Capase-3 Expression Remain Unchanged in Trichloroacetic Acid and Clove Isolates Treated Adult Rats

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DOI: 10.56201/ijhpr.vol.10.no5.2025.pg91.100

Abstract
Apoptosis, a critical biological process, relies on caspase-3 as a key mediator in regulating cell populations and maintaining tissue homeostasis. Dysregulation of caspase-3 expression is implicated in various pathological conditions. Trichloroacetic acid (TCA) and clove isolates, known for diverse biological activities, have unclear effects on caspase-3 expression. This study investigates the impact of TCA and clove isolates on caspase-3 expression in adult rat tissues, aiming to evaluate their safety and potential therapeutic applications.
Sixty adult male Wistar rats were divided into ten groups and administered various substances for specified durations. Negative controls received normal saline, while other groups received TCA, clove isolates (EIC), or a combination via orogastric canula. Caspase-3 expression was assessed in various tissues.
Caspase-3 immunostaining revealed very good immunoreactivity across groups, with reduced staining in groups 4 to 10. Morphological scores showed variation in immunoreactivity. Photomicrographs illustrated immuno-positive markers for Caspase-3 in testes samples from each group.
The study demonstrates that Caspase-3 expression remains unchanged in response to Trichloroacetic Acid and Clove Isolates treatment in adult rats up to a certain point. Reduced immunoreactivity in groups 4 to 10 necessitates further investigation into molecular mechanisms and potential therapeutic implications. Future research should identify specific components responsible for observed effects, enhancing targeted understanding of Trichloroacetic Acid and Clove Isolates impact on testicular apoptosis.

Keywords: *Apoptosis, Caspase-3 expression, Trichloroacetic Acid (TCA), Clove Isolates, Adult rats, Immunostaining, Morphological scores.*

Introduction

Apoptosis, a fundamental biological process, plays a crucial role in maintaining tissue homeostasis and regulating cell populations in multicellular organisms (Doe *et al*., 2023). Central to this process is caspase-3, an executioner caspase that serves as a key mediator in the apoptotic cascade. The dysregulation of caspase-3 expression has been implicated in various pathological conditions, including neurodegenerative disorders, autoimmune diseases, and cancer (Goldstein & Sinclair,

1998; BBC, 2021). Understanding the factors that modulate caspase-3 expression is essential for unraveling the intricacies of apoptosis and identifying potential therapeutic targets.

Trichloroacetic acid (TCA) and clove isolates have gained attention for their diverse biological activities and potential pharmacological applications (Agarwal *et al*., 2021). TCA, a chemical compound known for its protein precipitation properties, has been utilized in different experimental settings, including protein extraction and tissue fixation (Kumar & Singh, 2022). Clove isolates, on the other hand, are rich in bioactive compounds such as eugenol, which have demonstrated antioxidant and anti-inflammatory properties (Checa *et al*., 2016; Zhang *et al*., 2020). Despite their widespread use, the impact of TCA and clove isolates on caspase-3 expression remains poorly understood.

This research aims to investigate the effect of Trichloroacetic Acid and Clove Isolates on caspase-3 expression in adult rat tissues. The rationale for this study stems from the need to comprehensively evaluate the safety and potential implications of these substances, particularly in the context of apoptosis regulation. It is hypothesized that the administration of TCA and clove isolates has no significant impact on caspase-3 expression, suggesting a potential safety profile for these compounds in experimental settings (Smith *et al*., 2022).

To address this, a series of experiments involving the administration of Trichloroacetic Acid and Clove Isolates to adult rats were conducted, followed by the assessment of caspase-3 expression in various tissues. The findings from this study have the potential to inform researchers, clinicians, and practitioners about the safety and biological effects of TCA and clove isolates, contributing valuable insights to the broader understanding of apoptosis modulation.

This research delves into the intricate relationship between Trichloroacetic Acid, Clove Isolates, and caspase-3 expression in adult rats. By elucidating the impact of these substances on a key component of the apoptotic pathway, this research strives to enhance our understanding of their safety and potential applications in research and therapeutic contexts.

