Biological and physical studies on the protective and therapeutic roles of ashwagandha seed extract against the potential toxic effect of amoxicillin in rats

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Background
Ashwagandha plant enhances the body's defense against toxicants through improving the cell-mediated immunity.
Objective
The present work was performed to assess the protective and therapeutic efficiencies of ashwagandha seed extract (ASE) against the harmful effects of amoxicillin (AM) treatment on biological and physical parameters in the brain, liver, and testes tissues of rats.
Materials and methods
Total RNA was isolated from brain, liver, and testes tissues to assess the gene expression of steroid 5 alpha-reductase 1 (5α-R1), multidrug resistant 1b (mdr1b), and luteinizing hormone receptor (LHR) genes, respectively. The cDNA was synthesized and real-time polymerase chain reaction (Real-time PCR) was performed, total antioxidant capacity (TAC) was measured. Histological examination and physiological tests for the cerebral cortex of brain tissue were recorded.
Results and conclusion
Our findings revealed that AM treatment (90 mg/kg. b.wt.) caused detrimental effects in all biological parameters, including up-regulation of gene expressions and reduction of TAC values in brain, liver, and testes tissues, in addition to severe damage to histological architectures in the cerebral cortex, including hemorrhage and neurodegeneration. ASE treatment at different doses (100, 200, and 300 mg/kg) significantly enhanced the biological and physical parameters. As the ASE dose level increased, it was observed that these improvements in gene expressions and TAC also increased. Gene expression enhancements were more pronounced in the therapeutic efficacy of ASE, whereas TAC enhancements were more pronounced in the protective efficacy of this medicinal plant extract, particularly in brain and liver tissues. In terms of histopathological parameters, the damage caused by AM was reduced by using 300 mg/kg of protective ASE than 200 mg/kg of therapeutic ASE. Biophysical investigation indicated that relaxation time and enthalpy were restored and improved, while DC conductivity was not recovered by ASE use against AM damages. The present investigation provided biological and physical evidence for protective and therapeutic efficiencies of ASE against lesion effects of AM in the previously mentioned tissues of rats.

Keywords:
antioxidant activity, biochemical analysis, dielectrics-biophysics, gene expression of 5α-R1, histopathology, mdr1b, and luteinizing hormone receptor genes, medicinal herbal plant

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Declarations

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Author Contributions: DMA shared in genetic work, data analysis and writing of paper. NEI shared in biochemical and genetic work and writing of paper. M M E shared in biophysical analysis. AME shared in histopatological analysis. M E E shared in biochemical analysis. MAM provide us with ashwaganda extract. HR shared in practical work and writing of paper. IG shared in data analysis. IF made a design of the experiment, writing the paper

The manuscript has been read and approved by all the authors and each author believes that the manuscript represents honest work, if that information is not provided in another form.

Introduction

Amoxicillin (AM) was demonstrated to be one of the most important prescribed antibiotics used for the treatment of bacterial infections in children, adolescents, and adults [1,2]. However, dangerous side effects due to AM treatment have been documented in different body tissues, including the brain, liver, and testes organs [1–4].

In brain tissue, the adverse effect of AM treatment was revealed to cause a significant reduction of serotonin level, superoxide dismutase, and catalase activities, in addition to a significant increase in glutamate and lipid peroxide levels, resulting in oxidative stress [1,4]. Oxidative stress was believed to be the primary cause of depression, convulsion, and other neurotoxicities, including neurodegenerative and neurobehavioral disorders [1,5,6]. Moreover, the oxidative stress attributed to AM treatment is considered the primary trigger for abnormalities in biomolecules and cellular constituents involving membrane lipids, cellular proteins, and nucleic acids (DNA and RNA), thereby impairing cellular biological functions. These lesions are the cause of liver and testicular toxicity [1,2,4,7]. Hepatotoxicity is characterized by increased levels of oxidation agents like lipid peroxidation (MAD) and liver enzymes, including alanine aminotransferase (ALT) and aspartate aminotransferase (AST), as well as histopathological cases such as cellular necrosis, phagocytic infiltration, and cirrhosis [2,4]. Reduced antioxidant enzymes levels, such as catalase (CAT) and glutathione peroxidase (GPx), and spermatogenic cell abnormalities are indicative of testicular toxicity [4]. The aforementioned reports demonstrated that the AM drug could cause damage to numerous body organs. Therefore, the AM treatment must be supplemented with natural materials capable of mitigating or preventing these damages. Medical plants (particularly ashwagandha) could be advantageous for this purpose.

Ashwagandha plant (Withania somnifera (L) Dunal), family Solanaceae was found to be commonly known as Indian Ginseng or Indian Winter cherry [8]. This plant is considered a perennial medicinal herb, generally used in traditional Indian, Unani, and Ayurveda medicines. Since it was discovered to be characterized by multiple pharmacological activities involving anti-inflammatory, antitumor, anticancer, anticonvulsive, antiaging, antidepressant, antiosteoarthritic, immune-stimulant, and antioxidant agents [9–11]. Moreover, the herb has beneficial properties for the treatment of insomnia and nervous exhaustion, as well as it is recommended for improving memory capacity and learning ability [9,12].

Ashwagandha (ASH) has been preclinical indicated to be an efficient promoter in improving cognitive impairment and hepatoprotection [11,13]. Moreover, it was demonstrated to ameliorate productive hormones and fertility [14]. Numerous investigations documented that ASH is considered a natural resource for safe and effective therapies against various diseases such as sexual deficiency, fetal disorders, cordites, cancer, rheumatism, arthritis, low stamina, leprosy, and stress [11,14–18].

The pharmacological and medicinal properties of ASH may be attributable to the high levels of its biologically active phytochemical components such as flavonoids, saponins, alkaloids, steroidal lactones, phenolic compounds, and withanolides [9,16,19]. These active ingredients present in ASH have been confirmed to be potent agents that suppress oxidative stress by enhancing the antioxidant enzymes, ameliorating the immune-stimulant properties, reducing and scavenging the different species of free radicals, and reducing the lipid peroxidation [11,16,20].

