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Neuroprotective Activity of Strawberry Tree (*Arbutus unedo* L.) against Formaldehyde-Induced Oxidative Stress in The Rat Hippocampus

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Abstract: The neurotoxic effects of formaldehyde (FA) on the hippocampus are well documented, highlighting its negative impact on the nervous system. Arbutus Unedo L. (AUL), which is rich in antioxidants, represents a potential countermeasure. The aim of our study was to investigate the immunohistochemical and biochemical effects of AUE fruit extract (AUE) on apoptosis and oxidative stress induced by FA exposure in rat hippocampus. Rats were divided into four groups: experimental (EG) received 20 mg/kg AUE extract by oral gavage and 10 ppm FA inhalation; formaldehyde (FG) received 10 ppm FA inhalation only; sham (SG) received 10 mg/kg AUE by oral gavage; and control (CG) underwent routine observation. Hippocampal tissue was isolated after blood sampling. Physical changes, such as yellowing of feathers and tail spots, were observed in FA-exposed groups. Biochemical analysis showed significant differences in brain-derived neurotrophic factor (BDNF), malondialdehyde (MDA), and nitric oxide (NO) levels between groups. AUE-treated groups had reduced MDA levels, suggesting a potential reduction in oxidative stress, along with lower apoptotic cell rates in caspase-3 assessments. FG rats had lower BDNF levels than CG. SG had the fewest apoptotic cells. These findings indicate AUE's potential to mitigate neuron damage and reduce oxidative stress.

Keywords: apoptosis; arbutus unedo L; formaldehyde; hippocampus; oxidative stress

1. Introduction

AUL is a shrub species that produces red fruits (Figure 1) and belongs to the Ericaceae family. The literature reports that AUL fruits contain numerous phenolic acid derivatives [1,2], as well as vitamins E and C and important minerals [3–6]. Therefore, it can be concluded that AUL fruits possess strong antioxidant properties.

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Figure 1. AUL fruits used in the experiment

Reactive oxygen species (ROS) are free radicals that originate from oxygen, while reactive nitrogen species (RNS) originate from nitrogen. The increase in ROS during metabolism disrupts the balance between antioxidants and oxidants. Endogenous antioxidants attempt to restore this balance by increasing their levels. However, if the amount of free radicals exceeds the capacity of antioxidants to balance them, oxidative stress occurs. Cell and tissue damage occurs when oxidative stress exceeds the tolerable level, which should be noted is a level that can be tolerated up to a certain extent [7].

Apoptosis is the process of eliminating cells that have completed their function or whose DNA has been damaged, without harming the surrounding cells and tissues. This process occurs throughout life. For instance, during embryonic development, the cells between the fingers and toes undergo programmed cell death, resulting in the separation of the digits. Similarly, the regression of the mammary gland after lactation and organ atrophy in old age are physiological examples of apoptosis. Cell deaths occurring in ischemic diseases, such as radiation, chemotherapy, hypoxia, and myocardial infarction (MI), are defined as pathological apoptosis [8–10]. Formaldehyde is a highly reactive chemical compound in the aldehyde group that causes oxidative stress.

The aim of this study was to investigate the positive effects of AUE containing antioxidant compounds in rat hippocampus induced oxidative stress and apotosis by FA, which is known to have negative effects on the nervous system.

2. Materials and Methods

2.1. Fruit Collection

Fruits of A.unedo L. were collected in November 2021 from Sakarya, Turkey. The plant was identified by Dr. Zuhal Sahin, an organic chemist at Sakarya University.

2.2. Extract Preparation

AUL fruits collected from the western black sea coast in season were dried and preserved by Freeze Drying method. AUL fruit was homogenised with methyl alcohol at 18000 rpm and extracted under room conditions. The filtrate was removed using a rotary evaporator after the extracts were filtered to dryness under vacuum in a Buhner funnel.

2.3. Determination of Phenolic Content

The HPLC (High Performance Liquid Chromatography) method was used to determine the analytical percentages of antioxidant phenolic compounds in the obtained AUE. The analysis was

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performed in triplicate using the Shimadzu LC-20A HPLC device. Table 1 shows the average values of gallic acid, epicatechin, catechin, and resveratrol obtained from the three repetitions.

