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Evaluation of the beneficial impact of *Annona squamosa* extract against nicotine-induced retina toxicity in female rats and their progeny

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ABSTRACT

Prolonged nicotine usage caused oxidative damage. Consequently, it is involved in numerous pathophysiological neurological conditions and induces neurodegeneration. This study examined the curative effects of *Annona squamosa* L. extract (ASE) against nicotine-induced neurotoxicity in the retina of female rats and their offspring. Forty pregnant rats were split into four groups (10/group): the control group, the ASE group (350 mg/kg body weight/day, given from gestation day 15 till weaning), the nicotine group (2 mg/kg body weight/day, administered from gestation days 7 to 14), and the nicotine + ASE group. The administration of ASE to nicotine-exposed maternal rats and their offspring demonstrated a significant mitigation of oxidative damage caused by nicotine, as evidenced by a notable reduction in serum malondialdehyde and a considerable increase in superoxide dismutase, catalase, and glutathione antioxidant. The levels of acetylcholine showed a significant decrease, while glutamate and gamma-aminobutyric acid levels were significantly elevated, comparable to the nicotine-treated group. This was linked to a distinct repair of the histological defects in the retina caused by nicotine in both the mothers of rats and their pups. Furthermore, ASE mitigated the apoptotic effects induced by nicotine in the retinal tissues as evidenced by reduced immunohistochemical expression of p53, overexpression of glial fibrillary acidic protein, and elevated flow cytometric expression of proliferating cellular nuclear antigen. ASE effectively mitigated retinal damage induced by nicotine in female rats and their offspring by counteracting oxidative stress, inflammation, and the pathophysiological alterations caused by nicotine.

Keywords: Apoptosis, Oxidative stress, Gestation, Lactation, Neurotoxicity, Smoking

1. Introduction

Tobacco smoking represents a major global public health concern linked to several chronic diseases (Kulkarni and Banait, 2023). It is linked to an increased risk of coronary heart disease, cerebrovascular diseases, and vascular disorders (Rahman et al., 2025). Chronic smoking induces oxidative stress, hence elevating the risk of macular degeneration (Velilla et al., 2013). Smoking, as a significantly alterable environmental factor, has been linked to various ocular disorders glaucoma, elevated intraocular pressure ischemic optic neuropathy (Kulkarni and Banait, 2023).

Nicotine is a principal active poisonous constituent of tobacco. It is a recognized liquid alkaloid derived from the dehydrated foliage of the tobacco plant. Nicotine is predominantly processed by the liver, lungs, and kidneys, with a half-life of two hours (Hossain and Salehuddin, 2013). Nicotine is the principal addictive substance in tobacco that drives persistent usage despite adverse effects and the intention to cease (Hall et al., 2014). Nicotine and its principal metabolite, cotinine, can exert detrimental effects

on the structure and functions of various bodily organs (Joo, 2023). Three primary adverse effects were identified as a consequence of exposure to two components. These encompassed toxic effects, immunomodulatory effects, and carcinogenic consequences (Mohammed et al., 2020). Nicotine was associated with ocular impairment, including conjunctivitis, irritation, redness, inflammation, and retinal dysfunction (Wang et al., 2016). Moreover, it may elevate the chance of conjunctival epithelial carcinoma, impede corneal wound healing, diminish endothelial cell density, and contribute to cataract formation (Ilhan et al., 2016)

Nicotine exposure during gestation is associated with fetal complications and increased risk of hazardous health outcomes in adult offspring (Liu et al., 2019). It had been documented that the prenatal nicotine exposure throughout gestation resulted in adverse neurodevelopmental deficits (Wells and Lotfipour, 2023). Exposure to tobacco smoke, both direct and indirect, during pregnancy increases the chance of premature birth, membrane rupture, intrauterine growth restriction, and sudden infant death syndrome (Holbrook, 2016). Besides the adverse effects on embryo and fetal development, maternal smoking exposure may result in prolonged and deferred negative outcomes on the cognitive development of nicotine-exposed infants (Balazs et al., 2018). Moreover, nicotine during lactation can be passed to offspring, leading to behavioral problems and anxiety in the progeny (Karimiankakolaki et al., 2019). Reducing or eliminating cigarette smoking during gestation can lessen harm to the developing fetus (Diamanti et al., 2019).

The retina represents an outgrowth from the central nervous system in terms of both structure and development (Hussey et al., 2022). It is a complicated neural network consisting of multiple layers of specialized neurons that display similar morphological and functional traits to brain neurons. Dopamine and acetylcholine are neuromodulators in the brain affected by regular nicotine smoking (Pistillo et al., 2015).

Any natural dietary approach that mitigates or decelerates the advancement and intensity of nicotine toxicity has substantial health advantages (Xu et al., 2025). Consequently, researchers are persistently striving to create natural substitutes for the deleterious effects of nicotine (Bhattacharya et al., 2021). *Annona squamosa* L. is commonly referred to as "custard apple". It is a tropical plant found in many countries. *A. squamosa* is renowned for its edible fruit and its use as a natural medication in many home therapies. Moreover, its significant utilization was recorded in the production of food. The nutrient profile of custard apple offers nutritional benefits that augment the value of other processed foods as well (Abou-El-magd et al., 2025). The pulp of Custard apple is rich in vitamin content, like Vit C and B1, together with a high number of fibers (Ma et al., 2017).

Extracts derived from multiple components of the *A. squamosa* plant, including its bark, roots, leaves, and fruit, have been employed in traditional medicinal practices across various nations to treat an array of ailments, including dysentery, epilepsy, hemorrhage, fever, and tumors (Anaya-Esparza et al., 2020). *A. squamosa* comprises various phenolic compounds, including proanthocyanidins, with 18 distinct phenolic entities, predominantly alkaloids, flavonoids, peptides, and assorted acetogenins (Moussa et al., 2024). The phytochemical constituents demonstrate a wide array of biological activities, including immunosuppressive, antineoplastic, cytotoxic, antimicrobial, anti-inflammatory, anticancer, antiulcer, antidiabetic, antidiarrheal, antiplatelet, antioxidant, hepatoprotective, neuroprotective, and cytoprotective effects (Zahid et al., 2018; Ibrahim et al., 2024; Mobasher et al., 2024; Al-Zubaidi et al., 2025). Accordingly, the current study aims to explore the adverse impacts of nicotine on the retinas of female rats and their offspring throughout gestation and lactation, as well as the possible protective role of *A. squamosa* pulp extract against these effects.

2. Materials and methods

Chemicals

Nicotine (99% purity) was acquired from Sigma-Aldrich Co., St. Louis, MO, USA, dissolved in a

suitable solvent, and administered via intraperitoneal (i.p) injection at a dosage of 2 mg/kg b.wt. The rest of the kits required for the estimation of research parameters were available and indicated with each method.

Preparation of *A. squamosa* extract

The fruit of *A. squamosa* was acquired from the local market in Damanhour city. The fruit was recognized by the botanist staff, Faculty of Science. Four kilograms of *A. squamosa* fruit were wrapped, and the seeds were gathered. The pulp samples were dried at 45°C, subsequently crushed into fine powder, and kept at 4°C for subsequent use. The powdered materials were extracted using a 1:20 w/v ratio of distilled water and thereafter heated at 70°C for 30 minutes while stirring. The extracts were subjected to centrifugation at 8385 xg for 25 minutes, followed by filtration, lyophilization, and storage in a freezer at –20°C until needed (Shehata et al., 2021). The yield percentage was roughly 9% (360 g).

Experimental design

Forty mature female Wistar-Albino rats, each weighing 160-170 g, were housed in caged laboratory conditions for a two-week acclimatization period. They were authorized to consume standard pellets. Rats were housed in special cages within a room with a regular light/dark cycle. The temperature and humidity were regulated. For two days, female rats were mated at a ratio of two females to one adult male rat. Following the confirmation of pregnancy via the vaginal smear method (Inaloz et al., 2004), the dams were randomly allocated into four equal groups, with ten pregnant rats in each group. In group 1 (Gp1) (Control), the pregnant rats were provided with a standard meal and administered distilled water. Gp2 (*A. squamosa* extract; ASE) received ASE (350 mg/kg b.wt /daily orally, 1/10 of LD_{50}) from gestation day 15 (GD15) until the completion of weaning (Mobasher et al., 2024). In Gp3 (nicotine), pregnant rats received i.p injection of nicotine at a dosage of 2 mg/kg b. wt /day, from gestational day 7 to gestational day 14 (Everekliogl u et al., 2004). In Gp4 (nicotine + ASE), the pregnant rats were given a dosage of nicotine (2 mg/kg b. wt) i.p daily from gestational day 7 to gestational day 14, followed by treatment with ASE (orally) daily from gestational day 15 till weaning. This study was approved and performed in accordance with the guidelines of the Ethical committee of the Faculty of Science, Damanhour University (DMU-SCI-CSRE-24-11-08).

Sample collection and tissue preparation

Ten progenies from each cohort were selected for examination. Two offspring from each group were euthanized at post-natal day (PND) 7 and two at PND14 for histological examination of the retina. At the end of the experimental period (PND21), the maternal rats (ten per group) and their offspring (six per group, aged 21 days) were euthanized by decapitation for the collection of blood samples, and retinal tissues were obtained for histological, immunohistochemical investigations, and flow cytometric analysis. Serum was obtained by centrifugation at 755 xg for 10 min and preserved at −80°C before biochemical analysis.