In this study, we utilized biophysical tests and dielectric measurements, such as the brain's impedance, relaxation time, DC conductivity, enthalpy change, and activation energy, to investigate AM interaction within brain tissue as well as the role of ASE in the protection and treatment of tissues. Their application is due to their sensitivity in tissue damage diagnosis and prognosis. Whether the cause of such injuries is a drug or some other effector, such as various diseases, dielectric parameters can distinguish between cancerous and normal cells, as well as monitor the deterioration of preserved blood, differentiate between high and low dose rate impacts of gamma radiation on bone marrow, and detect the deleterious effects of ionizing radiation on bone marrow, hemoglobin, and red blood cells [21–29]. This work extends the biophysical examination of biological systems to include brain tissue. Physical parameters are measured to investigate the interactions of AM within brain tissue and cells versus the use of ASE-supplemented brain tissue to repair such damage.

Therefore, the present work was designed to investigate the protective and therapeutic efficacy of ASE against the potential toxic effect of AM in rats. This investigation included the evaluation of expression of 5α-R1, Mdr1b, and LHR genes, biochemical analysis for assaying the TAC in the brain, liver, and testes tissues of rats, histopathological examination of the brain as well as biophysical tests of the brain.

Materials and methods

Plant material

Ashwagandha seeds were obtained from the Department of Horticultural Crops Technology, National Research Centre, Dokki, Giza, Egypt.

Drug

AM drug (capsules) was obtained from a local pharmacy. AM capsules were dissolved in distilled water to form a dose of 90 mg/Kg.b.wt [4] and gavaged orally once daily to rats for 12 days.

Ethics approval and consent to participate

This study was approved according to Ethics of Medical Research Committee of National Research Centre, Al Buhouth Street Dokki, Cairo, Egypt (Date 3.11.2022/No 14511122022).

Experimental animals

In this study, male Sprague-Dawley rats weighing about 120 g were utilized. Animals were obtained from Animal House, National Research Centre, and they were housed in stainless-steel cages on a bedding of wood shavings. The ambient temperature was adjusted to 25±3.2°C, with a relative humidity of 50%±15. The rats were subjected to a light/dark cycle of 12/12 h. Food and water were provided ad libitum.

Preparation of the ashwagandha extract

Aqueous extract of Ashwagandha seeds was on hand according to Salama *et al.* [16] Maheswari *et al.* [30]. 50 gm of seed powder were placed in 500 ml of distilled water (1/10 w/v). This mixture was homogenized well, filtered through qualitative No.1 Whatman filter paper (Whatman International Ltd, Maidstone, England), and subjected to the lyophilization process through a freezer drier (Snijders Scientific-Tilburg, Holland) under a pressure of 0.1 to 0.5 mbar and temperature degree ranged from −35 to −41°C, After that, the dry extract had been stored at −20°C until usage.

Experimental design

A total of 56 rats were divided into eight groups (7 animals each) as follows:

Control group (G1): the animals of this group were fed only on a basal diet for 12 days.

AM group (G2): In this group, the rats received AM in distilled water orally at a dose of 90 mg/kg.b.wt. daily for 12 days.

Protection groups (G3-5): rats gavage AM in distilled water in the same dose and way as mentioned previously. Starting on the first day of AM administration, the rats in these groups (G3-5) had been received Ashwagandha extract (ASE) (100 mg/kg, 200 mg/kg, and 300 mg /kg, respectively) orally every day and these groups were used for estimating the protective role of ASE against the toxicity of AM.

Therapeutic groups (G6-8): rats in these groups took AM in distilled water orally as mentioned previously for the same period, then the rats were administrated (orally) with ASE (100 mg/kg, 200 mg/kg, and 300 mg/kg, respectively) for 7 days to evaluate their therapeutic role against the toxicity that produced by AM intake.

At the end of the experiment, the surviving animals were sacrificed. Liver, brain, and testes organs were collected for gene expression, as well as biochemical, biophysical, and histopathological studies.

Gene expression analysis

RNA isolation

Total RNA was extracted from the brain, liver, and testes tissues of male rats using TRIzol reagent kit (Invitrogen, Germany) according to the manufacturer's protocol. Briefly, 50 mg of each of the brain, liver, and testes tissues were homogenized in 750 µl of TRI reagent in the autoclaved mortar for 5 min at room temperature to dissociate the nucleoprotein complexes into the TRI reagent. After incubation, 140 µl of chloroform was added to the homogenized samples. The mixture was shaken vigorously, and then centrifuged at 12000 xg for 15 min at 4°C. Subsequently, the RNA in the aqueous phase was carefully separated, precipitated by adding 600 µl of 100% isopropyl alcohol, and centrifuged at 12000 xg for 10 min at 4°C.

The RNA pellet was washed with 70% DEPC ethanol and dissolved in RNase-free water at 60°C, RNA concentration and quality were measured using nanodrop-1000 spectrophotometer (Thermo Scientific, Rockford, IL, USA). The absorbance ratio (purity) at 260/280 nm was determined between 1.8 and 2.1 for the samples. All samples of

RNA were treated with DNase I (grisp research solutions, Portugal) to degrade any contaminating DNA and then stored at −80°C until usage.

cDNA synthesis

The complementary DNA (cDNA) was synthesized by reverse transcription of isolated RNA from the liver, brain, and testes tissues using oligo (dT) 15 primer Maxime RT PreMix Kit (iNtRON Biotechnology, Korea, Cat. No. 25081/96 tubes). The reaction volume was carried out in 20 µl and prepared according to the kit's instruction. The reverse transcription (RT) reaction was performed for 60 min at 45°C then the RTase inactivation step was terminated for 5 min at 95°C. The cDNA were kept at −20°C until using for DNA amplification [31,32]. Primer sequences of the brain 5α-R1 gene, liver Mdr1b gene, and testes LHR gene are mentioned in Table 1.

Evaluation of mRNA expression using real-time PCR

To study the relative mRNA expression of 5α-R1, Mdr1b, and LHR genes, Fast SYBR Green Master Mix (Topreal TM PCR 2X pre-Mix (SYBR green with low ROX, enzynomics Korea) was used, the final concentration of the primers was 10 pmol and the total volume of the reaction consisted of 20 µL [1 µl of cDNA, 1 µl of each forward and reverse primer, 10 µl of SYBR green master mix, and 7 µl water]. Real-time PCR was performed in triplicate using QuantStudio 5 DX Real-time PCR System (Applied Biosystems, Carlsbad, CA, USA), the amplification instructions consisted of a denaturation step at 95°C for 3 min followed by 40 amplification cycles of denaturation at 95°C for 10 s, and annealing/extension at 60°C for 15 s. The expression of studied genes was normalized to β-actin housekeeping gene. The relative gene expression was quantified using the ($2^{-\Delta\Delta Ct}$) method. Primers used for the amplification of LHR and β-actin genes were intended according to Shi *et al.* [33], whereas, primers of 5α-R1 and mdr1b genes were designed using Primer 3 software [34] and Hokkaido System Science [35], respectively.

