2013).

The results represented in Fig 3 indicated a significant (P<0.001) increase in MDA level in mice injected with nicotine as compared to their control counterpart. However, treatment with TPE showed a significant (P<0.01) decrease in MDA level when compared with the nicotine-injected group.

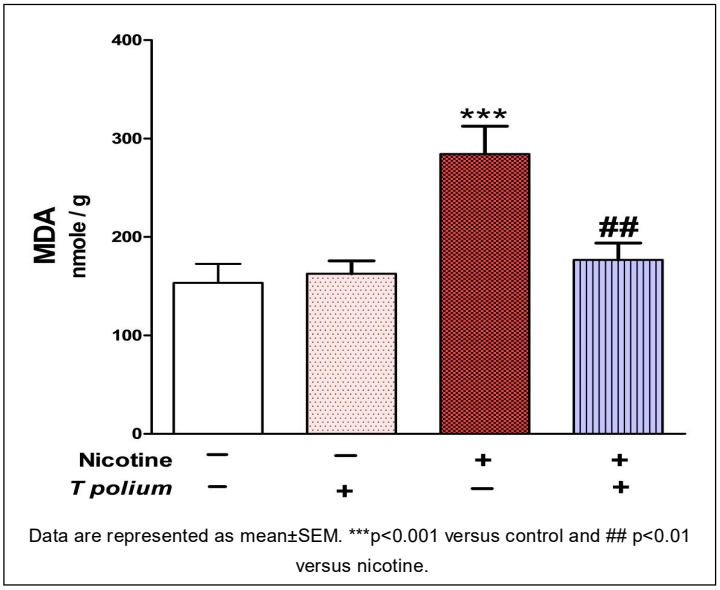
The overproduction of reactive oxygen species and imbalance of antioxidant defense systems represent the primary mark in liver toxicity (Khadrawy *et al*., 2021). The increased free fatty acids in hepatic tissues serves as the substrate for lipid peroxidation (Balakrishnan *et al*., 2007). Malondialdehyde is a reliable biomarker for assessing oxidative stress and monitoring the extent of lipid peroxidation in the liver. MDA also reacts with DNA forming MDA-DNA adducts leading to endogenous DNA damage (Singh *et al*., 2014). Herein, nicotine-injected mice displayed significantly increased lipid peroxidation as indicated by high MDA levels. Previous studies reported that nicotine caused hepatic damage as a result of oxidative stress initiation (El-Sokkary *et al*., 2007; Mahmoud and Amer, 2014). Kalra *et al*., 1991 explained that nicotine increased ROS production through activating polymorphonuclear leucocytes to produce oxygen free radicals through responsiveness to activated complement C5a.

Mice exposed to nicotine showed a significant (P<0.001) decline in GSH content when compared with the control



Data are represented as mean ± SEM. ** p<0.01 versus control and ##p<0.01 versus nicotine.

Fig 2: *T. polium* ameliorated serum aspartate aminotransferase (AST) in nicotine-administered mice.



Data are represented as mean±SEM. ***p<0.001 versus control and ## p<0.01 versus nicotine.

Fig 3: *T. polium* leaves extract ameliorated hepatic malondialdehyde (MDA) level in nicotine-administered mice.

mice. Treatment of nicotine administered mice with TPE showed a significant (P<0.01) rise in GSH content when compared with the untreated group as shown in Fig 4.

The activity of the enzymatic antioxidants; SOD (Fig 5) and CAT (Fig 6) decreased significantly (P<0.01) in the nicotine-administered group compared with the control group. Concurrent treatment with 100 mg/kg TPE significantly increased hepatic SOD (P<0.01) and CAT (P<0.05) in nicotine-administered mice.

Nicotine also affects antioxidant enzymes by oxidative inactivation of enzyme proteins or a reduction in enzyme protein synthesis (Oyeyipo *et al*., 2014). Enzymatic and non-enzymatic antioxidants inhibit oxidant attack and defense against free radicals. SOD and CAT are important antioxidant enzymes. SOD transforms superoxide anion to hydrogen peroxide. Then, CAT transforms hydrogen peroxide into water and oxygen (Birben *et al*., 2012). The superoxide anion and hydrogen peroxide are considered the main causes of the nicotine-induced free radical origination and exhaustion of the cellular antioxidants (Chattopadhyay and Chattopadhyay, 2008). Also, perturbation of GSH by nicotine injection has been reported to increase lipid peroxidation (Uday *et al*., 1999). GSH, a non-enzymatic antioxidant, protects against free radicals and ROS in several ways. Its amino acid constituent, cysteine, neutralizes the hydroxyl radical and has an important role in oxidative stress (Circu and Aw, 2011). GSH also protects the cellular constituents by its ability to form S-conjugates with the products of lipid