Investigated parameters

Body weight changes

Female rats and their pups were weighed weekly after birth at PND7, 14, and 21.

Estimation of serum antioxidants/oxidant parameters

The catalase (CAT) activity was quantified spectrophotometrically using the method established by Koroliuk et al. (1988). The activity of superoxide dismutase (SOD) was assessed by measuring the superoxide radicals (Bahrami et al., 2016). The activity of reduced glutathione (GSH) was assessed in accordance with the procedure established by Ellman (1959). The thiobarbituric acid reaction method developed by Placer was employed to measure malondialdehyde (MDA) levels (Placer et al., 1966).

Determination of acetylcholine, gamma-aminobutyric acid and glutamate in the retina

The activity of acetylcholine (Ach) was assessed in the retinal tissue homogenates of maternal rats and their progeny at PND-21 using ELISA kits (cat. number: MAK056). The assay process was conducted according to the manufacturer's specifications. The glutamate (Glu) activity in

retinal tissue homogenate was assessed using the glutamate assay kit (Abnova, Taipei City, Taiwan) in accordance with the manufacturer's technical guidelines. Gamma-aminobutyric (GABA) was quantified according to the previous method of Bak et al. (2006).

Histological technique for hematoxylin and eosin stain

The maternal rats and their progeny at PND 7, 14, and 21 were euthanized, and the entire eyeballs were excised, subsequently rinsed in normal saline, and preserved in 10% neutral buffered formalin. Following fixation, the specimens underwent dehydration using an ascending sequence of ethanol, were cleaned with xylene, and subsequently embedded in paraffin (Sakr et al., 2013). A 5-µm circular segment was created perpendicular to the corneal edge and parallel to the optic nerve. The ocular sections were stained with Mayer's hematoxylin and eosin, thereafter processed for examination of the retina using a bright-field light microscope, and then photographed.

Immunohistochemical labeling of glial fibrillary acidic protein and p53

An immunohistochemistry analysis for glial fibrillary acidic protein (GFAP) and p53 was conducted on the retinal sections of maternal rats and their progeny at PND21. Five-micrometer-thick paraffin slices of the eye were prepared, affixed to positively charged slides, and rehydrated via decreasing concentrations of ethyl alcohol. Endogenous peroxidase activity was inhibited with 3% H_2O_2 in methanol for 40 min at ambient temperature. The tissue sections were maintained at ambient temperature and subjected to antigen retrieval by digestion in 0.05% trypsin. Following extensive washing in TRIS-buffered saline (TBS) at pH 7.6, the sections were incubated with normal swine serum.

To demonstrate GFAP activity, the sections were treated for 30 min with a polyclonal rabbit anti-GFAP antibody (1:300 dilutions, DAKO Corporation, California, USA). Following three washes in TBS, they were treated for 30 min with an anti-rabbit antibody produced in swine. Following extensive washing with TBS, the sections were treated with a horseradish peroxidase-rabbit anti-horseradish peroxidase combination (PAP) for 30 min and subsequently rinsed for 30 min. For demonstration of p53 activity, additional sections were incubated for 45 min with a diluted 1:10 monoclonal primary antibody (Anti-p53; clone DO-7 Dako) (Bonsing et al., 1997). The slides were washed in PBS and then treated with secondary antibody for 20 min. In all sections, the complex sites were depicted in brown utilizing 3,3'-diaminobenzidine tetrahydrochloride with a fresh hydrogen peroxide substrate. The sections were counterstained with Mayer's hematoxylin, mounted, and imaged using phase-contrast optical microscopy. The occurrences of cellular accumulations of GFAP and p53 proteins were assessed for each group utilizing image analysis assays.

Flow cytometric analysis of proliferating cellular nuclear antigen

Cell suspensions from the retinas of mothers and their 21-day-old offspring were produced using Tris-EDTA buffer (pH 7.4) (Sigma-Aldrich Co.). The cell suspension was preserved in ice-cold 96-100% ethanol (Sigma) at 4 ºC overnight, centrifuged at 189 xg for 10 min, and subsequently re-suspended in PBS containing 50 µg/ml propidium iodide (PI) (Sigma-Aldrich Co.). Each sample was analyzed based on the measurement of 10,000 cells. Single cell suspensions were generated from the retinas of a minimum of six rats per group, and 1.5-3 × 10^6 cells were stained to assess the expression of specified lineage markers.

Following the washing of retinal cells in PBS, the cells were re-suspended in 10% normal goat serum in PBS for 20 minutes at room temperature to diminish nonspecific antibody binding to the cells. An indirect immunofluorescence technique was employed for the staining of PCNA, utilizing a monoclonal anti-PCNA antibody (PC10, Novocastra Lab, UK, cat number: 14-9910-82) diluted 1:25 in PBS, along with a FITC-conjugated anti-mouse IgG antibody (Tago, Burlingame, CA) also diluted 1:25 in PBS. Upon concluding the immunofluorescence technique, RNase (50 pg/ml, Sigma) was introduced to the cell suspensions. Nuclear DNA was stained with 5 µg/ml of propidium iodide (PI, Sigma). Controls were established by pre-treating and fixing cells as previously described, followed

solely by exposure to the fluorescent secondary antibody. Bivariate DNA/PCNA analysis was conducted using a FAC Scan flow cytometer (Becton Dickinson, San Jose, CA). The bivariate DNA/PCNA distributions were represented in dot plots (1,023 x 1,023 channel array) by enumerating over 10,000 cells. The cell cycle study was conducted utilizing Cell FIT computer software (Becton Dickinson). We quantified PCNA levels in specific cell cycle phases by gating analysis of data derived from DNA content. All trials were conducted a minimum of three times.

Statistical analysis

The data had been put into the computer and analyzed using IBM SPSS software version 20.0. IBM Corporation, Armonk, New York. The Kolmogorov-Smirnov test was utilized to evaluate the distribution's normality. Quantitative data were described using the mean, standard deviation, and media. The results' significance was evaluated at the 5% threshold. The applied tests comprised: 1- F-test (ANOVA) for normally distributed quantitative variables to compare several groups, and Post Hoc test (Tukey) for pairwise comparisons.

3. Results

1. Changes in body weight

In the ASE-supplemented group, the mean body weight of maternal rats and their offspring at PND7, 14, and 21 exhibited no significant differences compared to the control. Conversely, in the nicotine-treated group, both maternal rats and their offspring demonstrated a significantly decreased body weight (p<0.001) compared to the control group. The treatment of nicotine-injected female rats with ASE during gestation and breastfeeding effectively restored the body weight of mothers and offspring to levels comparable to the control, with a minor significant reduction (p<0.05) observed at PND7 in comparison to the control (Fig.1).

2. Changes in serum antioxidants

In nicotine-injected female rats and their pups, the blood activities of CAT, SOD, and GSH were dramatically reduced, whereas MDA level was significantly elevated compared to the control (p<0.001). Following the administration of ASE to nicotine-exposed maternal rats, the activities of SOD were restored to control values, although the activities of CAT and GSH were markedly elevated compared to the nicotine group, yet still significantly lower than those of the control group. The MDA level was dramatically reduced compared to the nicotine group, although it remained significantly elevated relative to the control group (Fig. 2). In pups exposed to maternal nicotine, serum activities of CAT, SOD and GSH were dramatically reduced (p<0.001). In contrast, MDA level was markedly increased compared to the control. In progeny subjected to maternal nicotine and ASE treatment, the SOD activity and MDA level were normalized to control values. However, the CAT and GSH activities exhibited a considerable increase (p<0.001) compared to the nicotine group, although they remained significantly lower than control levels (Fig. 2).

