# Effect of *Cordia myxa* fruit alcohol extract on AST, ALT, and AP seminal plasma leakage in cooled semen of Awasssi ram

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## Abstract

The chilling semen process induces cold shock injury to sperm membranes, increasing permeability and leading to leakage of intracellular enzymes into the seminal plasma. The current research aimed to evaluate the effect of adding *Cordia myxa* fruit alcohol extract to chilled Awassi ram semen on the leakage of AST, ALT, and AP enzymes. Semen samples were collected from five Awassi rams using an artificial vagina. Following collection, samples were pooled and extended with two extenders (Tris and skim milk). Following dilution, samples were divided into five groups for each extender as follows: Control (without additives); 20 µL/mL; 50 µL/mL; and 100 µL/mL Cordia myxa alcohol extract. All samples were cooled to 5°C and stored for 0, 24, 48, and 72 hours following centrifugation at 4000 rpm for 30 minutes. The results showed that the addition of the extract significantly ($P < 0.05$) reduced leakage of all three enzymes compared with the control group. It can be concluded that *the alcohol extract of Cordia myxa* exerts a protective effect against cold-induced membrane damage in Awassi ram spermatozoa. This results in additional experiments to evaluate the antioxidant activity and semen viability.

**Keywords:** *Cordia myxa*, Semen cooling, Enzyme leakage, Sperm membrane integrity

## Introduction

Sheep, particularly the Awassi breed, play a vital role in Iraq's livestock industry, particularly contributing substantially to providing red meat, milk, and wool production [1]. Artificial insemination is an important assisted reproductive technology in sheep because it facilitates the rapid dissemination of desirable genetic traits and improves reproductive efficiency [2]. Although chilled semen preservation has advantages, such as lower cost and easier field transport compared to cryopreservation, its short storage life remains a major limitation for practical artificial insemination programs [3].

During cooling and storage, spermatozoa are exposed to cold shock and oxidative stress due to excessive production of reactive oxygen species (ROS), and simultaneously to a decrease in seminal plasma antioxidants [4]. Sheep sperm membrane is highly susceptible to lipid peroxidation due to its richness in unsaturated fatty acids. This oxidative

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stress-induced damage to the sperm membrane may impair sperm motility, viability, membrane integrity, and mitochondrial function, thereby increasing membrane permeability and the leakage of intracellular enzymes into the seminal plasma. Consequently, measuring intracellular enzymes such as aspartate aminotransferase (AST), alanine aminotransferase (ALT), and alkaline phosphatase (AP) may provide vital biochemical biomarkers for evaluating sperm plasma membrane injury and structural preservation during storage [5],[6].

Aspartate aminotransferase (AST), alanine aminotransferase (ALT), and alkaline phosphatase (AP) are among the enzymes associated with sperm metabolism and reproductive-tract function [7][8] Increased AST and ALT activities, as well as alterations in AP activity, may reflect changes in sperm plasma-membrane stability, cellular damage, or enzyme leakage into the extracellular seminal fluid [9][10]. Therefore, evaluating these enzymes alongside semen characteristics may help assess the functional integrity of spermatozoa during chilled storage and provide indirect biomarkers of membrane damage. The use of natural antioxidants is a promising strategy for reducing ROS-mediated damage during semen preservation [11],[12]. Cordia myxa is a particularly relevant candidate because it contains phenolic compounds, flavonoids, tannins, phenolic acids, xanthones, terpenes, and saponins, which may contribute to its antioxidant activity [13]. These bioactive constituents may scavenge free radicals, reduce lipid peroxidation, and help preserve sperm membrane integrity. Thus, adding Cordia myxa fruit extract to ram semen extenders may offer a natural approach to improving semen stability during chilled storage.

Several natural antioxidants have been evaluated in ram semen preservation to mitigate cold-induced and ROS-mediated sperm membrane damage. Although numerous natural antioxidants have been broadly used in sheep semen preservation, evidence remains limited regarding the use of Cordia myxa fruit alcoholic extract in chilled ram semen and its effects on AST, ALT, and AP as indicators of sperm membrane damage and cellular leakage. In addition, comparative assessment of sperm stability in different dilutions has not been carefully investigated.