Table 1. Analytical percentages of antioxidant compounds in A. Unedo L extract

	Gallic acid (mg/kg)	Catechin (mg/kg)	Epicatechin (mg/kg)	t-Resveratrol (mg/kg)
AUL-1	2.276,57	325,11	366,94	0,092
AUL-2	2.124,37	313,10	385,19	---
AUL-3	1.986,86	209,56	345,77	0,085
Mean	2.129,27	282,59	365,97	0,09

2.4. Experimental Design and Animal Treatments

The study used adult female Wistar rats (Experimental Medical Research and Application Center, Kocaeli University, Kocaeli, Turkey) weighing 250-300 g. The rats were housed in an animal colony at a density of approximately 8 to 9 per cage for 2 weeks prior to the experiments. Before starting the experiment, the rats' body weights were measured and recorded (239.13± 13.26 g). The study's experiments were conducted in compliance with the Regulation of Animal Research Ethics Committee in Turkey (July 6, 2006, Number 26220). The Kocaeli University Animal Research Ethics Committee granted ethical approval (Project number: HADYEK 2021/17, Kocaeli, Turkey).

The study randomly selected animals and divided them into four groups, each containing nine rats. The tails of the rats in each group were painted different colours. Group I was designated as the Control Group (CG), Group II as the Experimental Group (EG), Group III as the Formaldehyde Group (FAG), and Group IV as the Sham Group (SG). The experimental period lasted for five weeks, accounting for inhalation and gavage losses.

Nine rats were assigned to each of the following groups: CG, EG, SG, and FAG. The rats in the CG group were exposed to normal air, while the rats in the EG group were given 20mg of lyophilized A.Unedo L. extract (AUE) by oral gavage along with 10ppm FA for 4 hours daily, five days a week. The rats in the SG group were given 10mg of AU extract by oral gavage daily for 30 days. The rats in the FAG group were exposed to subacute FA (10 ppm FA) for 4 hours a day, five days a week throughout the experiment.

A glass experimental chamber was prepared, with dimensions of 100cm (length) x 70cm (width) x 35cm (height), following Matsuoka's methodology [11]. The chamber was divided into two compartments: the experimental chamber and FA evaporation, as shown in Figure 2.

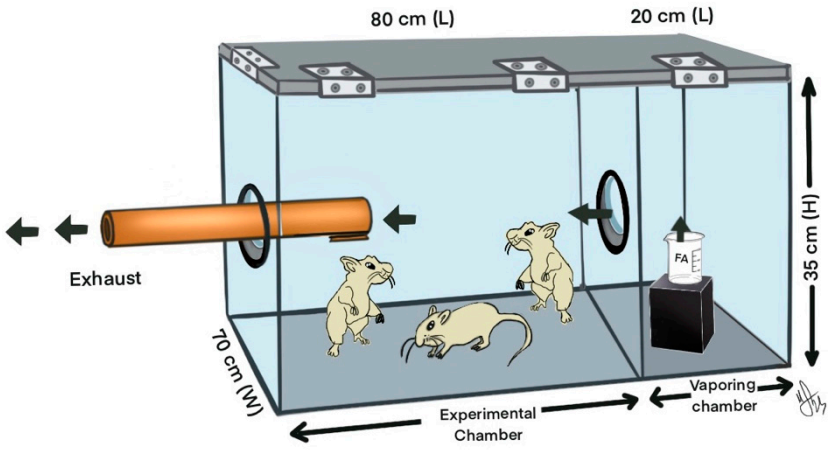

Figure 2. Illustration showing the experimental chamber system used for FA exposure (drawn by Mehtap ERDOGAN)

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Formaldehyde is passed through a small hole of 6 cm diameter between the two compartments. The reason for preferring the glass chamber is to be able to monitor the adverse situations that can develop in rats due to exposure to FA, which is highly toxic.