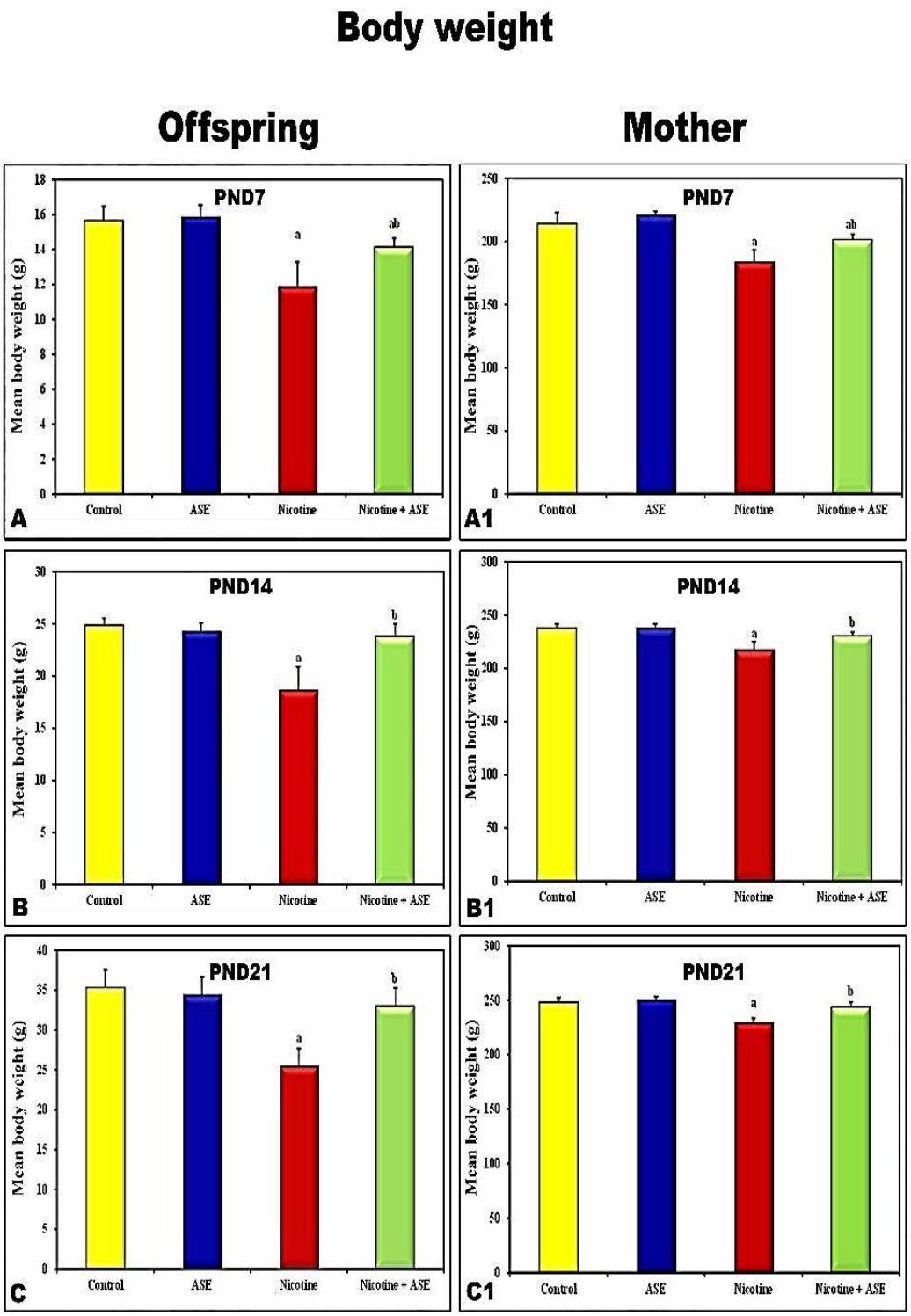

Fig. 1. The mean body weight (g) of rats' pups (A, B, C) and their dams (A1, B1, C1) at PND 7, 14, and 21. (a; significance with control, b; significance with nicotine).

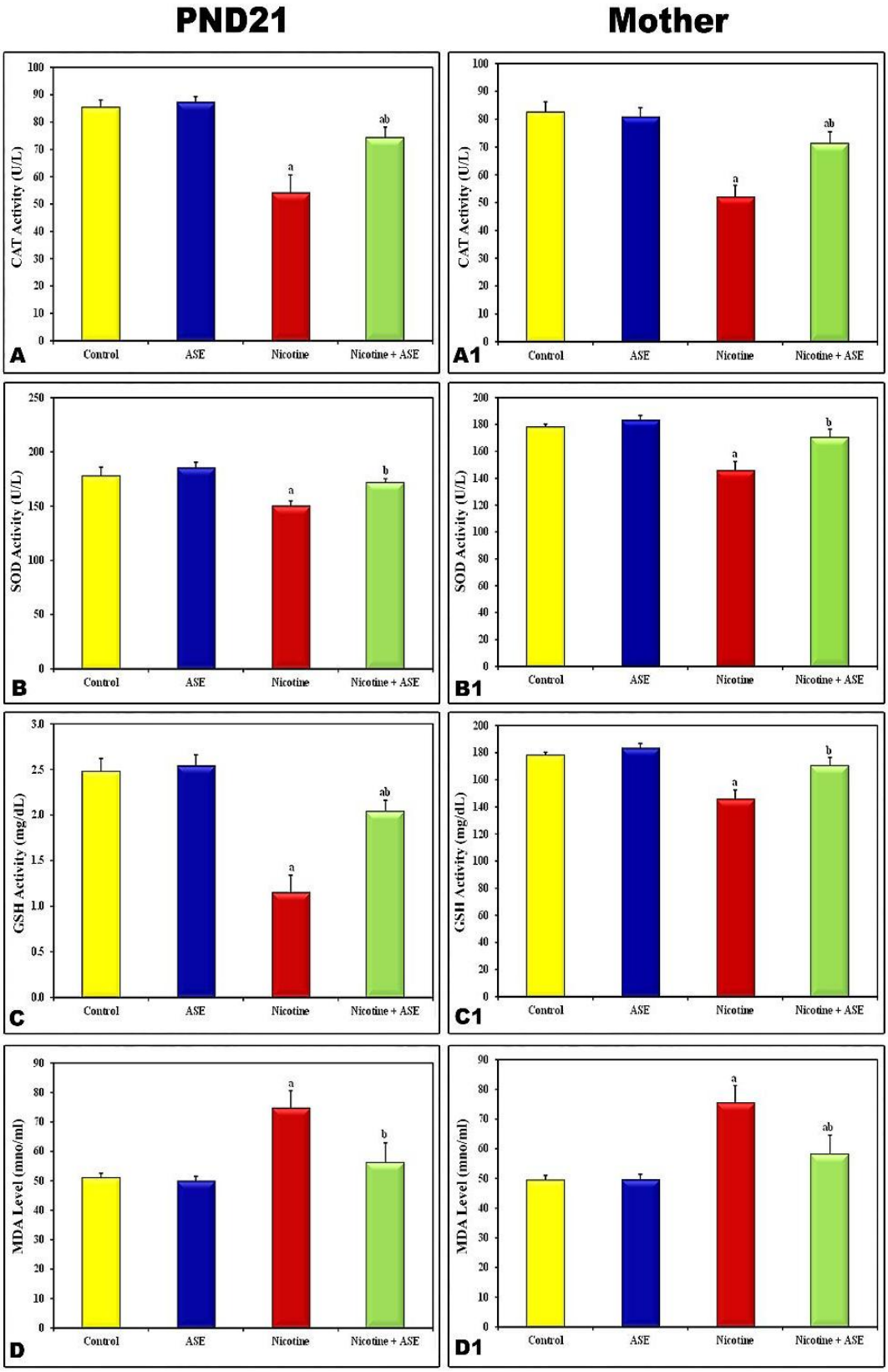

Fig. 2. Serum CAT (U/L) (A, A1), SOD (U/L) (B, B1), GSH (mg/dL) (C, C1), and MDA (nmol/mL) (D, D1) among the studied groups of female rats and their pups at PND 21. (a; significance with control, b; significance with nicotine)

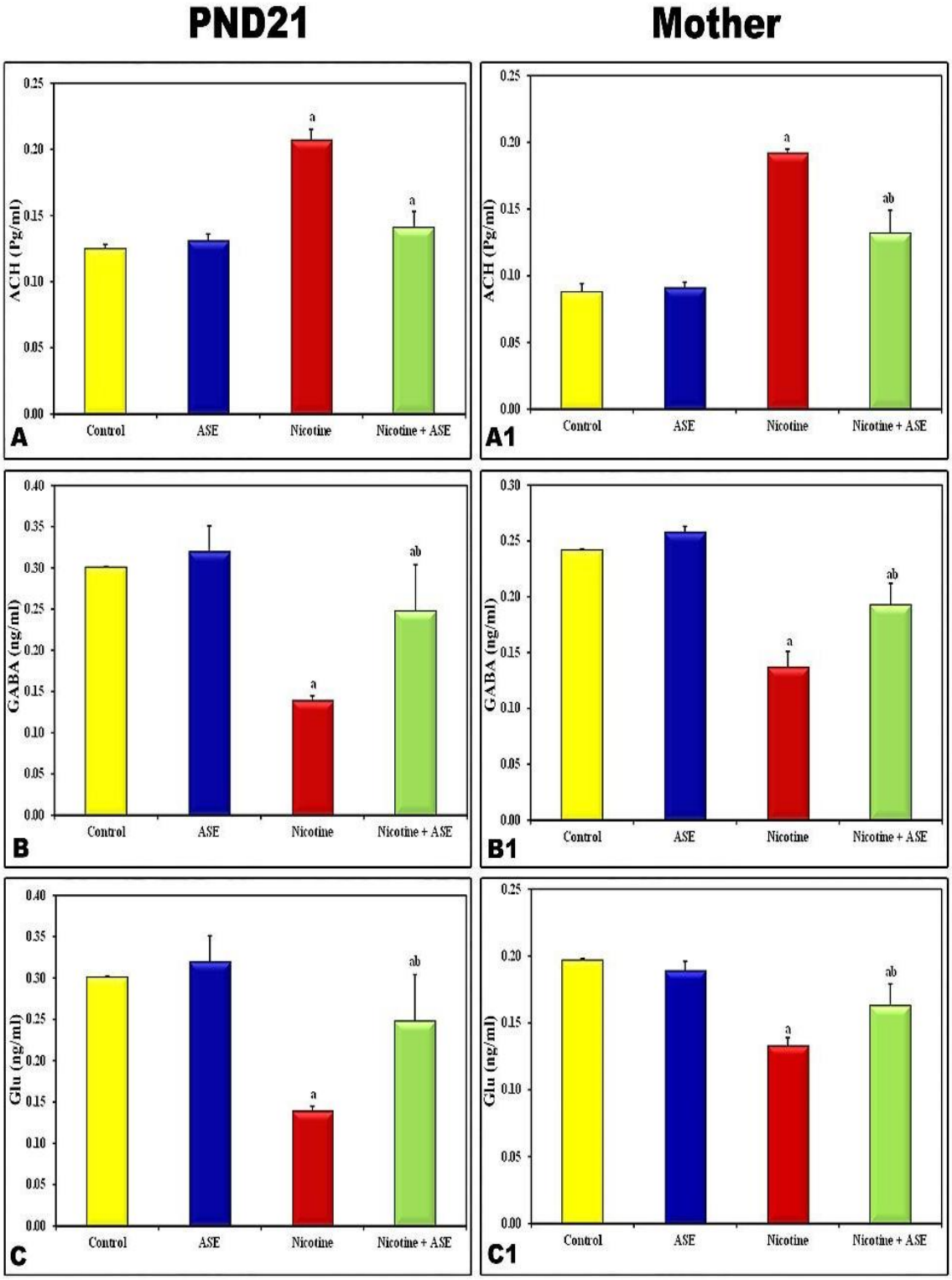

Fig. 3. The levels of Ach (Pg/ml), GABA (ng/ml) & glutamate (Ug/ml) in the retina tissue homogenate among the studied groups of mother rats and 21-day-old offspring (a; significance with control, b; significance with nicotine)