Therefore, this study aims to investigate the effects of supplementing Tris egg yolk or skim milk extenders with different concentrations of Cordia myxa fruit alcoholic extract on seminal plasma AST, ALT, and AP activities in chilled Awassi ram semen at different storage time points.

**Materials and Methods:**

The experiment was conducted as part of a research project affiliated with the Holy Husayni Shrine in the Al-Da'oum area of Karbala Governorate. Animal use protocols were approved by the ethical committee of Al-Qasim Green University (qgec.24.2025). Five adult local rams aged 2–4 years, weighing 40–60 kg, were used in the study. Five ejaculates were pooled to form a single sample, and the collection and evaluation process was repeated independently 6 times. Each pooled sample underwent a preliminary assessment of semen quality, including the evaluation of both mass and individual sperm motility; mass motility was 90%, while individual motility was 85%. Subsequently, the samples were diluted using either egg yolk or skim milk extenders at a 1:10 ratio. Samples were divided into four groups as follows: Control group (without additives); 20 µL/mL, 50 µL/mL, and 100 µL/mL of *Cordia myxa* fruit alcoholic extract. All samples were cooled to 5°C and stored for

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0, 24, 48, and 72 hours following seminal plasma centrifugation at 4000 rpm for 30 minutes, and stored at -20 until analysis.

**Extract Preparation in the laboratory:**

Five grams of fruit powder were added to 100 mL of 70% methanol at room temperature in a conical flask plugged with medical-grade cotton wool. After 24 hours, the mixture was filtered—first through cloth, then through Whatman No. 1 filter paper [14].

**Biochemical assessments:**

**Alkaline phosphatase (AP) activity**

Alkaline phosphatase (AP) activity was determined using a commercial AP kit (Biosam, IFCC method). The assay was performed at 37°C using a wavelength of 405 nm and a 1-cm light path. Briefly, 0.8 mL of buffer reagent was mixed with 0.02 mL of seminal plasma and incubated at 37°C for 1 min. Then, 0.2 mL of substrate reagent was added. After thorough mixing, the initial absorbance (A□) was measured after 30 s, followed by absorbance readings at 1-min intervals for 2 min. The mean change in absorbance per minute (ΔA/min) was calculated to determine AP activity.

**ALT activity**

Alanine aminotransferase (ALT) activity was determined in the seminal plasma using a commercial ALT NADH kinetic UV reagent kit. The assay was based on the decrease in NADH absorbance at 340 nm resulting from the ALT-catalyzed conversion of L-alanine and α-ketoglutarate to pyruvate and glutamate, followed by the conversion of pyruvate to lactate by lactate dehydrogenase. Briefly, 1.0 mL of working reagent was mixed with 100 µL of seminal plasma and incubated for 1 min. The absorbance was measured at 340 nm at 25, 30, or 37°C, with measurements taken at 1-min intervals for 3 min. The mean change in absorbance per minute (ΔA/min) was calculated, and AP activity was expressed as U/L according to the manufacturer's instructions.

**AST activity**

AST activity was determined by a kinetic UV method using a commercial AST reagent kit. The assay is based on the reduction of oxaloacetate to malate-by-malate dehydrogenase in the presence of NADH, with the decrease in NADH absorbance monitored at 340 nm. The working reagent was prepared by mixing one volume of reagent R2 with four volumes of reagent R1. For each assay, 1.0 mL of working reagent was mixed with 100 µL of seminal plasma and incubated for 1 min at the selected reaction temperature (25, 30, or 37 °C). Absorbance was then recorded at 340 nm at 1-min intervals for 3 min, and the mean change in absorbance per minute (ΔA/min) was calculated. AST activity was calculated as ΔA/min × 1750 and expressed as U/L.