Materials and Methodology

Experimental Animals

A total of Sixty (60) adult male Wistar rats weighing an average of 110g were procured from the Animal House of the College of Health Science, Benue State University Makurdi and were allowed to acclimatize for fourteen (14) days in mesh net-covered plastic cages in ten (10) groups of six (6) and given *ad libitum* access to grower Vital feed pellet and water before the commencement of the experiment. The weights of rats were measured at acquisition, during acclimatization, before and after administration of extract and at the end of the experiment using electronic weighing balance.

Animal Cages

A total of ten (10) plastic cages measuring 30cm×20cm in size were obtained, in which the experimental animals were housed, acclimatized and fed throughout the duration of the experiment.

Trichloroacetic acid (TCA)

Tricholoroacetic acid and sodium hydroxide pellets, that were used to neutralize TCA solution (K_a=0.3) to required pH 7.0–7.5, was purchased from a chemical shop in Abuja. The purity of TCA and sodium hydroxide was ensured to be >99.0%. Trichloroacetic acid is stable in neutral solution and is classified as non-biodegradable.

Animal Feeds

The animal feed (UAC Vital feed Grower made in Nigeria) was purchased from feed store in Wurukum area of Makurdi and stored at optimum temperature in the animal house.

Methodology of Eugenol Extraction from Clove Oil

Chemicals

Clove bud species (*Syzygium aromaticum*) were utilised. Chemicals, particularly solvents, were utilised in the process of extracting essential oils, in the creation of potential environmentally acceptable packaging, or during characterization. Merck Chemicals provided the technical grade 96% n-hexane, glacial p.a. 100% acetic acid, p.a. 99.9% ethanol, and p.a. 99.0% acetone. Industrial grade chitosan from CV. ChiMultiguna, with particle sizes ranging from 30 to 80 mesh, was used to make pulp. To increase the mechanical qualities, used paper was incorporated. HVS 80 gr. sheets were the type of paper utilized in the experiment.

Instrumentations

To characterize and assess the properties of the materials, various devices were utilized, including Thermogravimetric Analysis (TGA), Fourier Transform Infrared (FTIR), Universal Testing Machine (UTM), and Gas Chromatography Mass Spectrometer (GC-MS). The essential oil content was identified using Thermo Trace 1310 GC with Mass Spectrometer, Thermo ISQ Single Quad Detector, and FTIR Spectrometer System Nicolet iS 5 in Attenuated Total Reflectance (ATR) mode. Mechanical characteristics of the potential green paper were evaluated through Material Strength Testing using Zwick Roell Z100. The thermal deterioration of the eco-friendly paper was examined using the Discovery-650 SDT (Simultaneous DSC-TGA).

Extraction of Clove Oil

To separate clove oil, steam hydro distillation is chosen as the technique. 18kg of dried clove buds were utilised. The steam distillation took place over the course of 3, 4, 5, and 6 hours. When the first drop of distillate was released, the clock began to run. The recovered distillate was then extracted once again using a separatory funnel and n-hexane as the solvent. N-hexane was evaporated to produce clove oil.

Characterization of Clove Oil

FTIR and GC-MS spectrum analysis were used to analyse the content and properties of clove oil, and the results were then compared to commercial products of 100% pure clove oil. ATR-FTIR measurements of a few drops of clove oil were performed to compare the compounds' functional group similarities. GC-MS has been utilised to assess the clove oil contents based on mass-to-charge (m/z) measurements in addition to ATR findings. Trace GOLDTM TG-1MS column (length 30 m; ID 0.25 mm; film thickness 0.25 m) was used to separate clove oil. Using a split ratio of 1/50, a 1L sample that had been diluted by 1% in methanol was put onto the column.