Changes in the levels of Ach, GABA and glutamate in the retinal tissues

Acetylcholine levels were markedly elevated in the retinal tissue of nicotine-treated mother rats and their progeny, but GABA and glutamate levels were dramatically diminished ($p<0.001$) relative to the control. The supplementation of ASE to nicotine-treated maternal rats significantly reduced Ach levels and elevated GABA and glutamate levels compared to the nicotine-treated group of mothers and their pups alone ($p<0.001$), but it still exhibited a significant difference from the control group (Fig. 3).

Histological observations of the retina

At PND7, the retinas of control and ASE maternally supplemented pups exhibited normal histological architecture. At this stage, the retinal segment had five separate layers: pigment epithelium (PE), poorly developed photoreceptors (PR), undifferentiated neuroplastic layer (NL), inner plexiform layer (IPL), and ganglion layer (GL). Furthermore, the outer plexiform layer remains undifferentiated at this stage. In offspring exposed to maternal nicotine at postnatal day 7, an increase in the thickness of the pigmented epithelium, accompanied by detachment from shattered photoreceptor cells, was observed. In children treated with nicotine and ASE, the retina exhibited significant histological enhancement, but the PE and PR seemed constricted with minimal detachment sites (Fig. 4 A-D).

At PND14, the retinal slices from the control and ASE groups exhibited normal histological architecture, featuring well-differentiated outer and inner plexiform layers. In kids exposed to maternal nicotine, retinal sections exhibited minor histological signs, including degeneration of the pigment epithelium and photoreceptor cell layer, accompanied by minimal retinal detachment. The post-supplementation of ASE to nicotine-treated women effectively repaired the histopathological indications in their children (Fig. 4 A1- D1).

At PND21, the retinal sections from control and ASE-supplemented animals had a well-organized histological architecture, but the retinal sections from maternally nicotine-treated offspring demonstrated significant retinal detachment of photoreceptor cell layers from the pigment epithelium. Furthermore, disarray and significant involution were observed in the photoreceptor cell layer and outer nuclear layer. The nicotine and ASE group exhibited significant recovery in retinal architecture (Fig. 5 A-D).

The retinal sections of the control and ASE groups exhibited well-organized layers of retinal cells, including the pigmented epithelium (PE), characterized by a single row of low cuboidal cells with flattened nuclei; the photoreceptor layer (PR), comprising the outer and inner segments of rod and cone cells; the outer nuclear layer (ONL), containing densely packed rod and cone cell nuclei; and the thin outer plexiform layer (OPL), which displayed a reticular structure between the outer and inner nuclear layers. The inner plexiform layer (IPL), also exhibiting a reticular structure, was thicker than the outer plexiform layer. The ganglion cell layer (GCL) consisted of large, rounded, variably sized, and dispersed cells with pale nuclei. On the other hand, in nicotine-treated mothers, the retinal sections displayed remarkable histopathological signs in almost all layers. These signs included degenerated PE, detached and vacuolated PR, and the nuclei of ONL and INL appeared lost in some areas of the section. Post-supplementation of ASE to nicotine-treated rats', rats was successfully restored the histological pattern of retinal cell layers to near-normal architecture (Fig. 5 A1-D1).

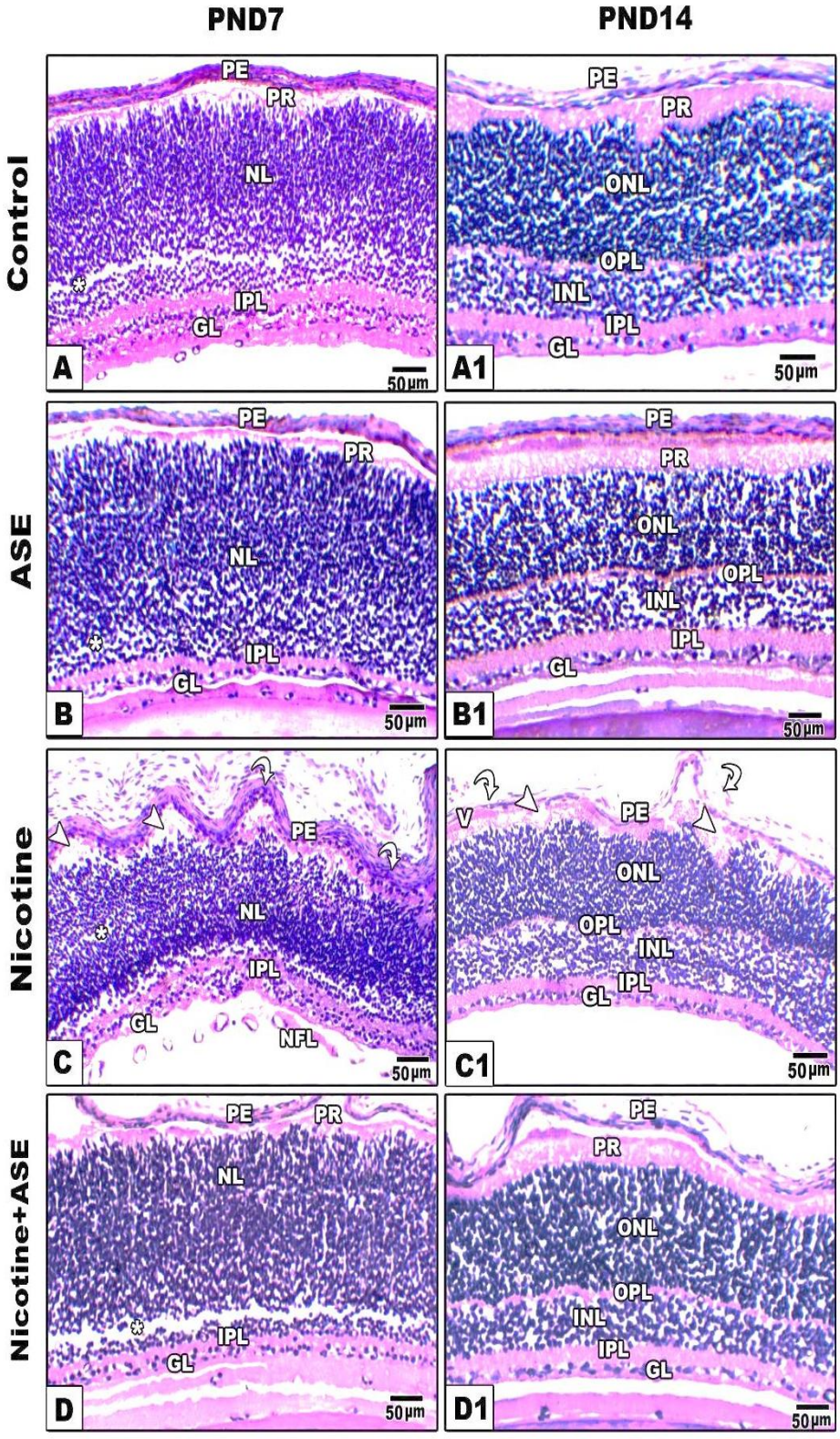

Fig. 4. Histological sections from the retinas form the studied pups at 7- and 14-days old Control (A-A1), *A. squamosa* extract (B-B1), Nicotine (C-C1), and Nicotine & *A. squamosa* extract (D-D1). The retinal sections from control and ASE supplemented rats exhibited normal histological architecture. In maternally nicotine treated offspring, the retinal sections showing wavy pigmented epithelium (curved arrows), detached (black asterisk), and vacuolated photoreceptors (arrows heads). In ASE and nicotine treated rats, the retina sections showed obvious recovery to normal histological architecture as control. (Stain: H&E, Scale bar: 50µm). Abbreviations: PND (post-natal day), Inner nuclear layers (INL), inner plexiform layer (IPL) and ganglion cell layer (GCL), outer nuclear layer (ONL), outer plexiform layer (OPL) pigmented epithelium (PE), and photoreceptor (PR)