**Statistical Analysis:**

Data were organized and analyzed using SPSS 26 software. One-way repeated-measures ANOVA was used to evaluate the effect of varying concentrations of the alcoholic extract of Cordia myxa on the activities of the enzymes AST, ALT, and AP. Duncan's Multiple Range Test [15] was used to compare means. The significance level was $P< 0.05$

**Results and Discussion**

**Effect of Cordia myxa alcoholic extract in Tris egg yolk extender on enzymatic leakage**

Statistical analysis revealed a significant effect of treatment with the alcoholic extract of the Cordia myxa plant, diluted with Tris egg yolk-based extender, on the concentration of aspartate aminotransferase (AST). The results showed a significant reduction ($P\leq0.05$) in the 20 µL group at 72 hours (Figure 1).

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These findings align with those of [16], who observed that the alcoholic extract of Cordia myxa reduces liver enzyme levels in healthy mice. The researcher notes that treatment with the alcoholic extract of Cordia myxa reduced the concentration of this enzyme; this result is consistent with findings showing that adding antioxidants to ram semen extender lowers AST levels [17]. The AST enzyme likely plays a role in regulating mitochondrial function and sperm metabolism [18].

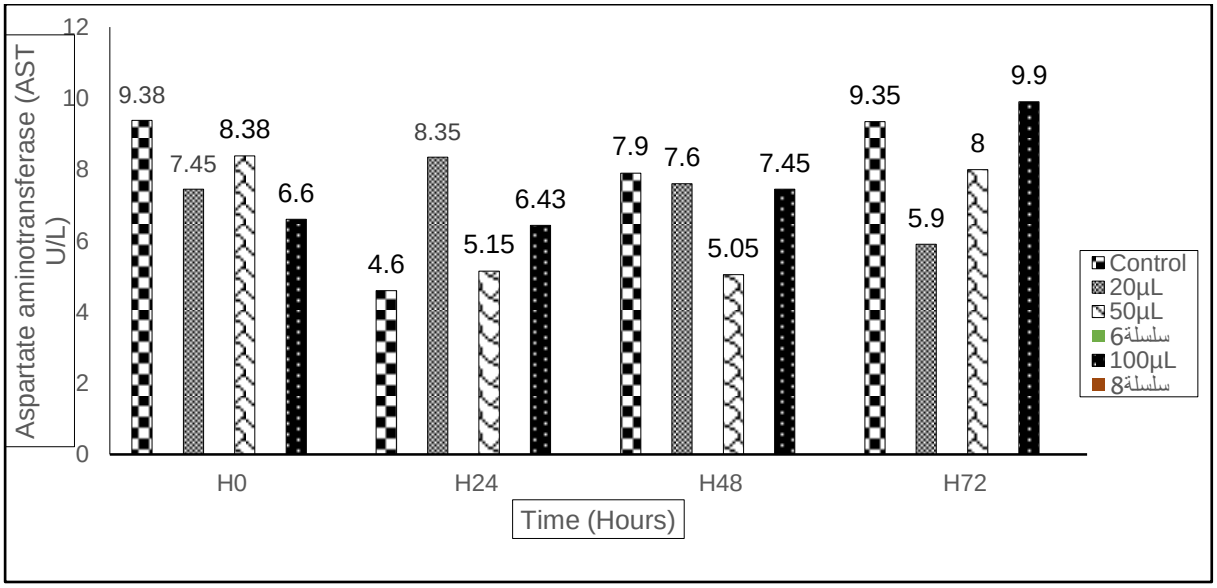



*Figure 1: Effect of adding different concentrations of the alcoholic extract of Cordia myxa to the Tris-egg yolk extender on aspartate aminotransferase (AST, U/L) activity in diluted and cooled semen from local male sheep over various time intervals (Mean ± Standard Error). Uppercase letters differ significantly (P ≤ 0.05) between groups within the same time. Lowercase letters differ significantly across time within the same treatment.*

Statistical analysis revealed a significant effect of treatment with the alcoholic extract of Cordia myxa, diluted with a Tris-egg yolk-based extender, on alanine aminotransferase (ALT) concentration (Figure 2). The results showed significant differences (P≤0.05) at 24 hours for the 50 µL group (Figure 2). The antioxidants in Cordia myxa inhibit free radical chain reactions by capturing free radicals or converting them into less reactive forms, and by inhibiting lipid peroxidation, thereby protecting the cell membrane from free radicals and preventing cell damage. It is believed that reduced membrane fluidity, with membrane interaction, inhibits lipid peroxidation, which can otherwise accumulate in the cell membrane and trigger adverse effects such as cell death [19]. The findings of the current study align with those of [20]. They observed a decrease in enzyme concentration in mice treated with the alcoholic extract of *Cordia myxa*.