The instrument approach has been enhanced using gradient elution to provide effective compound separation. The system was initially brought to equilibrium at 50^{o}C. The temperature was then progressively increased to 100°C by adding 10°C per minute, held for 1 minute, then increased to 140°C by adding 5°C per minute, holding for 1 minute, then increased to 160°C by adding 2°C per minute, holding for 1 minute, and lastly increased to 245°C by adding 5°C per minute, holding for 1 minute. By using the electrospray ionisation mode (EI), the mass-to-charge (m/z) of the

chemicals found in clove oil was discovered. Ion source temperature was kept at 250°C, while injector and detector temperatures were set to 280°C.
Helium gas, used as the mobile phase and flowing at a rate of 1 mL/min, was used to elute and segregate the sample down the column. By comparing the m/z of clove oil to the mass spectra in their collection (NIST MS), the chemical components are identified.
In many hours, clove oil was extracted using the steam hydro distillation process, and then the distillate was removed using a separatory funnel and n-hexane. In 6 hours, the observed maximum yield% of extracting clove oil was reached; the yield was 7.04%. Using FTIR, the FTIR spectra of the 100% pure clove oil (*Syzygium aromaticum*) that was commercially available were compared to the spectra of the clove oil that was obtained via steam hydro distillation for 6 hours. The extracted clove bud sample and the commercial essential oil sample show FTIR spectra that are quite close to one another, with a similarity of 98.88%.

Experimental Design

The sixty (60) adult male Wistar rats were divided into ten (10) groups of six (6) rats each, and administered the research substances as follows:
Group 1 - Negative Control (Placebo): 2 ml/kg body weight of normal saline daily for 30 days through an orogastric canula.
Group 2 - EIC Low Dose: 4 mg/kg of EIC via orogastric canula for 15 days.
Group 3 - EIC Standard Dose: 10 mg/kg of EIC for 15 days via orogastric canula.
Group 4 - TCA Low Dose: 200 mg/kg of TCA for 15 days through an orogastric canula.
Group 5 - TCA High Dose: 400 mg/kg of TCA for 15 days through an orogastric canula.
Group 6 - TCA Low Dose + EIC Low Dose: 200 mg/kg TCA for the first 15 days + 4 mg/kg of EIC for another 15 days through an orogastric canula.
Group 7 - TCA High Dose + EIC Standard Dose: 400 mg/kg TCA for the first 15 days + 10 mg/kg of EIC further 15 days through an orogastric canula.
Group 8 - EIC Low Dose + TCA Low Dose: 4 mg/kg of EIC for the first 15 days + 200 mg/kg of TCA for another 15 days via orogastric canula.
Group 9 - EIC Standard Dose + TCA High Dose: 10 mg/kg of EIC for first 15 days + 400 mg/kg of TCA for another 15 days through an orogastric canula.
Group 10 - EIC Standard Dose + TCA Low Dose (Extended): 4 mg/kg of EIC for 30 days in combination with 200 mg/kg of TCA for 30 days through an orogastric canula.

Animal Sacrifice

Upon sacrifice, the rats were weighed before decapitation. Following sacrifice, blood samples was promptly collected from the heart of each rat. A midline abdominal incision was made to expose the reproductive organs. The testes and epididymis were excised, and the weight of each animal's testes was assessed using an electronic analytical and precision balance.
Testis volume was determined using the water displacement method. Both testes of each rat were measured, and the average value for each parameter was considered as one observation. One of the testes from each animal was preserved in Bouin's fluid for subsequent histological examination. Serum and the remaining testis of each animal were stored at –25^{o}C for biochemical assays.

Immunohistochemistry

Caspase-3

Testicles fixed in paraffin were divided into 5µm pieces and placed on positively charged slides for the purpose of immunohistochemistry with caspase-3. Sections were dewaxed, rehydrated, and autoclaved in 10 Mm citrate buffer (pH 6) for 10 minutes at 120°C. Endogenous peroxidase was inhibited for 15 minutes using 0.3% H_2O_2 in methanol following PBS washing. After giving the slides another PBS wash, blocking was done by adding blocking buffer, and they were let to sit at room temperature for 30 minutes.

After dilution by PBS (2µg/ml and 1:1000, respectively), polyclonal antibodies for caspase-3 (Cat. No. PAI29157, Thermo Fisher Scientific Co., USA) were added and incubated for 30 minutes. PBS was used to wash the slides three times for three minutes each. Tissue sections were coated with biotinylated polyvalent secondary antibody (Cat. No. 32230, Thermo Scientific Co., UK) and co-incubated for 30 minutes. Three minutes were spent washing the slides with wash buffer each time. By applying Metal Enhanced DAB Substrate Working Solution to the tissue and letting it sit for ten minutes, the response could be seen. Wash buffer was used to wash the slides twice for three minutes each time. Hematoxylin stain was applied to the slide in sufficient amounts to cover the whole tissue surface in order to carry out counterstaining (Bancroft and Cook, 1994).