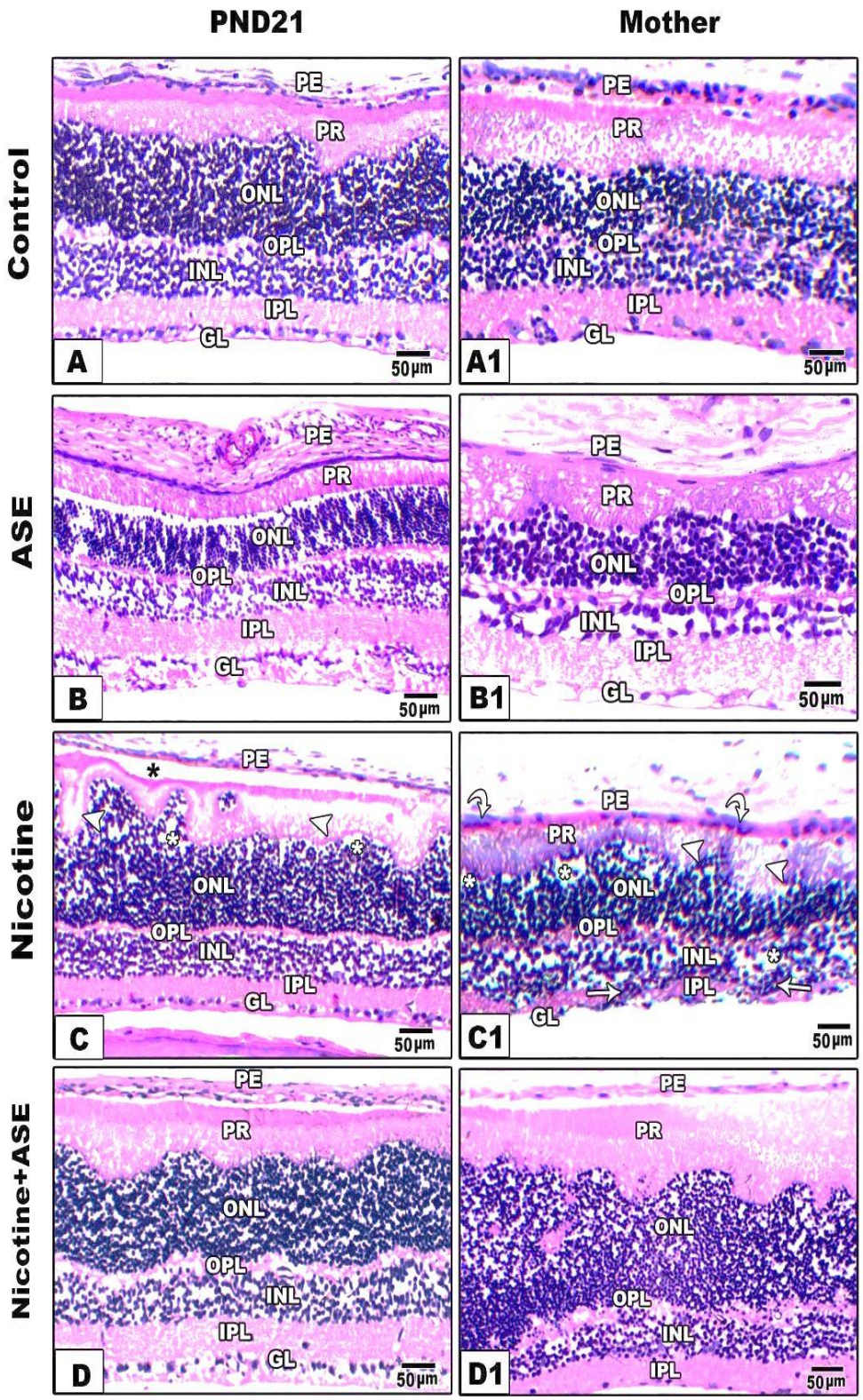

Fig. 5. Histological sections from the retinas of female rats (A1- D1) and their 21-day-old offspring (A- D). Control (A-A1), *A. squamosa* extract (B-B1), Nicotine (C-C1), and Nicotine & *A. squamosa* extract (D-D1). The retinal sections from control and ASE-supplemented rats appear with normal histological architecture. In nicotine-treated rats and 21-day-old pups, the retinal sections showed hypertrophied pigment epithelium (curved arrows), detachment of photoreceptors (black asterisk), and vacuolated (arrowheads) photoreceptors, involuted ONL (white asterisk). In ASE and nicotine-treated rats, the retina sections showed significant recovery to normal histological architecture as in controls. (Stain: H&E, Scale bar: 50 µm). Abbreviations: PND (post-natal day), Inner nuclear layers (INL), inner plexiform layer (IPL) and ganglion cell layer (GCL), outer nuclear layer (ONL), outer plexiform layer (OPL), pigmented epithelium (PE), and photoreceptor (PR).

Immunohistochemical observation

Immunohistochemical reaction for p53 in the retina of mother rats and their offspring

The retinal sections from control and ASE supplemented mother's rats (Fig. 6 A1-B1) and their offspring (Fig. 6 A-B) displayed negative or very weak expression for P53. In contrast, a strong reaction for p53 was shown in the retinal sections from nicotine-treated mothers' rats and their offspring when compared with the control group (Fig. 6 C1, C). In nicotine-treated mother rats followed by ASE treatment, the retinal sections showed negative to very weak expression for P53 however, the retinal sections from their offspring appeared very weakly stained by P53 antibody especially in the OPL, IPL, and GL (Fig. 6 D1, D). The quantitative analysis for the degree of immunoreactivity of P53 protein for offspring among the control, ASE, and nicotine and nicotine &ASE were represented by 0.06, 0.11, 4.32, and 0.65 respectively, while for mothers, these values represented by 0.15, 0.14, 3.75, and 0.41 respectively (Fig. 7 A, B).

Immunohistochemical reaction for GFAP in the retina of mother rats and their offspring

In the retinal sections of control and ASE-supplemented mother rats (Fig. 8 A1-B1) and their pups (Fig. 8 A-B), the immunoreactivity of GFAP seemed to be high compared with the other groups. However, the retinal sections from nicotine treated mothers' rats showed a weak reaction with GFAP antibody compared to control (Fig. 8 C1) whereas, the retinal section from offspring maternally treated with nicotine showed negative to weak GFAP expressions (Fig. 8 C). Additional findings showed that the retinal sections from nicotine treated mother rats followed by supplementation with ASE showed GFAP immunoreactivity that was moderate to strong and approximately comparable to the control, while their offspring showed a weak to moderate reaction (Fig. 8 D1, D). The immunoreactivity of GFAP was more noticeable in PR, OPL, and IPL than in the other layers for all groups under study. The quantitative image analysis for the degree of immunoreactivity of GFAP is illustrated on histograms (Fig. 9 A, B).

Changes in the PCNA levels by flow cytometry

The retinal tissues in the control and ASE groups of maternal rats exhibited a standard percentage of PCNA-positive cells (control = 64.8%, ASE =62.9%). In nicotine-treated mother rats, the mean % value of positively PCNA-reacted cells of retinal tissues appeared significantly lower when compared with control (38.4%). In nicotine mothers' rats post-supplemented with ASE, the mean % value of positively PCNA-reacted cells of retinal tissues appeared significantly higher (45.2%) than the nicotine-treated group alone, but still considerably lower than the control (Fig. 10).

The retinal tissues from offspring of control and maternally ASE-supplemented groups showed a normal standard range (control=71.8%, ASE= 66.7%) of mean % value of positively PCNA-reacted cells. However, in offspring maternally exposed to nicotine, the retinal tissues appeared with a lower % value of positively PCNA (45.1%) if compared with the control. In offspring maternally administered with nicotine and post-supplemented with ASE, the retinal tissues appeared with a higher % value of positively PCNA-expressed cells (60.9%) if compared with the nicotine group and still markedly lower than the control (Fig. 10 and Table 1).