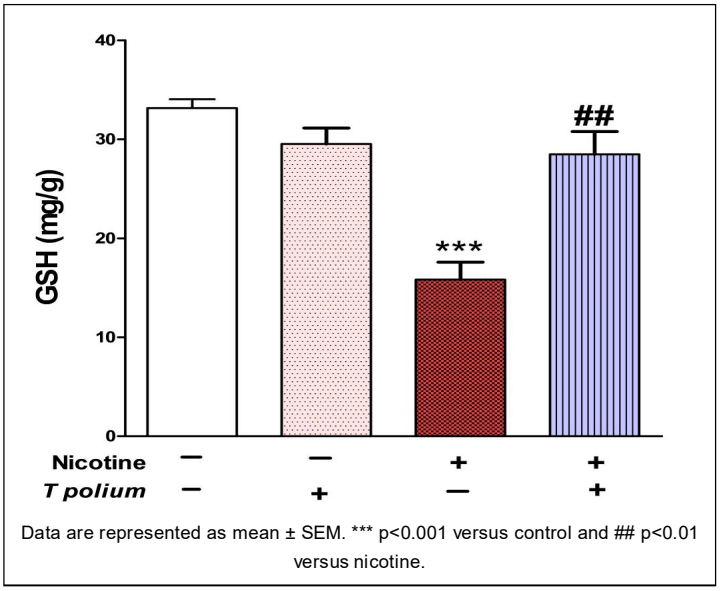


Fig 4: *T. polium* leaves extract increased hepatic glutathione reduced (GSH) content in nicotine-administered mice.

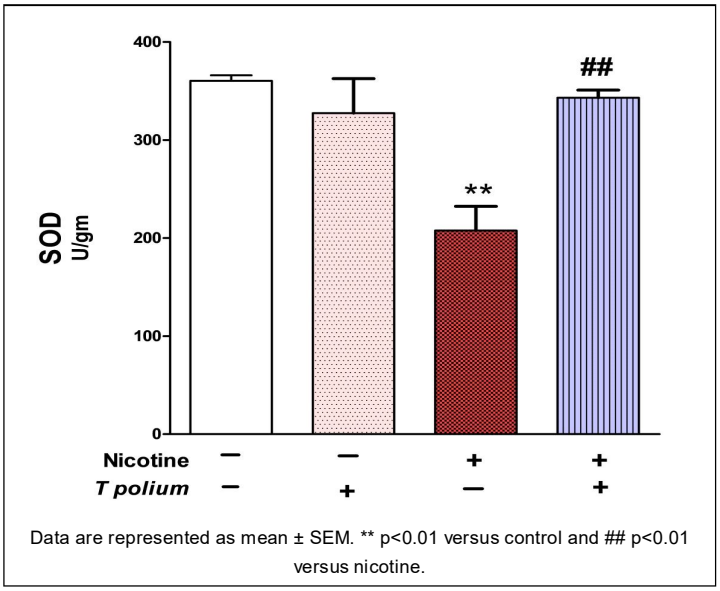
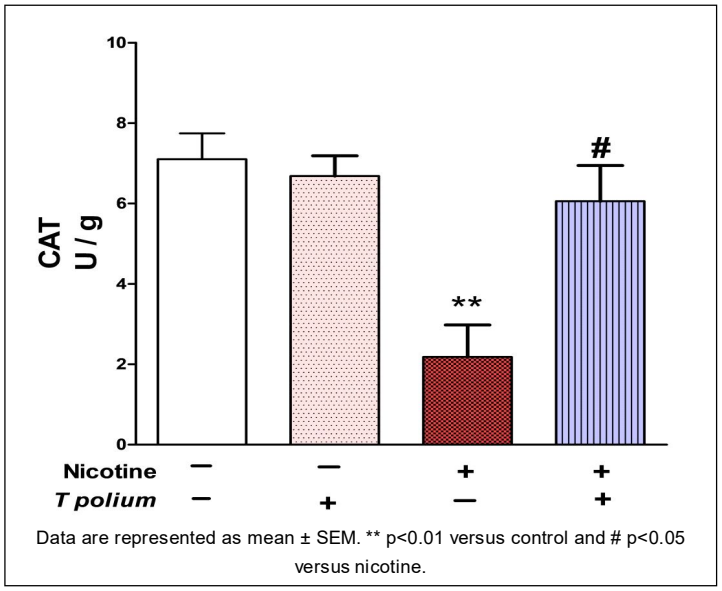


Fig 5: *T. polium* leaves extract increased hepatic superoxide dismutase (SOD) activity in nicotine-administered mice.

peroxidation (Laurent *et al*., 2000), reduce peroxides and maintain protein thiols in the reduced state (Sies, 1986).