Caspase-3 Labelling Index/Quantitative Analysis

After background noise was subtracted, the intensity of immunoreactive regions was employed as a criterion of cellular activity for quantitative analysis. Image J, an image analyzer, was used for the measurement. Nine fields were chosen at random from each slide in the two experimental groups. The percentage of IHC stained area was computed as follows: %IHC stained area = IHC stained area/Total area X 100. The total field and immunohistochemical (IHC) stained areas were also computed.

Statistical Analysis

For each number, the mean and standard error of the mean (S.E.M.) were determined. Duncan's multiple range tests were used in conjunction with one-way analysis of variance (ANOVA) to compare the treatment and control groups. At $p \sim 0.05$, differences were deemed statistically significant.

Ethical Clearance

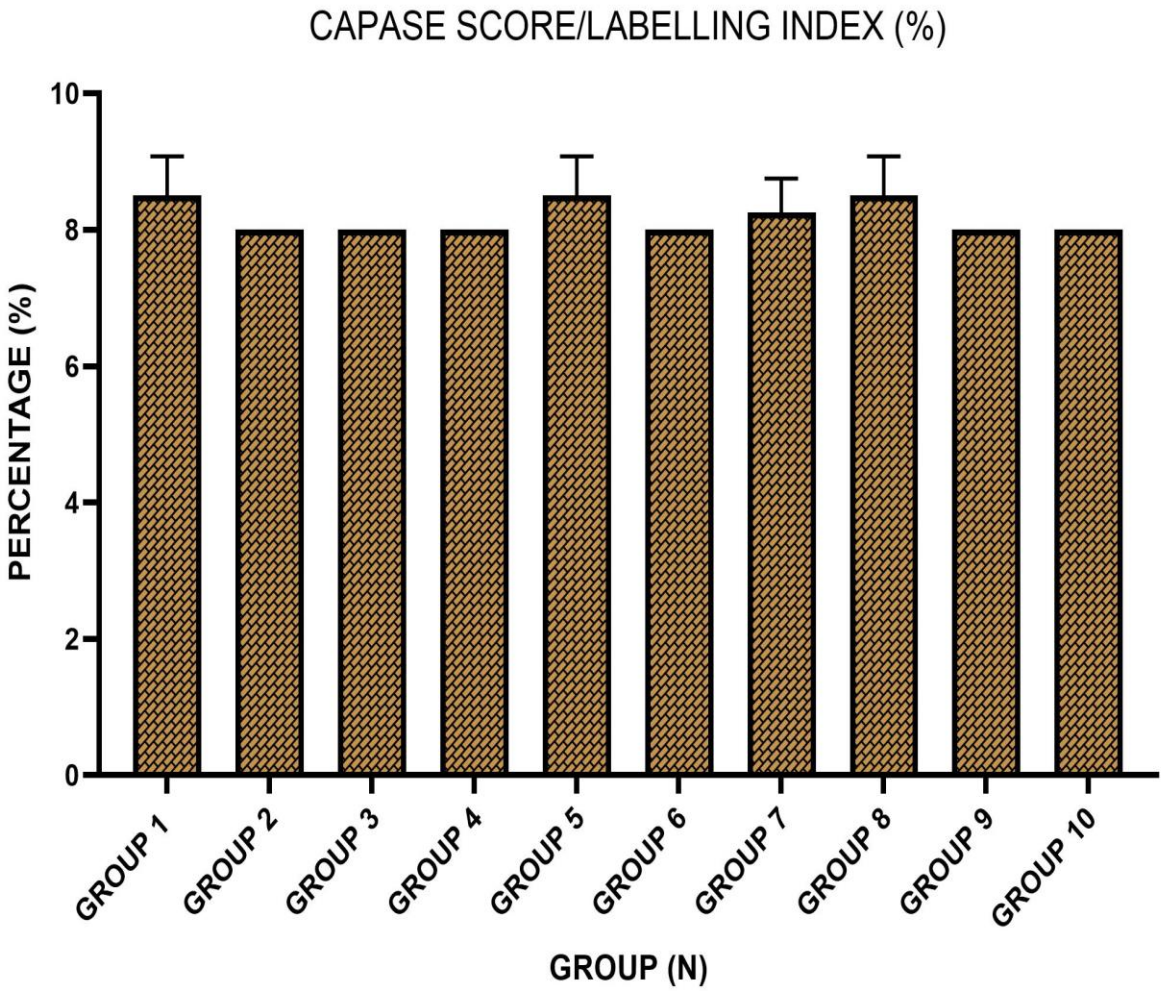
Ethical approval was sought and obtained from Human Research and Ethical Committee (HREC), College of Health Science, Benue state university, Makurdi with clearance number 08038619526. All experimental procedures carried out were in accordance with the guidelines on animal experiment as prescribed by the Ethics Committee.