A Honeywell ToxiRAE PRO (HCHO) dosimeter was used to monitor levels of the volatile gas FA throughout the experiment, which lasted an average of 4 hours per day. When the level of FA in the environment fell below 10 ppm, the container of FA was replaced with a new one. To obtain reliable values, the dosimeter was calibrated each day before the start of the experiment.

The experiment was terminated at the end of the fifth week. At the end of the experiment, all rats were sacrificed after blood sampling under deep anaesthesia. Brain tissue from the sacrificed rats was removed using the isolation technique according to the procedure (Figure 3).

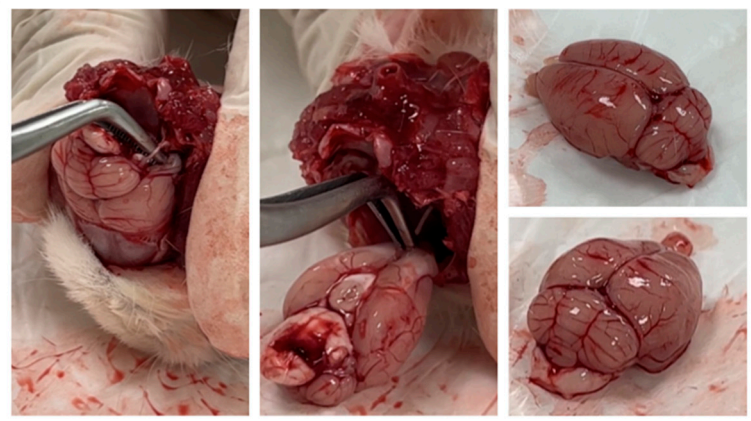

Figure 3. Rat brain isolation stages

2.5. Oxidative Stress Biomarkers

GSH, NO determination and BDNF and MDA analysis were performed on the blood samples obtained. 4 μm thick sections were cut from brain tissue embedded in paraffin blocks. Immunohistochemical labelling with caspase-3 antibody was performed to detect apoptosis in the obtained hippocampal sections. H-score analysis was performed using the cell numbers obtained from caspase-3 labelling of the groups in the experiment.

2.6. Statistical Analysis

Data were analysed using SPSS 21 statistical software. One-way analysis of variance (ANOVA) was used to compare response groups. Statistical analysis was performed using the Kruskal-Wallis test to examine significant differences in mean histopathological lesion scores.

3. Results

3.1. Histopathological Findings of Hippocampus Tissue

In the hippocampus of rats that we induced oxidative stress and apoptosis with FA; neuronal cells of brain tissue were morphologically examined to show the effects of Arbutus Unedo L. extract. In anti-caspase-3 positive staining, differences were observed between the rate of apoptosis in the experimental and sham groups and the rate of apoptotic cells between the FA and control groups (Figure 4).

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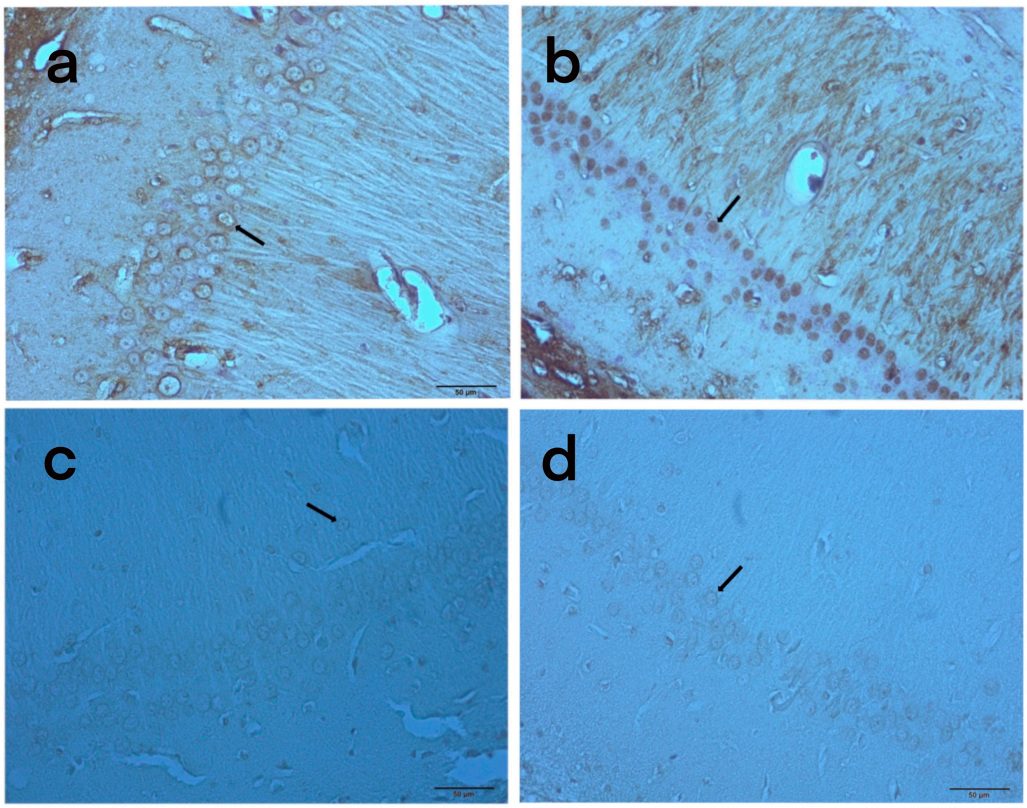