Table 1 Forward and reverse primers for 5 α-R1, mdr1b, LHR and β-actin genes

Gene	Primer	Sequences 5′ to 3′
5α-R1	Forward	GAGATATTCAGCTGAGACCC
	Reverse	TTAGTATGTGGGCAGCTTGG
mdr1b	Forward	ACAGAAACAGAGGATCGC
	Reverse	AGAGGCACCAGTGTCACT
LHR	Forward	CATTCAATGGGACGACTCTA
	Reverse	GCCTGCAATTTGGTGGA
β-actin	Forward	TCGTGCGTGACATTAAAGAG
	Reverse	ATTGCCGATAGTGATGAC

Biochemical analysis

Tissue samples from the brain, liver, and testes were separately excised, weighed, cut into small pieces, and washed with isotonic saline (100 mM potassium phosphate buffer, pH 7.4). These tissues were homogenized in 10 volumes of ice-cold normal saline (0.9% sodium chloride) to produce 20% (w/v) and centrifuged at 3000 rpm for 10 min, the supernatant was used immediately for determination of the TAC. The measurement of TAC was determined according to the method of Koracevic *et al.* [36], using a commercially available Bio-diagnostic Kit.

Histopathological examination

After anesthesia, samples of brain tissue were collected from rats and fixed in 10% formaldehyde solution for 24 h, then treated by a standard protocol in a histological lab [37,38] then the slides were inspected under a light microscope.

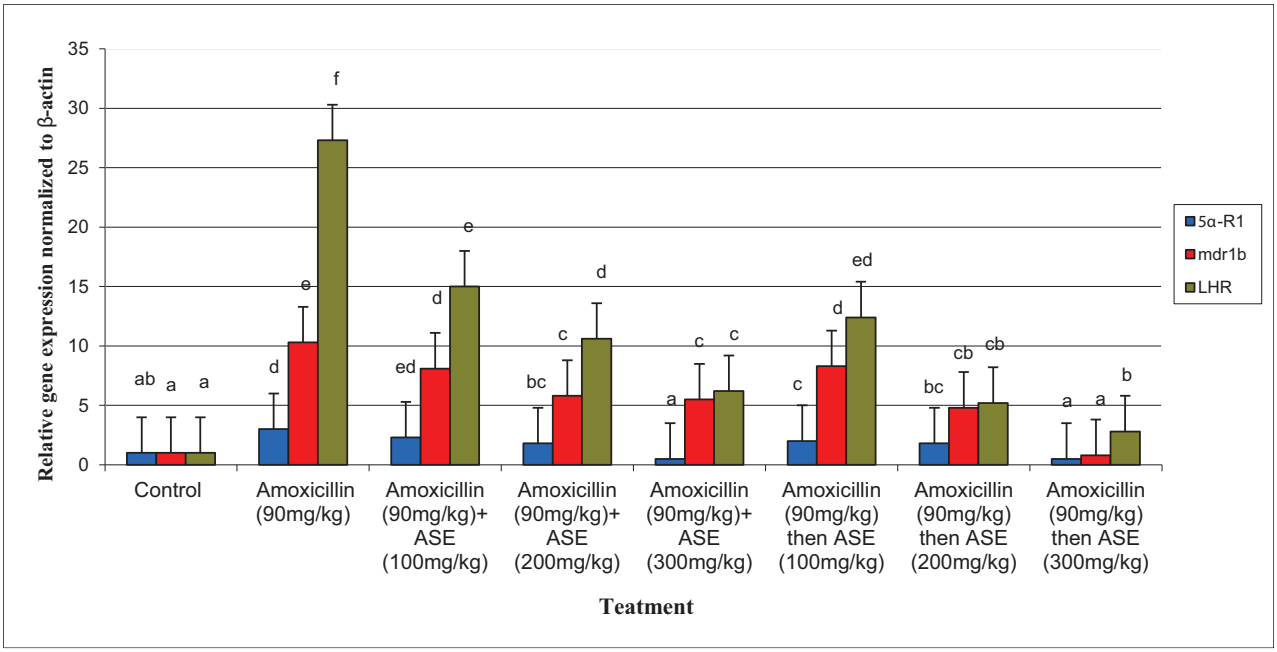
Biophysical studies

Rat brains were stored in 10% formaldehyde. Rat brains were sliced into 1–2 mm thick slices and transferred to distilled water for 24 h. They were then dehydrated in a series of ethyl alcohol concentrations of 50, 70, 90, 95, and 100% for 30 min period at each concentration. Finally, brain tissue slices were measured by Broad band Dielectric Spectrometer, Concept 40, from Novo Control, Germany. The impedance relaxation peak was from about 104 Hz to 107 Hz along the frequency range from 10-1 to 2×107 Hz and under temperature range of 10°C–40°C. The measured and calculated parameters were the impedance, relaxation time, DC conductivity, enthalpy change, and activation energy [24–28].

Statistical analyses

The obtained data were expressed as the mean±SE, and the statistical software SAS 9.1.3 (SAS Institute Inc, USA) was utilized for data analysis. Statistical significance was determined using the data had been analyzed using one-way analysis of variance (ANOVA) followed by Duncan's post hoc for comparison between different groups. The values had been expressed as mean ± SE and the differences had been considered significant at P less than 0.05.

Figure 1



Gene expression levels of 5α-R1, mdr1b and LHR genes in brain, liver and testes tissues, respectively, of male rats that had been treated with ashwagandha seed extract against Amoxicillin.

Results

Gene expression

The results of 5α-R1, mdr1b, and LHR gene expressions are summarized in Fig. 1. In rats treated with AM, the expressions of the genes mentioned in the testes, liver, and brain tissues were verified using Real-time PCR. Aside from, the influence of different doses of ASE as a therapeutic or protective agent on the expression levels of 5α-R1, mdr1b, and LHR genes has been identified. The expressions of the mentioned three genes were successfully revealed in tissues of three organs within the treated groups and normalized with the housekeeping β-actin gene expression.

The findings of Real-time PCR demonstrated that in AM-intoxicated rats, LHR (in testes tissues), mdr1b (in liver tissue), and 5α-R1 (in brain tissue) were significantly over-expressed in comparison to the normal control group.