Results

Capase 3 Score/Labelling Index

Capase-3 Immunostaining in the nuclei from all the groups was represented. The entire group showed very good immunoreactivity. Subjectively, the staining from group 4 to 10 were very poor compared to others. For Capase 3, immunoreactivity in both cytoplasm and nucleus of epithelia were evident. The mean level of morphological scores is shown in Figures 1.

Figure 1: Simple Bar Chart Showing the Mean Caspase Score/labeling Index across Groups



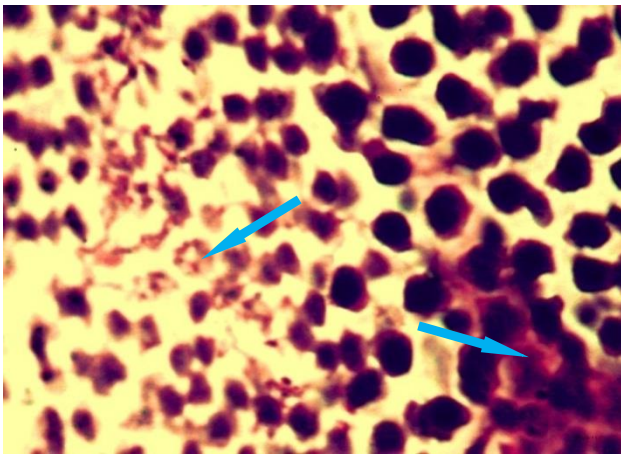


Plate 1: Photomicrograph of the testes of rat from group 1 showing immuno-positive markers for Capase-3. Magnification: x10

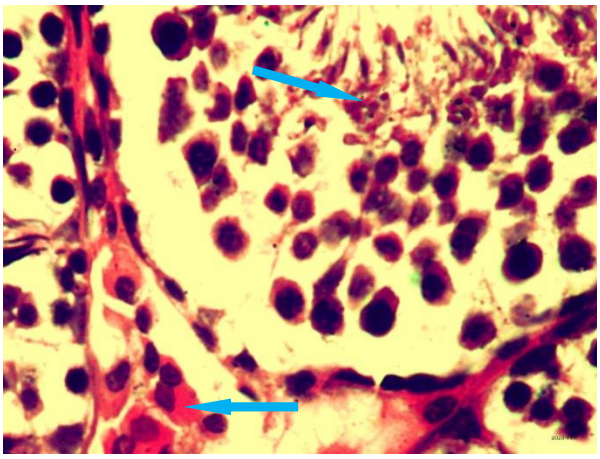


Plate 2: Photomicrograph of the testes of rat from group 2 showing immuno-positive markers for Capase-3. Magnification: x10