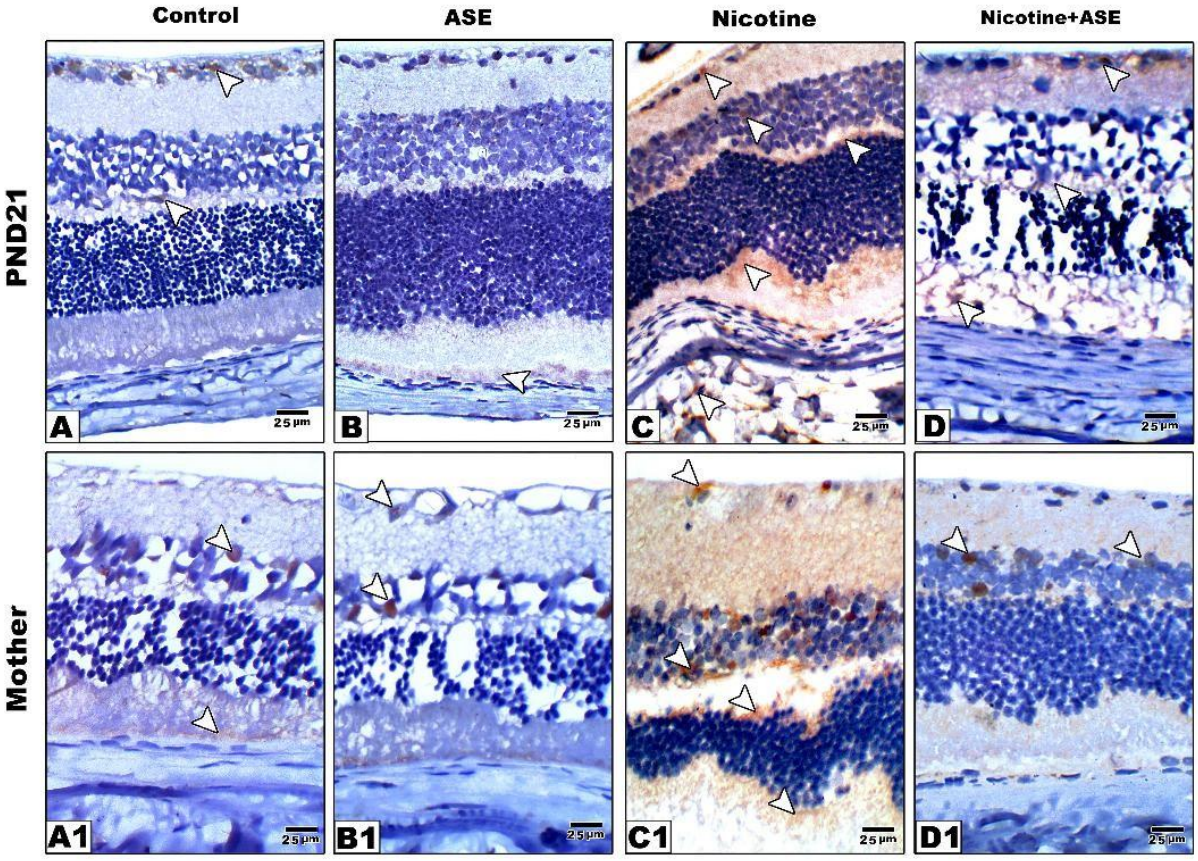

Fig. 6. Images through the retina of female rats (A1- D1) and their 21-day-old progeny (A- D) stained with anti-P53 antibody (Scale bar: 25 µm).

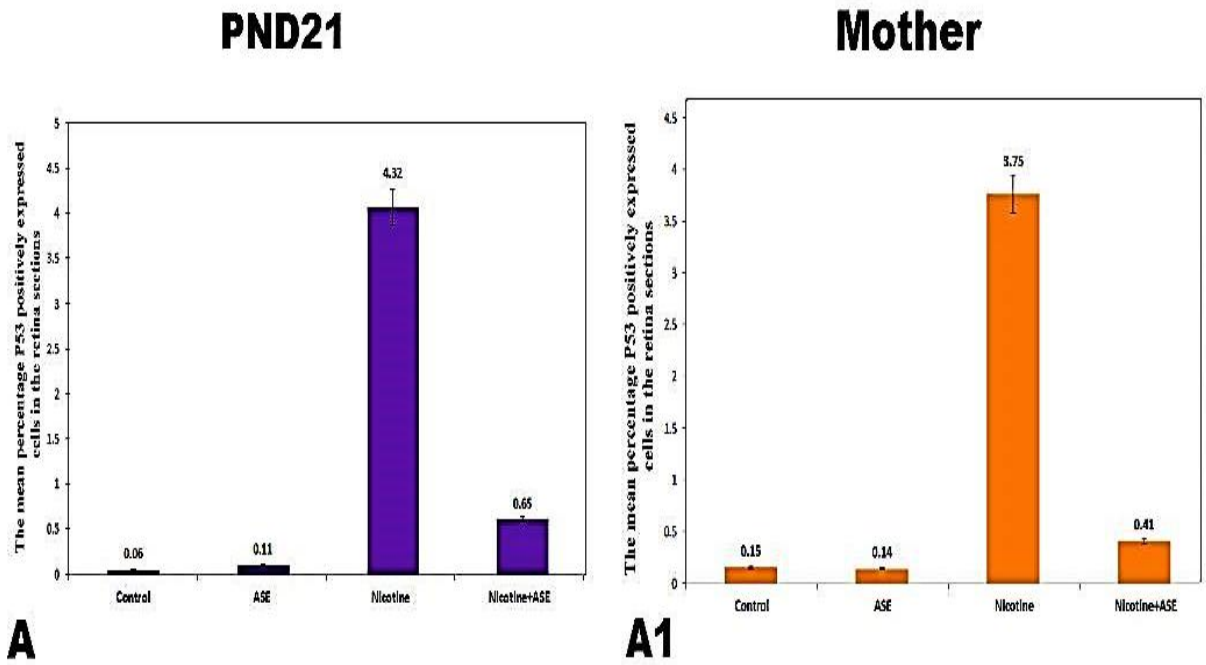

Fig. 7. Histograms illustrate the quantitative image analysis for the degree of P53-immunoreactivity within the retinal sections of mother's rats (A1) and their offspring (A).

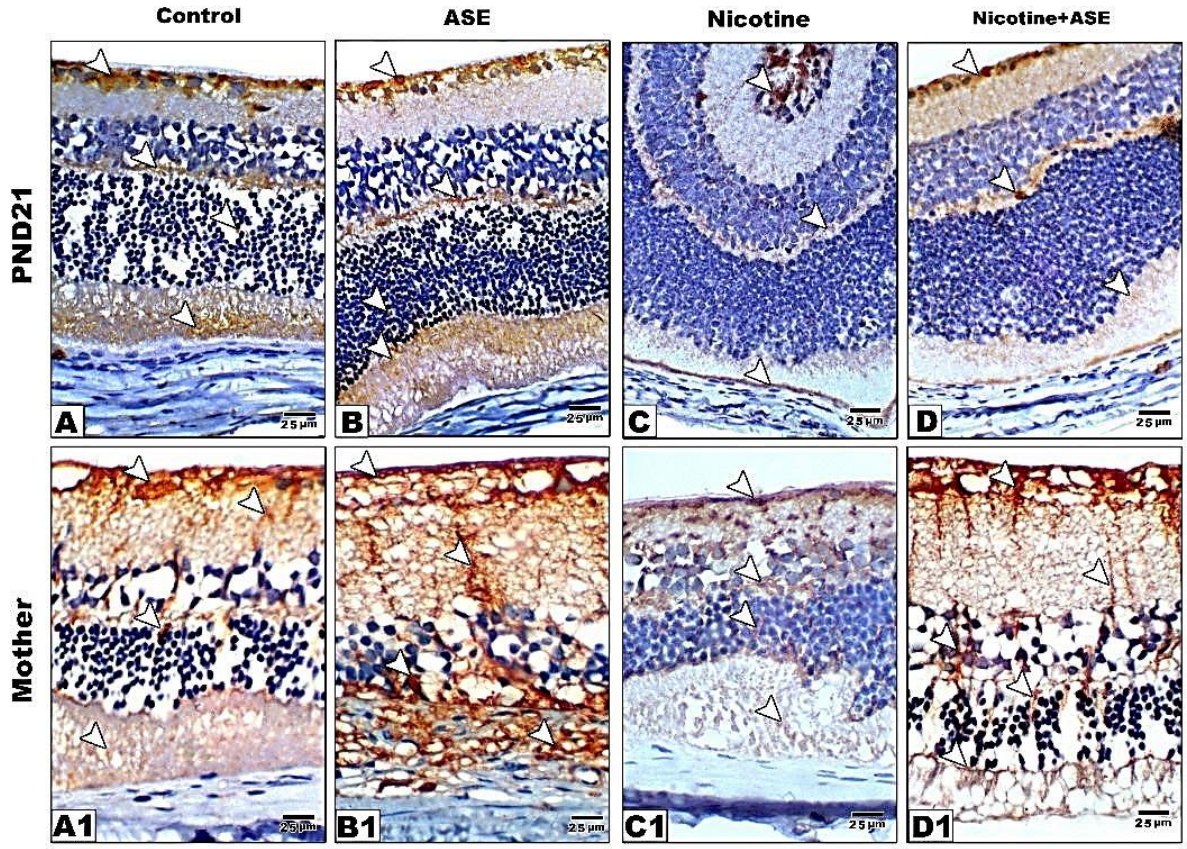

Fig. 8. Images through the retina of female rats (A1- D1) and their 21-day-old progeny (A- D) stained with anti-GFAP antibody (Scale bar: 25 µm).

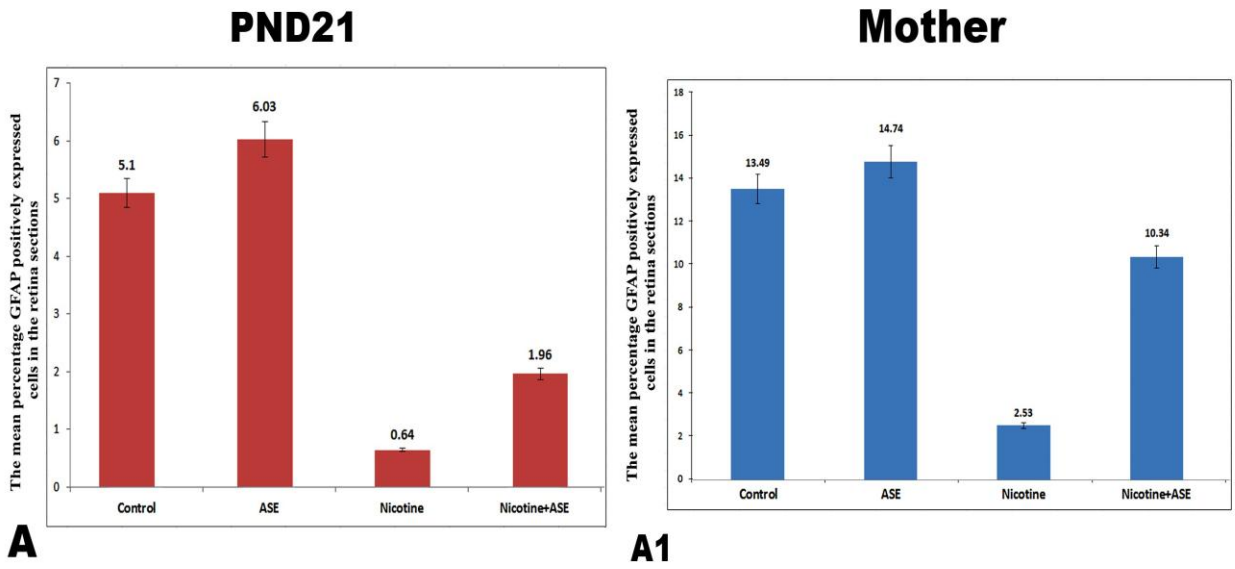

Fig. 9. Histograms illustrate the quantitative image analysis for the degree of GFAP-immunoreactivity within the retinal sections of mother's rats (A1) and their offspring (A).