Figure 4. Representative light microscopy of the CA3 of the hippocampus from rats from the control, sham, experiment and formaldehyde groups. (40X) (Arrows are examples of immunopositive cells) [a.Experimental group; b.Formaldehyde group; c.Sham group; d.Control group].

In the immunohistochemical images obtained, apoptotic cells surrounded by shrunken membranes are visible in the hippocampal sections of FG and EG rats (Figure 4a, Figure 4b). The changes in the nuclear structure of these groups in the microscopic images confirmed apoptosis and oxidative stress. When the microscopic images of FG and EG rats were compared, it was observed that the cell shape with the specified characteristics was present in FG. According to the H-score values, the number of apoptotic cells is higher in FG than in DG, indicating that AUE has a neuroprotective effect (Figure 5).

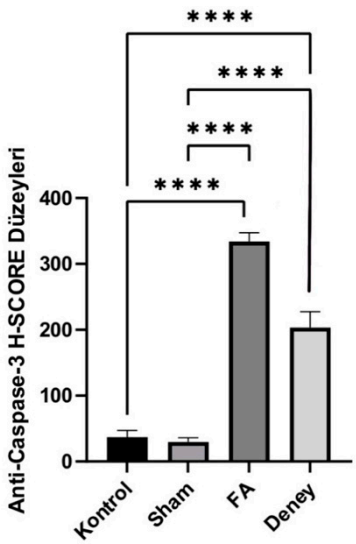

Figure 5. Anti-Caspase-3 H-Score Levels of the Groups (**** = p<0.0001)

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3.1. Biochemical Analysis Results

Serum BDNF, MDA, GSH and NO values were analysed in the biochemical analysis of rat sera.

In the comparison of MDA, BDNF, NO, GSH and H-score results according to the groups, a significant difference was found between the groups in MDA, NO and H-score ($p<0.05$, $p<0.05$ and $p<0.001$) (Table 2).

Table 2. Comparison of MDA, BDNF, NO, GSH and H-Score results by groups

	CG (n=9) mean ± SS	**EG (n=9) mean ± SS**	**FA (n=9) mean ± SS**	**Sham (n=9) mean. ± SS**	**p**
MDA	79,32 ± 27,48	84,24 ± 15,14	92,64 ± 34,88	71,55 ± 15,66	**p<0,05**
BDNF	4,43 ± 1,44	4,17 ± 1,37	3,51 ± 0,49	3,38 ± 1,27	p>0,05
NO	16,04 ± 5,42	12,62 ± 0,65	12,39 ± 0,95	12,83 ± 0,61	**p<0,05**
GSH	0,16 ± 0,01	0,15 ± 0,02	0,16 ± 0,03	0,16 ± 0,01	p>0,05
Hscore	37,11 ± 10,01	203,3 ± 24,08	334,22 ± 13,132	29,78 ± 6,52	**p<0,001**

In the pairwise group comparisons of MDA, NO and H-score values, no significant difference was found between the groups for BDNF and GSH ($p>0.05$, $p>0.05$) (Table 3).