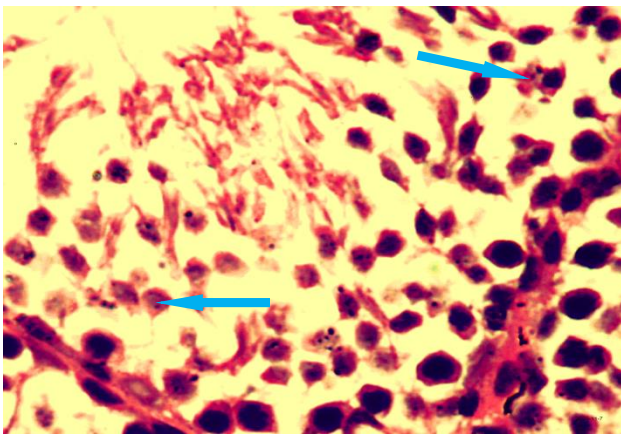


Plate 3: Photomicrograph of the testes of rat from group 3 showing immuno-positive markers for Capase-3. Magnification: x10

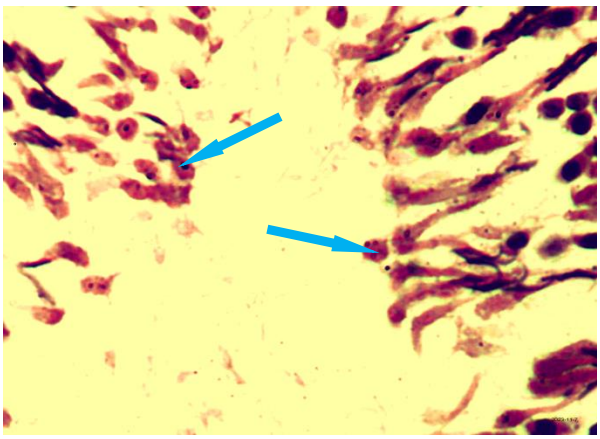


Plate 4: Photomicrograph of the testes of rat from group 4 showing immuno-positive markers for Capase-3. Magnification: x10

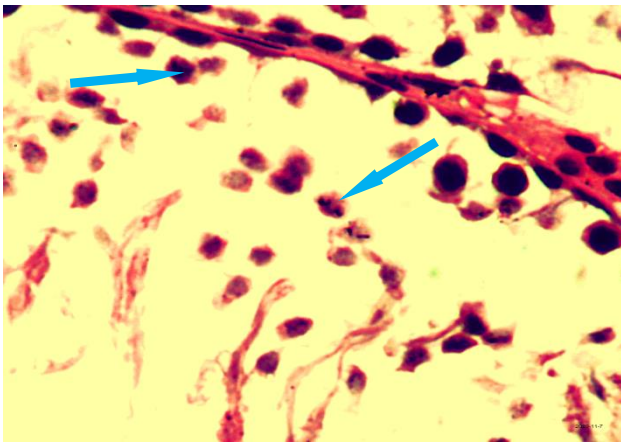


Plate 5: Photomicrograph of the testes of rat from group 5 showing immuno-positive markers for Capase-3. Magnification: x10

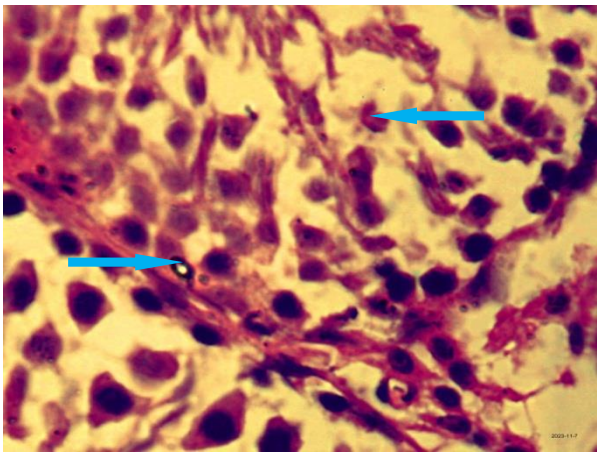


Plate 6: Photomicrograph of the testes of rat from group 6 showing immuno-positive markers for Capase-3. Magnification: x10