Table 1. The mean percentage values of PCNA-positively expressed cells in the retinal tissues among the studied groups of mother rats and their pups

	Control	ASE	Nicotine	Nicotine & ASE
Pups	71.8% ± 2.98	66.7% ± 1.92	45.1 %± 1.49	60.9% ± 2.70
Mother	64.8% ± 1.53	62.9% ± 1.68	38.4% ± 1.81	45.2 %± 1.97

A low % value of PCNA was positively expressed in retinal cells in nicotine nicotine-treated group of mother rats and their pups compared with the control.

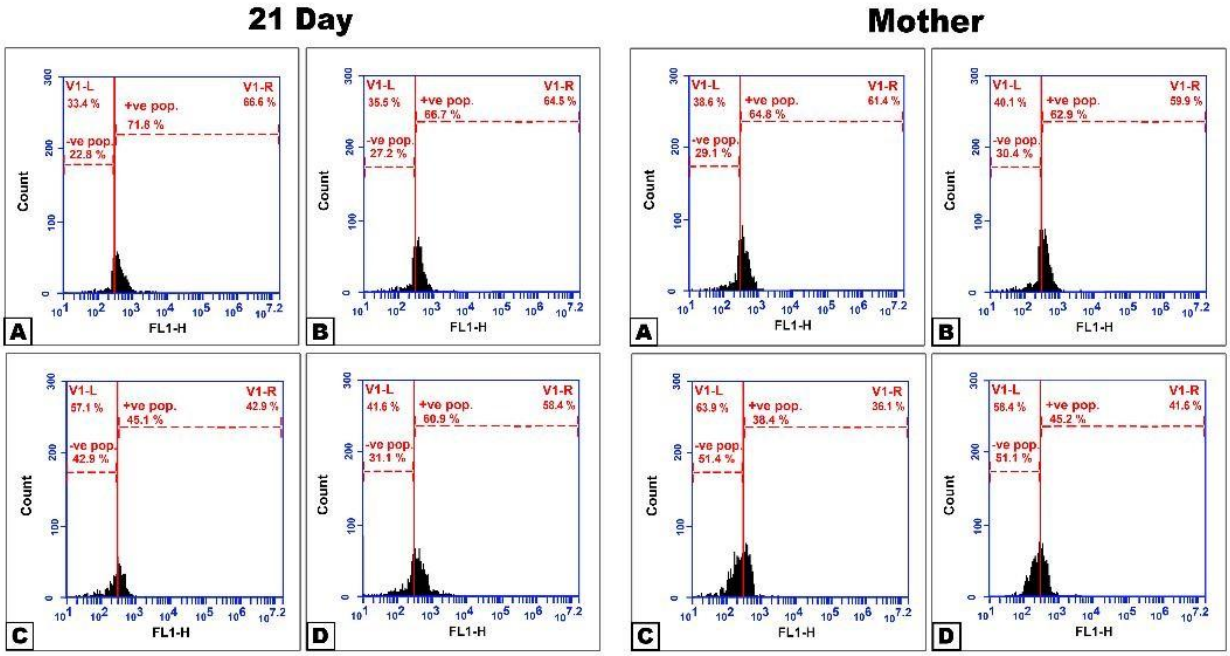

Fig. 10. A flow cytometric analysis illustrating the mean % of PCNA-positive cells by FAC Scan analysis via FL1 H in the retinal tissues of female rats and their pups. A: Control, B: ASE, C: Nicotine & D: Nicotine & ASE. A low % value of PCNA was positively expressed in retinal cells in nicotine nicotine-treated group of mother rats and their pups in comparing with the control.

4. Discussion

Exposure to nicotine during prenatal development leads to detrimental neurodevelopmental changes and neurobehavioral impairments (Wells and Lotfipour, 2023). This is mostly because nicotine included in tobacco smoke may traverse the placenta with an efficiency of 88% and may enter fetal circulation (He et al., 2022). Moreover, smoking during pregnancy has been approved to cause vision impairments in offspring (Fernandes et al., 2015). The hazardous effects of nicotine have been documented to exacerbate the onset and advancement of several ocular disorders, including age-related ocular degeneration (Perez-de-Arcelus et al., 2017), glaucoma (Pakravan et al., 2017), and cataracts (Galor and Lee, 2011). The custard apple fruit has been recorded to promote the development of the brain and neurological system in the fetus. This is mainly attributable to its abundant copper content and vitamins C and A, which significantly aid fetal growth (Kumar et al., 2021).

This study sought to assess the potential protective effect of *A. squamosa* fruit extract against nicotine-induced retinal impairment in rat dams and their offspring. The findings of the current study indicated that the average body weights of nicotine-exposed maternal rats and their progeny at PND7, 14, and 21 were considerably reduced compared to the control. Nonetheless, following treatment with ASE, body weight was effectively restored to levels comparable to the control group. These findings align with those of Chen et al. (2015), who discovered that maternal nicotine exposure during gestation and breastfeeding causes substantial body weight reduction in both dams and pups. Friedman et al. (2012) revealed that nicotine delivery has been shown to enhance lipolysis and fat utilization, without alterations in total food consumption, exercise, or heat output (Friedman et al., 2012; Rupprecht et al., 2018). Furthermore, nicotine enhances the secretion of circulating catecholamines, which may partially facilitate enhanced lipolysis (Rupprecht et al., 2018). The restoration of body weight to near-normal levels following treatment with ASE is primarily ascribed to the regulatory role of phenolic-rich components in nutritional absorption present in this extract (Alkhalidy et al., 2023). The current study has demonstrated that nicotine exposure significantly impacts the oxidative stress scavenging system by elevating the MAD level, an indication of lipid peroxidation, while markedly reducing the activity of CAT, SOD, and GSH in the sera of maternal rats and their offspring. This indicates that nicotine induces oxidative stress in retinal tissues. Consistent with our findings, prolonged nicotine exposure was shown to provoke lipid peroxidation in brain tissues through increased MDA levels, leading to cerebral injury (Taha et al., 2021). This observation aligns with the study by Oyeyipo et al. (2014), which identified a significant decline in SOD and CAT activity in nicotine-treated rats. This reduction in serum SOD activity may result from diminished production of enzyme proteins or oxidative inactivation of the enzyme protein (Elsonbaty and Ismail, 2020). A reduction in CAT activity in serum following nicotine administration suggests an inadequate removal of harmful H_2O_2 by GPx in tissues (Naidu et al., 2007). The reduced concentration of GSH may be ascribed to its utilization in the nicotine detoxification process (Mons et al., 2016). Notably, *A. squamosa* fruit extract mitigated oxidative stress generated by nicotine through the regulation of SOD, CAT, GSH, and MDA levels. Antioxidants are widely recognized for their essential function in preventing diseases linked with oxidative stress (Koksal et al., 2011). Prior research confirms that *A. squamosa* fruits are rich in antioxidant components such as acetogenins, ascorbic acid, flavonoids, and polyphenolics, which significantly contribute to the scavenging of free radicals generated by oxidative stress (Kumar et al., 2021). This finding elucidates the potential function of ASE in enhancing antioxidant enzymes, which are diminished due to oxidative stress generated by nicotine. Furthermore, Albuquerque et al. (2016) documented the antioxidant properties of *A. squamosa* pulp in relation to various disorders. Subsequent research indicated that ASE comprises a range of antioxidant chemicals, including tannins and flavonoids, as well as many of the polyphenols. Phenolic chemicals and flavonoids are vital due to their antioxidant capabilities. Phenolics, the most varied category of secondary metabolites, function as antioxidants by neutralizing free radicals and demonstrate anti-inflammatory properties, potentially preventing chronic diseases like cancer, diabetes, and cardiovascular disorders. Flavonoids confer anti-inflammatory advantages and safeguard against vascular illnesses (Tungmunnithum et al., 2018). Tannins mitigate lipid peroxidation, DNA mutations, and the suppression of inflammatory mediators (Fraga-Corral et al., 2021). Terpenoids augment antioxidant activity and ameliorate inflammatory symptoms (Yao and Liu, 2022). Neurotransmitters are crucial for the optimal functioning of the body's intricate neurological system. ACh is a principal retinal neurotransmitter that regulates visual processing via a diverse array of cholinergic receptors present on several retinal cell types. Similarly,

glutamate serves as the primary excitatory neurotransmitter, whereas GABA functions as the principal inhibitory neurotransmitter in the retinal neurons of mammals (Bringmann et al., 2013).