Table 3. MDA, NO, H-score post-hoc pair group comparison tests.

	MDA	**NO**	**H-score**
Sham-Control	p>0,05	p>0,05	p>0,05
Sham-Experiment	**p<0,05**	p>0,05	**p<0,01**
Sham-FA	**p<0,05**	p>0,05	**p<0,001**
ControlExperiment	p>0,05	**p<0,05**	**p<0,01**
Control-FA	p>0,05	**p<0,01**	**p<0,001**
Experiment-FA	p>0,05	p>0,05	p>0,05

As a result of pairwise group comparison tests, the MDA values of the Sham group were found to be significantly lower than both the Experiment and FA groups ($p<0.05$, $p<0.05$). When the average MDA values of the groups are examined, the lowest MDA value is found in the sham group, while the highest MDA value is found in the FA group. The oxidative stress inducing effect of FA is clear. It was observed that the average MDA values tended to decrease in the sham and experimental groups to which A unedo L extract was applied. It was found that A unedo L extract applied to the experimental group tended to reduce the oxidative stress caused by FA, but this reduction was not significant. The MDA level of A unedo L extract applied to healthy rats was lower than the control group average, resulting in a significant difference in MDA level between the experimental and FA groups.

BDNF plays an important role in the plasticity, regeneration and memory functions of cells in the brain. On average, BDNF was lowest in the sham group and highest in the control group. However, there was no significant difference between the groups. ($p>0.05$) The lower average level of BDNF in the FA group compared to the control group may be related to the impairment of memory functions due to FA. It can be said that A undeo L extract partially corrects the negative effects of FA on BDNF. However, studies with larger samples are needed to confirm this.

When NO levels are examined, the group with the lowest average is the FA group and the highest is the control group. The NO levels of the experimental and FA groups were found to be significantly lower than those of the control group ($p<0.05$, $p<0.01$). However, as there is no significant difference in NO between the experimental and FA groups, it can be said that A undeo L extract does not contribute to the formaldehyde-induced decrease in NO levels.

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The group means for GSH were very close to each other and there was no significant difference between the groups (p>0.05). It can be said that GSH levels were not affected in rats in the FA environment and A undeo L extract did not have a regulatory effect on GSH levels.

When the H-scores, which express the number of apoptotic cells in the brain, were analysed, it was found that both the experimental and FA groups were significantly higher than the control and sham groups. The highest average number of dead cells in the FA group and the fact that both the sham and control groups contained significantly fewer dead cells than the experimental and FA groups suggest that formaldehyde damage to the hippocampus. Although the experimental group had a lower average number of dead cells than the FA group, this difference was not found to be significant. Although the average number of dead cells in the sham group was lower than in the control group, this difference was not found to be significant. However, it can be said that A undeo L extract tends to slow down cell death in the brain.

Correlation analysis revealed a strong positive correlation between MDA and H-score (p<0.001) (Table 4). The strong positive correlation between oxidative stress indicator MDA and H-score, which expresses cell death in brain tissue, shows that oxidative stress induces apoptosis in the brain. However, no significant difference was found between BDNF, NO and GSH and H-score. (p>0.05, p>0.05 and p>0.05)

Table 4. Correlation of MDA, BDNF, NO, GSH and H-score.

	H-Score
MDA	**KK: 0,558**
	p <0,001
BDNF	KK: -0,085
	p>0,05
NO	KK:-0,264
	p>0,05
GSH	KK: -0,079
	p>0,05

4. Discussion

Inhalation shows acute and chronic effects, particularly in the respiratory tract, eyes, CNS and skin [12]. FA can be found endogenously in living organisms as well as through environmental exposure [13].

Studies with FA have shown yellowing of the fur of rats exposed to inhaled FA [14] and movements such as avoiding left and right and closing in on each other during exposure [15]. In our study, in parallel with the literature, similar avoidance and yellowing of feathers, increased eye blinking and initial avoidance of movement were observed; slowing of movement in the following minutes was among the physical observations noted.