In contrast, AM-intoxicated rats treated with different doses of ASE as a protective or therapeutic agent exhibited a significant reduction in the expression of these genes induced by AM alone. The LHR, mdr1b, and 5α-R1 gene expression levels were significantly improved by increasing the ASE dose levels. These suppressions of gene expressions were more pronounced when ASE was used as a therapeutic agent, resulting in better outcomes than when ASE was used as a protection agent. In addition, the highest dose of ASE (300 mg/kg) produced the best results when compared with the low (100 mg/kg) and medium (200 mg/kg) doses. Since the highest dose caused a further reduction in the up-regulation of gene expression levels induced by AM treatment alone, the expression rates, particularly of the 5α-R1 and mdr1b genes, were found to be relatively recovered to normal levels in comparison to normal controls. Using ASE as a protective or therapeutic agent against AM toxicity resulted in approximately comparable expression levels of the 5α-R1 gene, as determined by Real-time PCR.

Biochemical results

The levels of TAC in the brain, liver, and testes tissues of AM-intoxicated rats that were treated with ASE as a protective or therapeutic agent are shown in Table 2. The present results markedly recorded a significant reduction in levels of TAC in the brain ($P<0.01$), liver ($P<0.01$), and testes ($P<0.05$) cells in AM-intoxicated rats regarding those revealed in the untreated control group. Contrarily, in brain cells, levels of TAC in the AM-intoxicated rats treated with ASE as a protective or therapeutic agent were observed to be significantly elevated compared with those found in AM-intoxicated rats alone. These elevations of TAC were shown to be increased by increasing the dose level of ASE. The best results were obtained using the highest ASE dose (300 mg/kg). The use of ASE as a

Table 2 Protective and remedial efficacies of Ashwagandha against Amoxicillin –induced total antioxidant capacity (TAC) changes in male rats

Group	Brain (μmol/g.)	Liver (μmol/g.)	Testes (μmol/g.)
Control (G1)	0.92^{a}±0.03	1.30^{ab}±0.03	0.79^{a}±0.05
AM (G2)	0.68^{c}±0.05	1.09^{d}±0.03	0.59^{b}±0.03
AM+ASE.L (G3)	0.85^{ab}±0.02	1.25^{bc}±0.03	0.71^{a}±0.02
AM+ASE.M (G4)	0.89^{a}±0.02	1.36^{a}±0.02	0.76^{a}±0.06
AM+ASE.H (G5)	0.91^{a}±0.023	1.34^{ab}±0.02	0.78^{a}±0.02
AM then ASE.L (G6)	0.80^{b}±0.012	1.15^{cd} ± 0.05	0.72^{a}±0.04
AM then ASE.M (G7)	0.85^{ab}±0.02	1.19^{c}±0.02	0.75^{a}±0.04
AM then ASE.H (G8)	0.85^{ab}±0.02	1.24^{bc}±0.03	0.78^{a}±0.03

*Data had been expressed as mean±SE. Means with different letters a, b, c, d were significant different ($P < 0.05$). AM, Amoxicillin; ASE. L: Low dose of Ashwagandha (100 mg/kg). ASE.M: Medium dose of Ashwagandha (200 mg/kg). ASE.H: High dose of Ashwagandha (300 mg/kg).

protective agent produced better results than its use as a therapeutic agent. In liver cells, the levels of TAC were also significantly increased in AM-intoxicated rats that received ASE as a protective agent or therapeutic agent. The use of ASE as a protective agent gave the best results, and the treatment with medium (200 mg/kg) or high (300 mg/kg) doses of ASE produced comparable results, while their results were higher than those recorded of treatment with low dose (100 mg/kg). Furthermore, the levels of TAC in AM-intoxicated rats that gavage ASE as a therapeutic agent were shown to be increased by increasing the dose levels of ASE, and the best results were observed by using the high dose (300 mg/kg). In testes cells, the obtained results clarified that the TAC values in AM-intoxicated rats treated with ASE as a protective or therapeutic agent were significantly upraised compared with AM-intoxicated rats alone. These elevations in TAC were demonstrated to be elevated by increasing the dose levels of ASE, and the highest dose (300 mg/kg) resulted in the best findings. Interestingly, the present data showed approximately identical results of TAC levels by using ASE as a protective agent or its use as a therapeutic agent against AM toxicity.

Brain histopathology

Figure 2a and b showed the effect of AM treatment with a toxic dose (90 mg/kg, b.wt.) on the cerebral cortex compared with control and the impact of using Ashwagandha extract as a protective or therapeutic agent to reduce the toxicity syndromes induced by AM. It was observed that AM led to a severe cerebral hemorrhage and neurodegeneration. The protected groups with 100 mg/kg and 200 mg/kg of ASE demonstrated a hemorrhage in the cerebral cortex and congestion of blood vessels in the hippocampus region, as well as mild vacuolar degeneration of hippocampal cells, which play a role in memory and learning. At 300 mg/kg of ASE, hemorrhage in the cerebral cortex and blood vessels was less than that in lower doses, with mild degeneration of nerve cells and Purkinje cells responsible for well-coordinated movement, emotion, and cognition. In contrast, a therapeutic ASE showed less hemorrhage at all doses, especially at 200 mg/kg of ASE, where this group showed less hemorrhage and apparent normal cerebellum with intact Purkinje cells. Nevertheless, the cerebral cortex of therapeutic groups was still displaying vascular congestion, nerve fiber demyelination, and mild neurodegeneration.