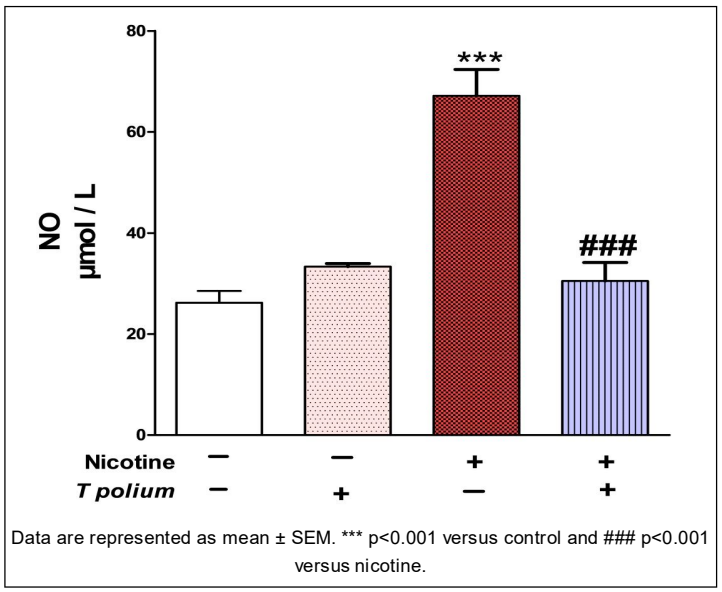
The current outcomes revealed that nicotine induction significantly reduced GSH, SOD and CAT levels indicating lowered cellular defense and tissue damage. Similar studies found a decline in glutathione levels and the activity of catalase and superoxide dismutase in nicotine-exposed animals (Al Anany *et al*., 2015 and Sudheer *et al*., 2008). In the same context, nicotine-administered mice in our study presented a significant increase in NO levels in consonance with previous studies (Salahshoor *et al*., 2016 and Oyeyipo *et al*., 2014). Nitrosative stress interferes with normal body functions since nitric oxide can react with the superoxide anion to form the cytotoxic peroxynitrite which decomposes into the highly reactive hydroxyl radical (Ohshima and Bartsch, 1994). Peroxynitrite can alter protein structure by nitration of proteins2 tyrosine residues. Also, it triggers oxidation of thiol compounds and enhances lipid peroxidation (Maeda and Akaike, 1998).

TPE decreased lipid peroxidation and nitrosative stress and restored GSH content and antioxidant enzyme activity in the liver of nicotine-administered mice. TPE has the potential to have antioxidant effects directly as a chemical antioxidant because of its capacity to scavenge reactive oxygen species (Alreshidi *et al*., 2020) and nitrogen free radicals (Sharifi *et al*., 2022) or indirectly by modifying intracellular antioxidant defense systems (Rahmouni *et al*., 2022). Also, it has been reported that TPE increases



Data are represented as mean ± SEM. ** p<0.01 versus control and # p<0.05 versus nicotine.

Fig 6: *T. polium* leaves extract increased hepatic catalase (CAT) activity in nicotine-administered mice.



Data are represented as mean ± SEM. *** p<0.001 versus control and ### p<0.001 versus nicotine.

Fig 7: *T. polium* leaves extract ameliorated hepatic nitric oxide (NO) level in nicotine-administered adult mice.