FA causes toxicological and carcinogenic effects by triggering the formation of reactive oxygen species [16]. A study has shown that FA, which has effects on many cellular pathways, slows motor activity by causing neurodegenerative changes in the CNS [17]. In our study, rats in groups exposed to FA; while motor movements were normal before exposure, the observation of slowed movements after exposure confirms this explanation.

In a study where MDA, an indicator of lipid peroxidation and oxidative stress, was used as a parameter, it was observed that the MDA level increased significantly in the FA exposed group [18]. Similarly, in our study the highest MDA level was found in the FA group. This can be interpreted as oxidative damage occurring most in the FA group.

It was observed that the MDA level tended to decrease in the experimental and sham groups where Arbutus Unedo L. extract, a powerful antioxidant, was applied. In this case, it can be explained that AUE is effective in balancing oxidative damage. The fact that the sham group had the lowest

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MDA value in the evaluation of all the groups can be interpreted as the fact that there is currently no factor affecting the balance between ROS and antioxidants and that AUE has positive effects on the changes that occur in the natural apoptosis process.

One study reported that oxidative stress in brain tissue increased in the group of rats exposed to FA and parallel DNA damage [19]. According to the results of our study, the highest oxidative damage was found in the FA group.

A study conducted in 2013 reported degenerations related to learning and memory and an increase in the number of apoptotic cells in the hippocampal region in rats exposed to FA [20]. In our study, a relationship was established between the apoptotic cell index and neuronal damage in the immunohistochemical evaluations of FG and DG due to FA exposure. Apoptotic cell indices in the CA3 region of the hippocampus were found to be significantly higher in FG and DG rats than in SG and CG.

In cells undergoing apoptosis, the cytoplasm begins to shrink and contract. With the degradation of some structural proteins, the nucleus begins to condense and usually the nuclear chromatin is displaced towards the inner side of the nuclear membrane, taking on a shape similar to a horseshoe or the letter C [21]. In the immunohistochemical images obtained in our study, apoptotic cells surrounded by shrunken membrane were visible in the hippocampal sections of FG and DG rats. The apoptotic changes in the nuclear structure of these groups in both microscopic imaging and H-score analysis confirmed the above statement. When the microscopic images of FG and DG rats were compared, it was observed that the cell shape with the specified characteristics was present in FG. According to the H-score values, the number of apoptotic cells is higher in FG than in DG, indicating that AUE has a neuroprotective effect.

There is a positive correlation between oxidative stress and GSH levels. If we look at the literature, animal studies show that when oxidative stress increases, so do GSH levels increase [22]. In contrast to the literature, in our study the mean values of the groups in terms of GSH levels were very close to each other and no significant difference was found between the groups. This can be interpreted as GSH levels are not affected in rats in the FA environment and A Undeo L extract does not have a regulatory effect on GSH levels.

5. Conclusions

According to the results of our study, there was a correlation between FA exposure and apoptosis, in line with the literature. A significant increase in the rate of apoptotic cells was observed in hippocampal tissues due to formaldehyde exposure compared to other groups.

Biochemical and histological data showed that cell death was reduced in the groups given Arbutus Unedo L extract compared to the groups not given the extract.

FA is a highly reactive free radical commonly used in cadaver embalming laboratories. According to literature data, occupational groups working with FA have negative effects on the nervous system, especially in groups with chronic exposure. We believe that a multi-faceted study of high antioxidant content, such as AUL, which can minimise the degenerative effects of FA on the hippocampus, which is involved in learning and memory, may help to prevent damage that may occur at the cellular level.

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Informed Consent Statement: Not applicable. Ethical review and approval were obtained from Kocaeli University Local Ethics Committee for Animal Experiments (KOU HADYEK) (protocol code: KOU HADYEK 7/2-2021, date of approval: [10.09.21]).

Data Availability Statement: The data used to support the findings of this study are included within the article.

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Conflicts of Interest: The authors declare no conflicts of interest.

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