Biophysical parameters

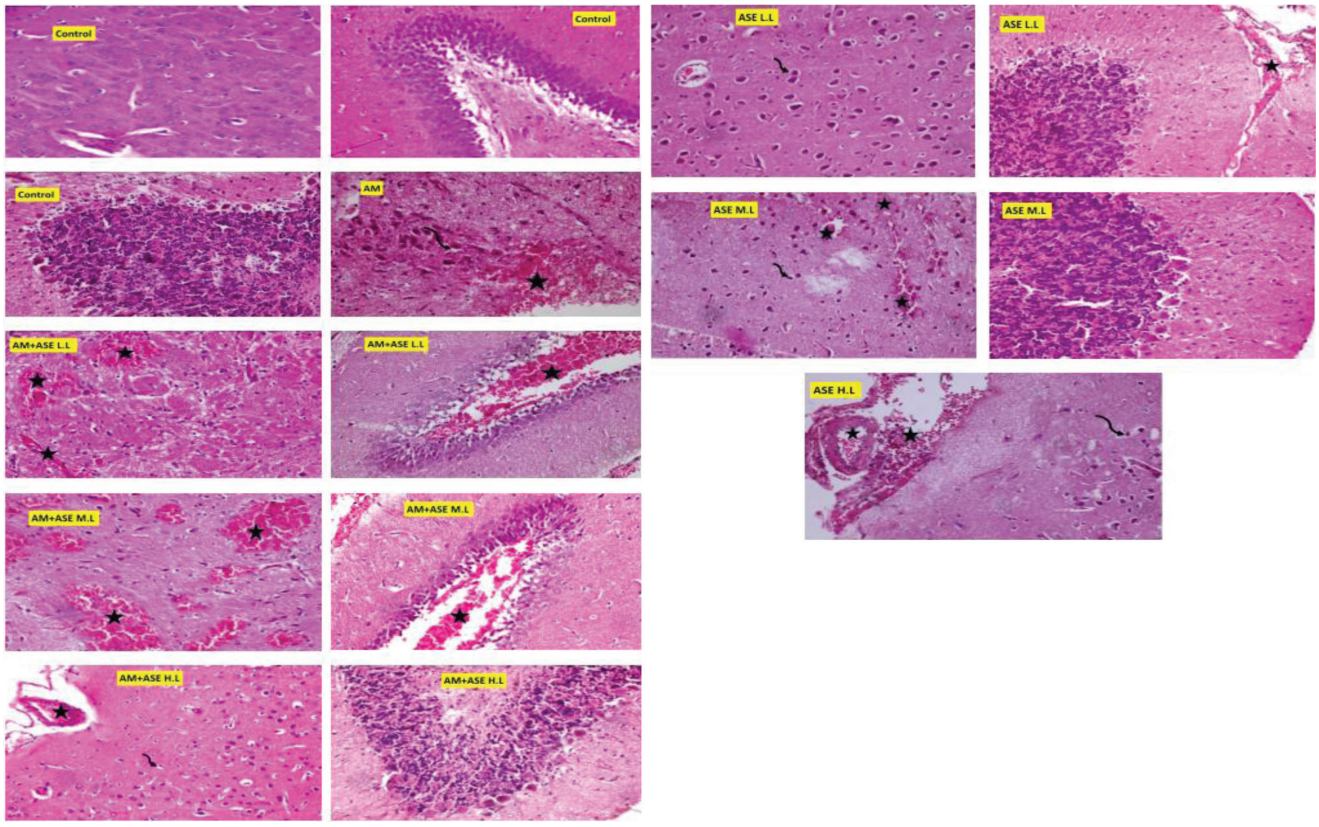
The impedance of brain tissue

Figure 3 demonstrates the impedance relaxation peak was from about 104 Hz to 107 Hz along the frequency range. The impedance peak was investigated in a temperature range from 10 to 40°C to provide a data platform from which results are extracted and calculated. The lower section describes the fitting of real and imaginary impedance components of brain tissue at 40°C, using the Havrilliak-Negami equation for one relaxation peak, HN 1 P. HN is the standard equation for fitting dielectric data to extract dielectric parameters. The activation energy of the relaxation process is then computed, and the change in enthalpy is deduced from dielectric results.

Relaxation time

The AM treatment suppressed the relaxation process to consume about double or more the usual time. Along with AM, protective ASE doses were administered (Fig. 4a). Lower doses of ASE produced better results than the other two doses, as the relaxation time was closer to that of the control group. Seven days after ceasing AM treatment, therapeutic doses of ASE were administered (Fig. 4b). Both the moderate and high doses of ASE were superior to the low dose; their relaxation time was comparable to that of the control, but also shorter.

Figure 2



(A) Sections of cerebral cortex for control and protective groups, (H&E X200). AM: Amoxicillin. AM+ASE L.L: Low dose of protective ASE (100mg/kg). AM+ASE M.L: Medium dose of protective ASE (200mg/kg). AM+ASE H.L: High dose of protective ASE (300mg/kg). The control group showed a normal structure of the cerebral cortex with normal neurons (arrow), hippocampus cells, and Purkinje cells, respectively. The amoxicillin group (AM) exposed severe hemorrhage in the cerebral cortex (star) with prineuoral edema and degeneration of neurons with pyknotic nucleus (curved arrow). The protective group with 100mg/kg of ASE (AM+ASE L.L) showed hemorrhagic patches in the cerebral cortex (star), nerve fibers demyelination, vacuolar degeneration of neurons (curved arrow), and hemorrhage in the hippocampus region (star) with vacuolar degeneration of its cells. The protective group with 200mg/kg of ASE (AM+ASE M.L) revealed hemorrhagic patches in the cerebral cortex (star) with vacuolar degeneration of neurons (curved arrow), neurodegeneration, and hemorrhage in the hippocampus region with vacuolar degeneration of its cells, The protective group with 300mg/kg of ASE (AM+ASE H.L) showed congestion of blood vessels (star), vacuolar neurodegeneration in the cerebral cortex (curved arrow), and degeneration of Purkinje cells in the cerebellum. (B) Sections of cerebral cortex for therapeutic groups, (H&E X200). AM: Amoxicillin. ASE L.L: Low dose of therapeutic ASE (100mg/kg). ASE M.L: Medium dose of therapeutic ASE (200mg/kg). ASE H.L: High dose of therapeutic ASE (300mg/kg). The therapeutic group, with 100 mg/kg of ASE (ASE L.L) displayed neuronal degeneration, vacuolization with pyknotic nucleus (curved arrow), mild vascular congestion, and moderate degeneration of Purkinje cells, the therapeutic group with 200mg/kg of ASE (ASE M.L) showed moderate hemorrhage in the cerebral cortex, mild vacuolar degeneration of neurons, and apparent normal cerebellum with intact Purkinje cells. The therapeutic group with 300mg/kg of ASE (ASE H.L) exposed submeningeal hemorrhages, congestion of blood vessels (star), neuronal degeneration (curved arrow), and nerve fibers demyelination.

DC conductivity

AM decreased the DC conductivity of brain tissue (Fig. 5a). However, the lower protective dose of ASE attained the nearest position toward the control value at 35°C, 37°C, and 40°C, followed by the high dose. Therapeutic doses of ASE could not retain DC conductivity to the control level (Fig. 5b). At physiological temperatures, only the highest dose of ASE caused the DC conductivity to approach the control level.