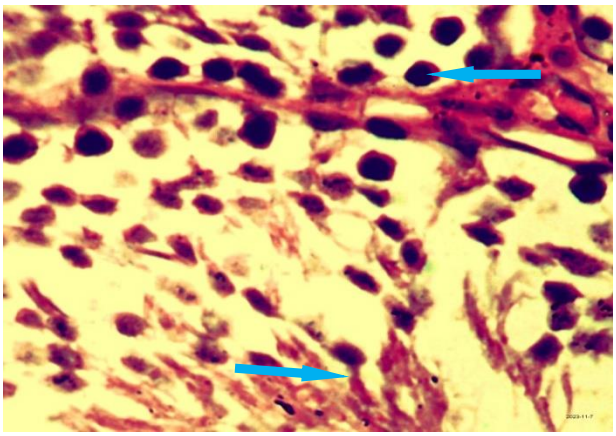


Plate 7: Photomicrograph of the testes of rat from group 7 showing immuno-positive markers for Capase-3. Magnification: x10

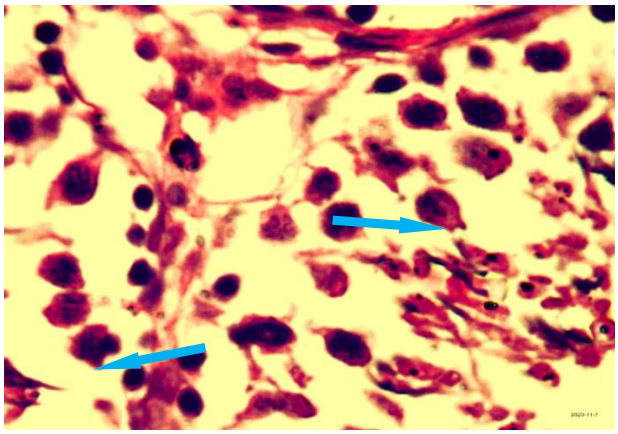


Plate 8: Photomicrograph of the testes of rat from group 8 showing immuno-positive markers for Capase-3. Magnification: x10

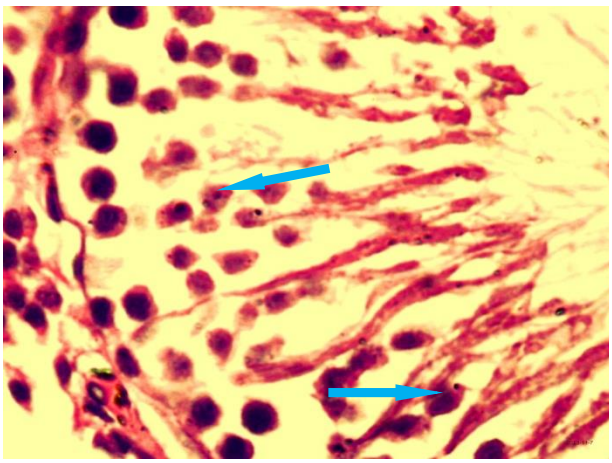


Plate 9: Photomicrograph of the testes of rat from group 9 showing immuno-positive markers for Capase-3. Magnification: x10

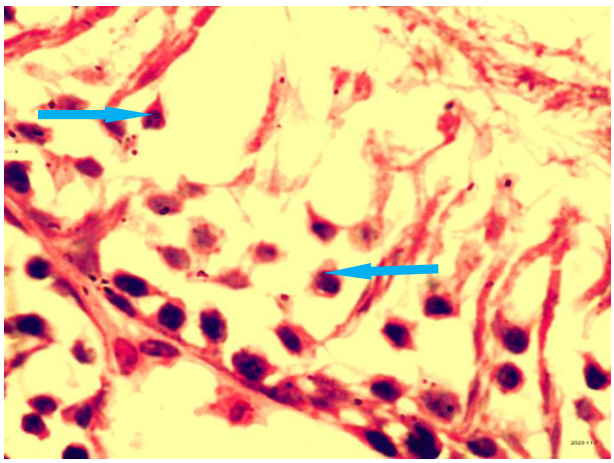


Plate 10: Photomicrograph of the testes of rat from group 10 showing immuno-positive markers for Capase-3. Magnification: x10

Discussion

The results of the Capase-3 expression in the testes of adult rats following treatment with Trichloroacetic Acid and Clove Isolates reveal interesting patterns of immunoreactivity. The immunostaining of Capase-3 in the nuclei across all groups displayed robust signals, indicating a widespread presence of this apoptotic marker in the testicular tissues. However, a notable divergence in immunoreactivity was observed from group 4 to 10, with staining intensity appearing significantly diminished compared to other groups.