The present study demonstrated a considerable elevation in Ach levels, accompanied by a reduction in glutamate and GABA levels in the retinal tissues of nicotine-exposed rats and their progeny. This indicates that nicotine induces disturbance in the neurotransmission pathways of retinal cells. Nicotine readily penetrates the blood-brain barrier and binds to the nicotinic receptors of acetylcholine in the retina, leading to an abnormal buildup of acetylcholine and inhibiting its metabolism (Hopkins et al., 2012). Nicotine increased the firing rate of dopaminergic and GABAergic neurons. The diminished robust response in the non-dopamine GABAergic neurons results in specific inactivation. It may ultimately lead to the desensitization of dopamine neurons, so facilitating a subsequent regulated increase in the response of mesolimbic dopamine neurons to nicotine (Paik et al., 2018).

Conversely, following treatment with ASE, the increased levels of acetylcholine and the reduced levels of glutamate and GABA induced by nicotine were markedly ameliorated, indicating the potential of ASE in the modulation of neurotransmitters across many retinal cells. The mechanism through which *A. squamosa* regulates neurotransmitter levels in the retina remains unidentified; however, prior studies have confirmed that polyphenolic compounds exert significant neuroprotective and modulatory effects on neurotransmitter pathways (Rebas et al., 2020; Guo et al., 2022). Additionally, several phytochemicals, including quercetin, resveratrol, and myricetin, have been utilized by various authors to alter essential neurotransmitters in humans (de Carvalho et al., 2011).

Extensive histopathological alterations were seen in retinal layers of nicotine-exposed maternal rats and their offspring at postnatal days 7, 14, and 21. The observed symptoms comprised hypertrophied and undulating pigmented epithelium, detached and vacuolated photoreceptors at all developmental stages, whereas significant vacuolation in the ONL was noted exclusively in the retina at postnatal day 21 (PND21) and in their mothers. Conversely, ASE supplementation significantly mitigated several of these histological alterations. Consistent with our findings, nicotine administration induced disruption in retinal pigment epithelial cells, resulting in the thinning of the outer nuclear layer, and impaired the photoreceptor-RPE interface (Yang et al., 2010; Yang et al., 2022). A separate study demonstrated significant inflammation in the retinal tissue of mice subjected to tobacco smoke (Wang et al., 2021). Furthermore, nicotine administration during gestation was seen to provoke significant detrimental histological alterations in the structural organization during the development of the retina in neonates, leading to the advancement of retinopathy (Evereklioglu et al., 2004; Spiegler et al., 2013; Fernandes et al., 2015). Fernandes et al. (2015) indicated that fetal exposure to nicotine is a substantial risk factor for vision impairments in later life, particularly affecting the intraocular muscles and retinal neurons more than other structures. Maugeri et al. (2017) indicated that nicotine may exacerbate damage to the blood-retinal barrier in a model of human diabetic macular edema. A cotinine (a major nicotine metabolite) exerts direct toxicity through lipid peroxidation in retinal photoreceptors (Zenzes, 2000), augment the production of free radicals, and diminish antioxidant levels in the bloodstream, aqueous humor, and ocular tissues (Cheng et al., 2000), while also inducing peripheral vasoconstriction and elevating peripheral vascular resistance (Cinar et al., 2019). Nicotine exposure during gestation was discovered to traverse the placenta, leading to fetal hypoxia and subsequent oxidative stress, which adversely impacts neurogenesis in the retina and brain (Inaloz et al., 2004).

The mechanism by which nicotine induces retinopathy during gestation and lactation remains unclear; however, it has been shown that maternal smoking may cause prostaglandin synthesis disruption, cadmium interference with calcium signaling, vasoconstriction, carbon monoxide-induced fetal hypoxia, and altered steroid hormone production, and modified responses to oxytocin, as articulated by Ion and Bernal (Ion and Bernal, 2015). These pathways

may decrease uterine blood flow, resulting in intrauterine growth limitation of the fetus and premature birth (Wagijo et al., 2017). Moreover, research has shown that nicotine exposure stimulates the production of pro-inflammatory cytokines like IL-1βTNF-α, which can compromise the structure and function of retinal cell layers, leading to pathological changes (Ma et al., 2004). Moreover, smoking correlates with an elevated risk of premature rupture of membranes, cervical incompetence, and chorioamnionitis (Hayashi et al., 2011) which has been proposed to heighten the likelihood of developing retinopathy (Kuon et al., 2015).

The qualitative examination of phytochemicals in the Custard apple fruit verified the existence of several anti-inflammatory and antioxidant components, including tannins, flavonoids, terpenoids, and saponins (Nhung and Quoc, 2024). This may elucidate the possible function of ASE in mitigating retinal histological alterations caused by nicotine. Consistent with our findings, Hendawy et al. (2019) discovered that *A. squamosa* extract can mitigate neuroinflammation caused by aluminum chloride in rats. Moreover, numerous investigations have indicated the possible therapeutic actions of *A. squamosa* fruit extract against various cellular damage, including testes (Mobasher et al., 2024) and kidneys (Shokr et al., 2024). It had been reported that the principal bioactive constituents of Annona are acetogenins. These chemicals possess antioxidant properties and facilitate the healing of cellular damage (Singh et al., 2024).

The protein GFAP is an interconnected filament expressed by various cell types within the central nervous system (Lewis and Fisher, 2003). GFAP participates in numerous critical central nervous system processes, encompassing cellular communication and the operation of the blood-brain barrier (Venkatesh et al., 2013). The present study observed reduced GFAP expression in the retinal cell layers of nicotine-treated dams and their offspring compared to controls, whereas treatment with ASE resulted in a significant increase in GFAP expression. This data indicates that nicotine contributes to retinal cell damage through disrupted cell communication and mitotic division, whereas ASE mitigates this impairment. Fares et al. (2023) indicated that nicotine administration diminished GFAP positivity in the cells of hippocampus in mice. Contrary to our findings, Abdel-Rahman et al. (2003) observed an increase in GFAP immunostaining in the hippocampus and cerebellum of both female and male pups at PND30 from mothers administered nicotine. This discrepancy in results may be ascribed to differences in nicotine dosage and exposure length.

The data on elevated GFAP expression in retinal tissues following ASE treatment indicates the potential of *A. squamosa* fruit ingredients in restoring retinal cell integrity through GFAP synthesis. The mechanism by which ASE can diminish GFAP activity remains unidentified; nevertheless, this may suggest the anti-inflammatory properties of its components, such as tannin and acetogenin (Hemlatha and Satyanarayana, 2015). p53 is a tumor suppressor gene whose inactivation is correlated with an elevated risk of some malignancies and the suppression of apoptosis (Kroemer et al., 2007). Furthermore, the majority of evidence indicates that the principal role of p53 in apoptosis is predominantly reliant on its transcriptional activity. p53 can stimulate the transcription of many pro-apoptotic genes (Jeffers et al., 2003). Consequently, heightened p53 activity can induce apoptosis by inhibiting anti-apoptotic genes, hence facilitating caspase activation (Hoffman et al., 2002). p53 overexpression was observed in the retinal cell layers of rats treated with nicotine, both in the mothers and their children, compared to the control. This indicates that nicotine triggers retinal cell death through the activation of p53. Smoking tobacco produces higher levels of reactive oxygen species, which lead to protein oxidation, DNA strand breakage (Chen et al., 2004), RNA oxidation (Kong and Lin, 2010), mitochondrial depolarization, and death. Mutations in p53 protein can induce apoptosis, are also linked to the toxicity of tobacco smoke (Rodin and Rodin, 2005).

PCNA was initially recognized in the cell nuclei during the DNA duplication (Leonardi et al., 1992). Moreover, PCNA is crucial for proper cellular growth and replication; yet its excess results in tumorigenesis, whereas reduced expression facilitates apoptosis (Wang et al.,

2018). The flow cytometric analysis results indicated that nicotine administration induces retinal cell apoptosis in dams and their pups by reducing the proportion of PCNA-stained cells relative to the control group. Comparable findings were documented in other bodily tissues stimulated by nicotine (Brooks and Henderson, 2021). The precise mechanism through which nicotine induces apoptosis remains ambiguous; however, new research shows that nicotine increases reactive oxygen species or free radical levels, which causes tissue damage, membrane damage, and oxidative stress (Addissouky et al., 2024). Interestingly, ASE therapy effectively mitigated the apoptotic effects induced by nicotine by reducing P53 expression and enhancing PCNA protein expression in retinal tissues. This indicates the anti-apoptotic properties of *A. squamosa* fruit. While the anti-apoptotic mechanism of *A. squamosa* remains ambiguous, prior studies indicate that its elevated levels of gallic acid, vitamin C, flavonoids, and cyclosquamosin D may mitigate oxidative stress and apoptosis (Abd-Elrazek et al., 2021).

Conclusion

Nicotine exposure during early gestation is associated with harmful histological and biochemical alterations in the retinas of dams and their progeny. The extract of *A. squamosa* fruits mitigates the harmful alterations in the retina caused by nicotine. This relief is primarily ascribed to the presence of essential antioxidants, anti-inflammatory, and anti-apoptotic constituents in *A. squamosa* pulp extract. *A. squamosa* pulp extract may be a new therapeutic approach for attenuation of retinal toxicity in clinical application.

Limitation of study

Further studies are needed to explore the mechanism of action concerned with nicotine induced retinal toxicity.

Conflict of interest

The authors declare that there is no conflict of interest.

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