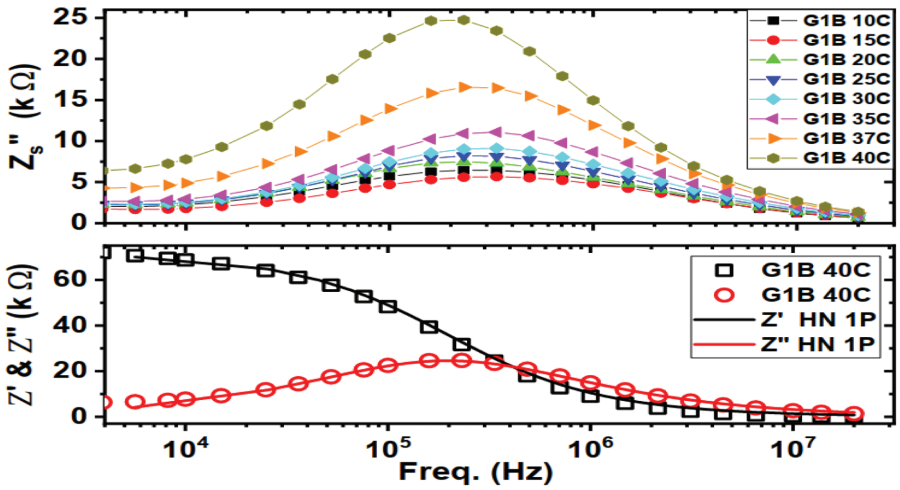
Enthalpy change

In enthalpy change (Fig. 6a), AM dose alleviated the enthalpy at physiological temperatures. ASE protective doses were able to reverse the AM effect. The intermediate and high doses of therapeutic ASE increased the enthalpy at physiological temperatures (Fig. 6b). The lower dose decreased the enthalpy severely, about five folds of the AM level.

Activation energy

AM in G2, caused a slight elevation of activation energy (Fig. 7) of the investigated process. Protective doses of ASE taken with AM of G3, G4, and G5 demonstrated good results as they all decreased the activation energy below the control level. The lower dose had the most effective effect. Therapeutic doses of ASE in G6, G7, and G8, could reduce activation energy as well, except for the lower dose, which increased activation energy significantly to about five

Figure 3



Displays impedance relaxation of brain tissues, upper part, and fitting of the complex relaxation (z', z'), lower part, where G1 is the control group.

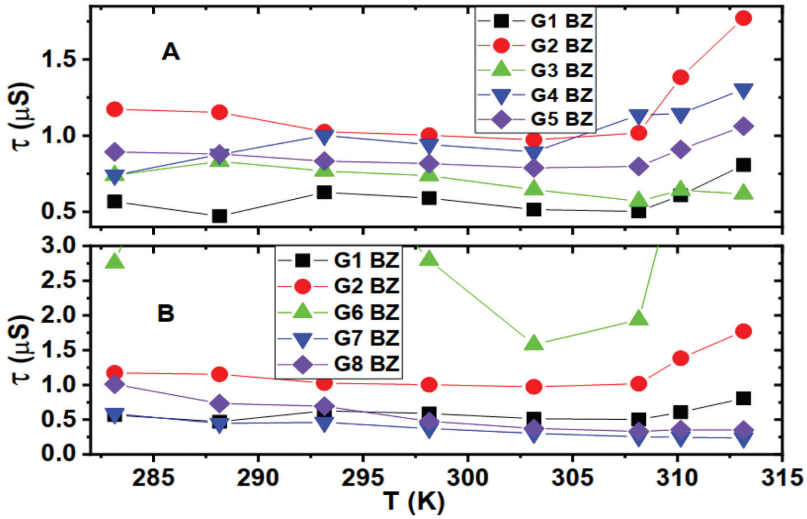
or more folds. The lower dose result of ASE requires more investigations to explain the result.

Discussion

Gene expression analysis

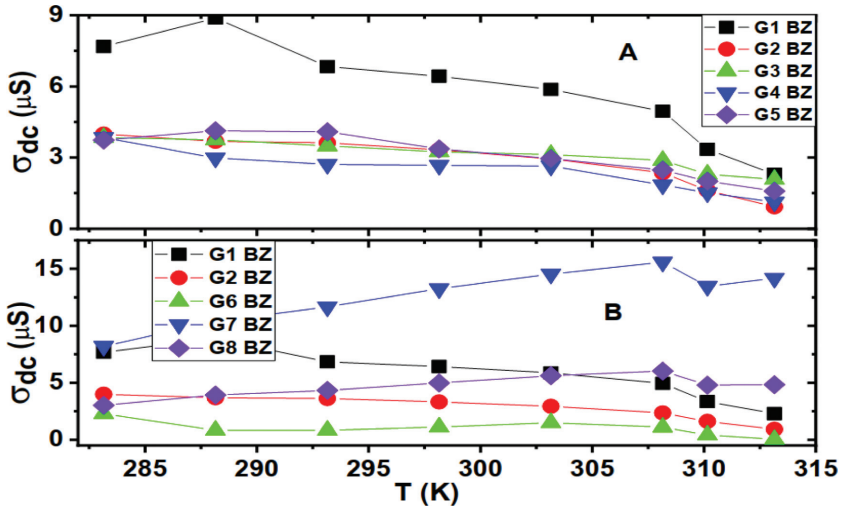
In this study, we examined three genes: LHR, mdr1b, and 5α-R1. These genes respectively regulate testicular, liver, and brain cell functions. LHR gene expression was predominantly found in testicular tissues [31,33,39] and plays a pivotal role in mammalian reproductive processes [39–41]. It accelerates testosterone production in Leydig cells, influences Sertoli cell proliferation and maturation, and is crucial for spermatogenesis [39,42–45]. Mdr1b mRNA was detected in liver tissue [35,46], encoding P-glycoprotein that confers drug resistance [35,47]. It facilitates xenobiotic transport and bile component absorption [35,46,47]. The 5α-R1 gene predominantly expresses in the brain, influencing sexual differentiation and protecting against neurotoxicity [34,48,49]. Regarding the significance of three genes in the functional processes of brain, liver, and testes tissues, a number of studies revealed that their expressions were influenced by exposure to various chemical substances (including antibiotics) and pathophysiological conditions [35,50–52]. We investigated the effect of AM treatment on their gene expression. AM-intoxicated rats displayed significant up-regulation of LHR, mdr1b, and 5α-R1 gene expression [53]. Other antibiotics like cefazolin and gentamicin also induced gene over-expression [50]. Conversely, AM treated mice exhibited down-regulation of genes linked to

Figure 4



Shows relaxation time of protective groups, A, and therapeutic groups, B.