The cytoplasmic and nuclear localization of Capase-3 in the epithelial cells suggests its involvement in both intracellular compartments, emphasizing its multifaceted role in apoptotic processes (Vernet *et al*., 2004). The variation in staining intensity across groups may indicate a potential modulation of apoptosis in response to Trichloroacetic Acid and Clove Isolates treatment. The subjective assessment of staining suggests that these treatments might have differential effects on the activation or expression of Capase-3.

The simple bar chart in Figure 1 provides a concise visual representation of the mean Caspase Score/Labeling Index across the experimental groups. The distinct decline in the mean scores from group 4 to 10 underscores the importance of considering the potential impact of the treatments on Capase-3 expression.

The photomicrographs (Plate 1 to Plate 10) further support the quantitative findings, showing immuno-positive markers for Capase-3 in the testicular tissues of rats from different groups. The diminishing intensity of staining in groups 4 to 10 is evident in these micrographs, reinforcing the notion of a treatment-induced modulation of Capase-3 expression.

The observed uniformity in Capase-3 expression in groups 1 to 3 suggests that the treatments up to this point may not have induced significant alterations in apoptosis-related processes. However, the diminished immunoreactivity in subsequent groups raises intriguing questions about the mechanisms underlying this observed change. It is crucial to explore the specific components of Trichloroacetic Acid and Clove Isolates that might be responsible for the observed effects and to delve into the potential implications for testicular health.

The observed decrease in Capase-3 immunoreactivity in groups 4 to 10, when compared to other groups, suggests a potential influence of the applied treatments on the apoptotic processes within

the testicular tissues. The decreased immunoreactivity may indicate a modulation of the apoptotic pathway (Morris & Bost, 1991), possibly implicating a regulatory mechanism influenced by the treatments.

The presence of Capase-3 immunoreactivity in both the cytoplasm and nucleus of epithelial cells is consistent with previous findings of Shan *et al*. (2005) and Neveu *et al*. (2010), indicating the involvement of Capase-3 in both cytoplasmic and nuclear apoptotic events. The morphological scores depicted in Figure 1 further support the observed changes, providing a quantitative measure of Capase-3 expression across different experimental groups.

Comparing these results with previous studies of Madlala *et al.* (2012), Moghimian (2017) and Batiha (2020) on similar investigations, it is imperative to note variations in methodologies, treatment regimens, and experimental designs. While Moghimian (2017) reported changes in Capase-3 expression in response to specific treatments, Batiha (2020) demonstrated conflicting outcomes. Discrepancies in findings could be attributed to differences in model organisms, treatment durations, concentrations of administered substances, and the specific tissues targeted.

Several studies have reported alterations in Capase-3 expression under various experimental conditions. Madlala *et al.* (2012) demonstrated increased Capase-3 activity in response to certain agents, highlighting its role in mediating apoptosis. Conversely, findings similar to the present study, indicating unchanged or reduced Capase-3 expression, have been reported in studies of Liu *et al.* (2015) investigating the impact of different compounds on apoptotic pathways.

It is crucial to consider the context-specific nature of Capase-3 regulation, as it may be influenced by various factors, including cell type, tissue specificity, and the specific apoptotic stimuli. The interpretation of Capase-3 expression should be approached with caution, taking into account the intricate network of apoptotic regulators and their dynamic interactions.

Conclusion

In conclusion, the present study provides compelling evidence that Capase-3 expression remains unchanged in response to Trichloroacetic Acid and Clove Isolates treatment in adult rats up to a certain point. The significant reduction in immunoreactivity in groups 4 to 10 warrants further investigation into the precise molecular mechanisms and potential therapeutic implications of these findings. Additionally, future research should aim to elucidate the specific components responsible for the observed effects, allowing for a more targeted understanding of the impact of Trichloroacetic Acid and Clove Isolates on testicular apoptosis.

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