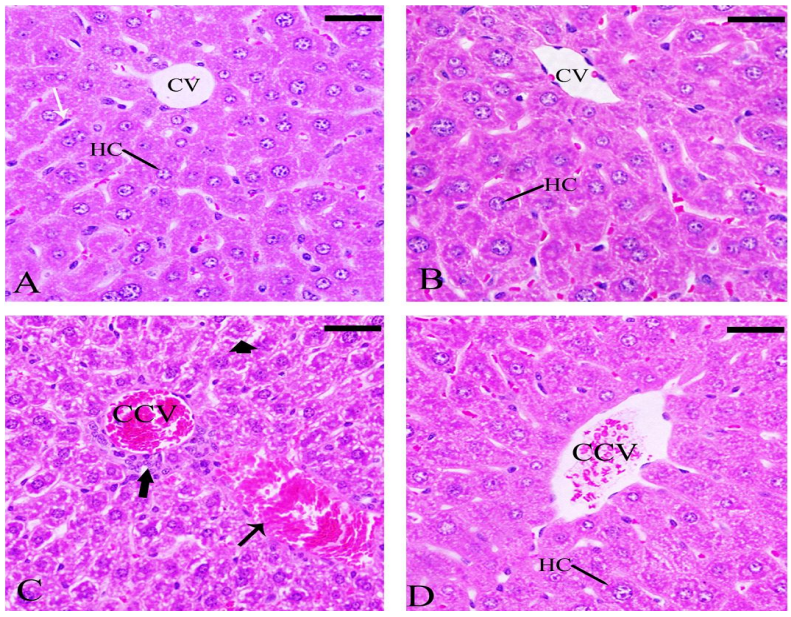
intracellular glutathione by stimulating the biosynthetic pathway of glutathione *in vitro* (Shtukmaster *et al*., 2010).

The results illustrated in Fig 7 showed a significant increase (P<0.001) in NO level in nicotine-administered mice when compared with the control group. Conversely, TPE supplementation considerably (P<0.001) reduced NO level as compared with nicotine-induced mice.

Movahedi *et al*. (2014) demonstrated that the beneficial properties of *T. polium* were attributed to the excessive content of total phenolics and flavonoids and the antioxidant competency. The same conclusion was supported by Sharifi *et al*. (2022) who stated that *T. polium* extract has different phytochemicals such as flavonoids, phenolics, saponins and alkaloids which showed antibacterial, antioxidant and anti-inflammatory properties. Phenolic compounds can compete with O2 to unite with NO molecules reducing free radicals' formation because of the conversion of NO into its reduced form (Möller *et al*., 2019). Additionally, Nasri and Shirzad, (2013) supposed that the antioxidant properties of the plant are attributed to the ortho-dihydroxy substitution found in the flavone B-ring. While Panovska *et al*. (2007) supposed that the hepatoprotective effect of *T. polium* extract was achieved via the elimination of free radicals and the prevention of GSH depletion.

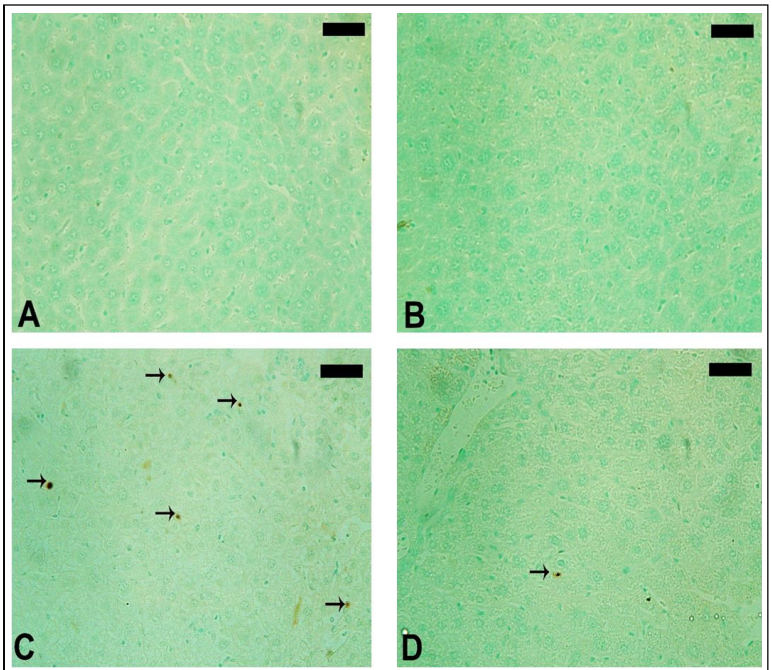
The control and TPE-treated group showed normal hepatic structure in H&E-stained liver sections. The hepatic lobules consist of hepatic cords of polygonal hepatocytes which radiate from the central vein and contain centric round nuclei and cytoplasm. The hepatic cords are separated by blood sinusoids lined by Kupfer cells (Fig 8 A, B). On the contrary, the nicotine-injected group showed severe degeneration of hepatocytes, congestion of the central vein, hemorrhage and inflammatory cell infiltration (Fig 8C). All previously indicated changes caused by nicotine were ameliorated with TPE treatment except congestion in the central vein was still observed. Most cells showed improvement and exhibited the normal histological structure (Fig 8D).