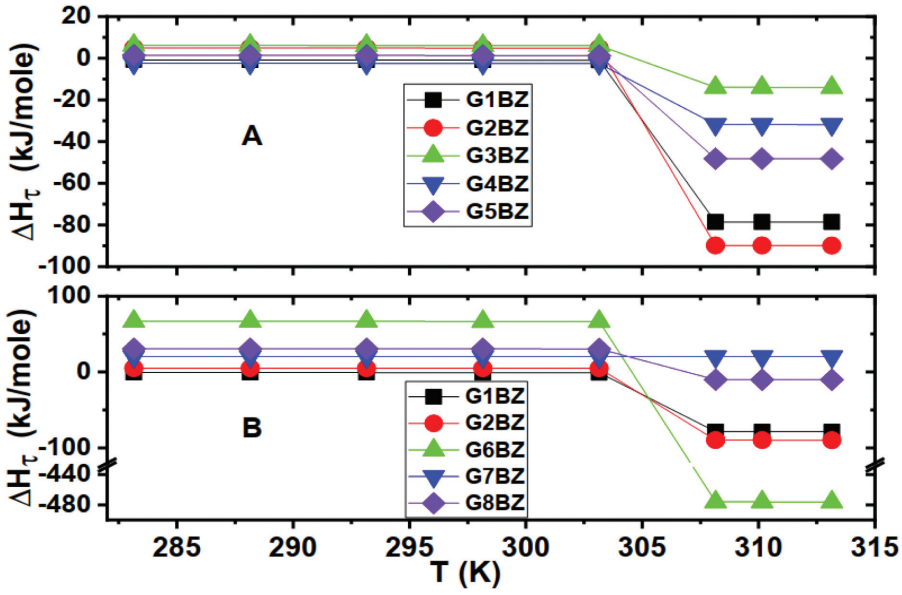
Figure 5



Pageants dc conductivity of protective groups, A, and therapeutic groups, B.

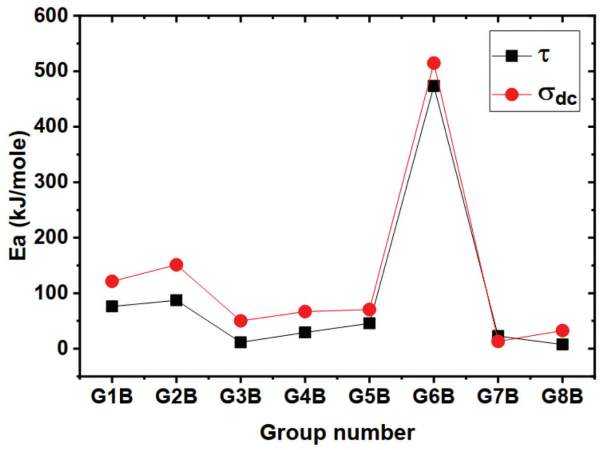
spermatotoxicity [53], while AM-exposed human cells displayed reduced expressions associated with cardiovascular disease [3]. AM's biomechanical action involves reactive radicals causing oxidative stress [3,50,51]. Our study delved into the ameliorative effects of ASE treatment on gene expressions. ASE significantly reduced AM-induced expression up-regulation of LHR, mdr1b, and 5α-R1 genes [11,15]. Similar findings were observed in mice exposed to scopolamine [15]. ASE's antioxidant properties mitigated expressions associated with hepatic encephalopathy [11] and aging [52], while also enhancing antioxidant capacity and sexual function in rats [14,53]. ASE's mode of action involves antioxidant components, which enhancing antioxidant enzyme activity, scavenging free radicals, and countering toxicant structural constituents [11,15,52]. Kazemi *et al.* [54] found over-expression with significant in HO-1 and GADD45B genes of BPA (bisphenol A)-intoxicated rats causing hepatotoxicity. Aboelhassan et al., [55] confirmed significant up-regulation of IKB α, mdr1a, and COX-1 gene expressions in BPA-intoxicated rats in different tissues of liver and kidney organs, causing histopathological cases. Additionally, Aboelhassan et al., [56] reported that overexpression of P450 aromatose and Tph2 and COX-1 genes in various tissues of the brain and testes organs caused

Figure 6



Highlights enthalpy change of protective groups, A, and therapeutic groups, B.

Figure 7



Displays activation energy of all groups.

extensive damage to their histological architectures. In addition, a significant increase in mdr1b mRNA was detected in the liver, brain, heart, and colon of mice treated with dexamethasone (a chemical belonging to the class of microsomal enzyme inducers) compared to controls [57]. Adesanoye et al. [58] reported evidence that the deleterious effect of AM treatment might be due to its β- lactam ring could interact and attack the protein membrane and enzyme thiol groups. Therefore, according to the aforementioned deleterious events of AM treatment, these events might be the primary agents for inducing the impairment of cellular functions, including gene expression changes for numerous genes..

Biochemistry

In this study, rats treated with AM demonstrated a significant decrease in TAC. Consistent with our findings, Atli *et al.* [1] reported a significant decrease in TAC in the brain tissue of AM-intoxicated rats due to a significant decrease in SOD and CAT as well as an increase in MDA as compared with the normal control group. In addition, Omodamiro, [2] and Paudel *et al.* [4] reported in the liver tissue of rats that were treated with AM significant increases in ALT and AST levels compared with untreated controls. By elevating the indicators of oxidative products (ALT and AST) and, as a result, decreasing the ratio of antioxidant products, these results are regarded as a strong marker for the reduction of TAC. Moreover, Karaman *et al.* [53] demonstrated a significant decrease of TAC in the testes tissues of AM-intoxicated mice with respect to normal control since the treatment with AM led to a significant decrease in antioxidant products such as catalase (CAT), glutathione reductase (GR), glucose-6-phosphate dehydrogenase (G6PD), and glutathione S-transferase (GST) and strikingly significant increase in oxidative product such as H_2O_2 level. In the present study, the treatment with ASE (as a protective or therapeutic agent) in AM-intoxicated rats caused a significant elevation of TAC compared with AM-intoxicated rats alone. Our findings are supported by Salamaa *et al.* [16], who reported a significant TAC improvement in pilocarpine-intoxicated rats treated with ASE compared with pilocarpine-intoxicated rats alone. Furthermore, Khalil *et al.* [11] confirmed that pretreatment of ASE in thioacetamide (TAA)-intoxicated rats resulted in amelioration of TAC by inducing a significant increase of GSH, NrF2, and H0-1 levels and significant reduction of MDA, GS, and iNOS, since these events showed upraise of the ratio of antioxidant products and reduction of the percentage of oxidative products. In addition, Priyanka *et al.* [29] found that in horses that were exposed to various oxidative stresses and fed ASE, significant minimization of oxidative products such as ALT and AST and significant enhancement of antioxidant factors such as GSH and SOD, leading to increase of TAC values as compared with control. Pradhan *et al.* [59] observed that HEK293 cells were injected with the H_2O_2 component and treated with ASE, enhancement of TAC through the significant improvement of expressions of FOXO3A and SIRT3 genes in comparison with cells injected with H_2O_2 alone. These ameliorations of Ali, [14] detected significant amelioration of TAC in sodium valproate (SV)-intoxicated rats that were treated with ASE by the presence of significant increases of SOD and GST, while significant decreases of AST and such gene expressions are responsible for increasing antioxidant capacity by elevating SOD and reducing H_2O_2 levels, as well as increasing the rates of antioxidant enzymes that have properties for scavenging the different species of free radicals. ALT were observed as compared with SV-intoxicated rats alone. As mentioned reported, the ASE is an abundant antioxidant component that was identified for having important properties *in vivo* via the increase and improvement of antioxidant products and minimization of oxidative products by modulating GSH, SOD, CAT, MDA, ALT, and AST equivalent [14,60].

Brain histopathology

Adverse effects of AM treatment were evidently apparent in brain histopathology, where it caused neurodegeneration and severe hemorrhage in the cerebral cortex, which can cause communication difficulties such as walking, speech, and cognitive

and vision problems as shown by Paudel *et al.* [4]. This mostly returns to the oxidative stress induced by AM treatment, leading to cellular damage, where oxidative stress is considered the main reason for neurotoxicity, whether neurodegenerative or neurobehavioral disorders [1,5]. The study of Atli *et al.* [1] revealed that AM treatment (25 and 50 mg/kg) induced oxidative stress and reduced SOD and CAT activities, in addition to a depletion of serotonin levels causing depression and anxiety.

The role of ashwagandha in alleviating oxidative stress is known as it contains potent antioxidants such as alkaloids, lactones, polyphenols, and flavonoids which scavenge and neutralize the free radicals [14,15]. In brain tissue, a slight improvement in tissue damage was noticed for both cases of protective and therapeutic ASE. Nevertheless, the effect of protective ASE at the highest dose (300 mg/kg) and therapeutic ASE at 200 mg/kg of ASE on the cerebral cortex was more pronounced in reducing the hemorrhage and repair of Purkinje cells.

The enhancement in therapeutic groups may return to the prevention of AM treatment, which means extended time for the biological system to create more antioxidants and coagulation factors for repairing cell damage and stopping hemorrhage. However, the brain tissue still needed more time in both cases of treatment to be able to recover the considerable damage caused by AM treatment, so it is recommended that the period of ASE treatment becomes more prolonged than that used in this work.

Biophysical parameters

The biophysical parameters explored in this study focused on the effectiveness of ASE as a protective supplement and its interaction with brain tissue in the presence of AM exposure. The observed trends in provide valuable insights into the mechanisms underlying the impact of ASE on brain tissue [61]. The usage of ASE as a protective supplement at low doses yielded promising outcomes, maintaining relaxation times at physiological temperatures [62]. This implies that ASE could counteract the impact of AM by preventing or mitigating its effects on relaxation. Interestingly, when ASE was introduced after stopping AM as therapeutic suplement, higher doses were required to sustain relaxation times. However, this approach displayed a unique advantage-relaxation times were lowered to levels below or equal to the control, suggesting that ASE not only neutralized AM effects but also aided in the brain recovery process [63]. This dual effect could be attributed to ASE role in both discontinuing AM actions and supporting brain tissue's recuperation. Regarding DC conductivity, the interactions between ASE and cells seemed to influence conductivity negatively, possibly impeding charge transfer [64]. Protective ASE doses, however, did not have a significant impact on brain tissue's DC conductivity in the presence of AM. In the context of therapeutic usage, higher ASE doses were needed to approach control levels at physiological temperatures. These findings suggest that the protective effects of ASE might not extend to improving brain tissue's conductivity significantly, especially when AM is present. The examinations of enthalpy change due to AM exposure revealed that AM-induced heat loss and energy consumption in brain tissue were mitigated by protective ASE doses. This implies that ASE helped the biosystem maintain equilibrium by counteracting AM's effects. Notably, an inverse relationship between ASE therapeutic doses and enthalpy change was observed, indicating that ASE might have a more pronounced effect in the absence of AM. This unexpected decrease in enthalpy with lower ASE therapeutic doses merits further investigation, potentially indicating a unique interaction mechanism between ASE and brain tissue. The study's findings related to ASE's effect on activation energy aligned with its behavior in terms of enthalpy and supported the notion of an independent influence of ASE on tissue repair. This independent effect could involve ASE aiding in repairing AM-induced tissue damage through mechanisms that modulate the impedance relaxation process. The biophysical parameters investigated in this study provide valuable insights into the complex interaction between ASE and brain tissue in the presence of AM exposure. ASE exhibits multifaceted effects, including neutralizing AM's impact, aiding in tissue recovery, and potentially influencing tissue repair mechanisms. Further research is warranted to unravel the precise mechanisms through which ASE exerts its effects on brain tissue and to determine the optimal dosages for therapeutic and protective applications.

Conclusion

This study provided biological and physical evidence for the protective and remedial efficacious of ASE against the lesion-causing effects of AM in various rat organs. The amelioration of gene expression was more pronounced in the therapeutic efficacy of ASE, whereas the TAC improvement was more pronounced in the protective efficacy of this medicinal plant extract, particularly in brain and liver tissues. In brain

histopathology, AM caused extensive damage to the cerebral cortex, including hemorrhage and neurodegeneration, while the protective and therapeutic ASE treatment partially reduced these damages. However, this improvement was noticed more at 300 mg/kg of protective ASE and 200 mg/kg of therapeutic ASE. The biophysical investigation revealed that brain tissue could retain some physiological functions before complete tissue repair, while others, such as enthalpy, were enhanced. However, the use of DC conductivity by ASE did not restore all physiological processes. The diversity of physical parameters that could be measured and deduced from dielectric measurements demonstrates the vast potential of their future application, and the difficulty in designing future experiments that accurately relate each parameter to its corresponding physiological process.

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The authors have no relevant financial or non-financial interests to disclose.

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