The protective impact of *T. polium* was also verified by the improved histological structure of the liver. Nicotine-induced mice showed different histopathological changes including inflammatory cells, congested blood vessels, haemorrhage and degeneration of some hepatic cells. These observations were in harmony with the results of Gawish *et al*. (2012). The damaging effect of nicotine on hepatic tissues has been previously reported (Salahshoor *et al*., 2016 and Munir *et al*., 2015). Syed and Shangloo, (2020) clarified that the changes in hepatic tissue architecture were directly attributed to nicotine metabolism into cotinine in the liver or indirectly attributed to ROS production which activated the macrophage monocyte system and lipid peroxidation. On the other hand, *T. polium* extract restored liver structure to the normal state. Similar results were observed in the liver of rats treated with *T. polium* extract after CCl4-induced liver injury (Panovska *et al*., 2007) and in rats with nonalcoholic steatohepatitis (Nosrati *et al*., 2010).



(A) Control and (B) *T. polium*-treated mice show normal hepatic cells (HC), central vein (CV) and Kupffer cells (white arrow). (C) Nicotine-administered mice show inflammatory cells infiltration (thick arrow), congested central vein (CCV), haemorrhage (thin arrow) and hepatic cells degeneration (arrowhead). (D) Nicotine-administered mice treated with *T. polium* show ameliorated liver structure with normal hepatic cells (HC), with absence of inflammatory cells. And the central vein mildly congested were seen (CCV). [Scale bar= 50 µm (A, B, C, D)].

Fig 8: Photomicrograph of mice liver sections stained with hematoxylin and eosin (H and E).



(A) Control and (B) *T. polium*-treated group with negative expression of apoptotic changes. (C) Nicotine-administered group with strong TUNEL positive expression. (D) Nicotine-administered group treated with *T. polium* show reduced TUNEL-positive expression. TUNEL-positive cells appeared brown. [Scale bar = 50 µm (A, B, C, D)].

Fig 9: TUNEL expression in the hepatic tissues of mice.

Apoptosis in the liver from all experimental groups, as demonstrated by TUNEL staining, was illustrated in Fig 9. TUNEL-positive cells were not detected in the liver of control mice (Fig 9A) and TPE-treated mice (Fig 9B). The number of positive cells was increased in the liver of nicotine-injected mice (Fig 9C). However, an observable decrease in the number of TUNEL-positive cells was observed in the liver of nicotine and TPE treated mice (Fig 9D).

TUNEL assay showed apoptotic changes in the group exposed to nicotine. Several previous studies supported our results and revealed an association between nicotine exposure and apoptosis (Galitovsky *et al*., 2004; Zha and Reece, 2005). ROS are involved in the mitochondrial apoptotic pathway and directly oxidize DNA and initiate genotoxicity (Sastre *et al*., 2000). Thus, it has been showed that nicotine prompted hepatic apoptosis via augmented lipid peroxidation, oxidative stress and DNA damage (Husain, 2001). A strong correlation has been found between hepatocyte apoptosis and the degree of oxidative stress (Singh and Czaja, 2007). Furthermore, NO production may be related to apoptosis and cell damage (Tamm *et al*., 2008). On the opposite side, few apoptotic cells were detected in the hepatic sections of mice treated with nicotine and TPE indicating the anti-apoptotic efficacy of the plant extract. Aghazadeh and Yazdanparast, (2010) reported the anti-apoptotic impact of *T. polium* extract in a non-alcoholic steatohepatitis model that was mostly attributed to its potent antioxidant capacity.

CONCLUSION

The current study demonstrated that TPE exerted antioxidant, anti-nitrosative and anti-apoptotic properties against nicotine-induced liver toxicity in mice. The hepatoprotective effect was exerted mainly through free radicals scavenging ability and enhancing the antioxidant defense system because of the high content of flavonoids and phenolics in the plant extract. Decreased liver enzymes in serum and improved histological structure of the liver were further indicators for abrogation of the liver toxicity by TPE treatment.

Therefore, efforts have to be made to cease smoking and avoid nicotine smoke exposure. Also, we encourage smokers to consume TPE to protect their liver from oxidative damage induced by nicotine. Nevertheless, further studies are necessary for the isolation and identification of the individual compounds in the extract and to define the exact mechanism of action.

ACKNOWLEDGEMENT

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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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