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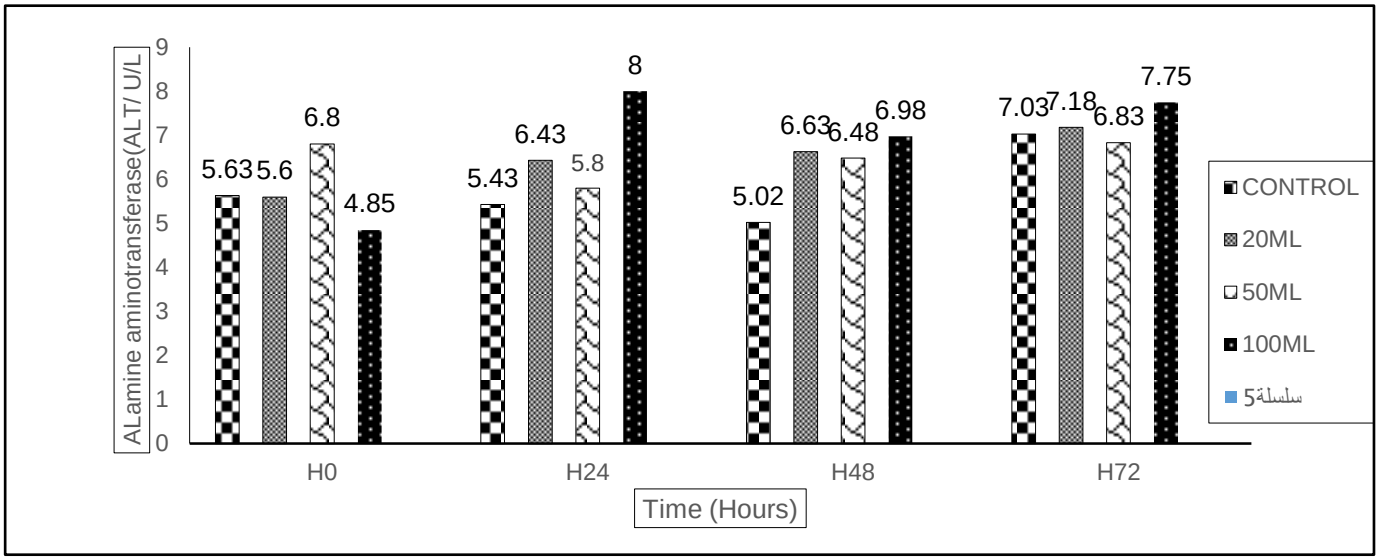



*Figure 2: Effect of adding various concentrations of Cordia myxa alcoholic extract to the Tris-egg yolk extender on alanine aminotransferase (ALT, U/L) activity in diluted and cooled semen from local male sheep over different time intervals (Mean ± Standard Error). Uppercase letters differ significantly (P ≤ 0.05) between groups within the same time. Lowercase letters differ significantly across time within the same treatment.*

Statistical analysis revealed a significant effect of treatment with the alcoholic extract of Cordia myxa diluted with a Tris-egg yolk-based extender on alkaline phosphatase (AP) enzyme concentration (Figure 3). At time zero, a significant decrease was observed in the 20 µL treatment group, while no significant differences were found among the other treatments. Alkaline phosphatase (AP) is an enzyme that catalyzes the transfer of phosphate groups from various substrates; it is produced in the epididymis and testes of the male reproductive system, as well as in the body fluids of numerous species [21]. The presence of this enzyme in the male reproductive system makes it crucial for sperm function in mammals, particularly for metabolic processes and fertilization [22]. Furthermore, [21] reported that AP levels in the seminal plasma of rams decreased following chilled storage when the plasma was treated with antioxidants, a factor that may influence the survival of stored sperm [23].

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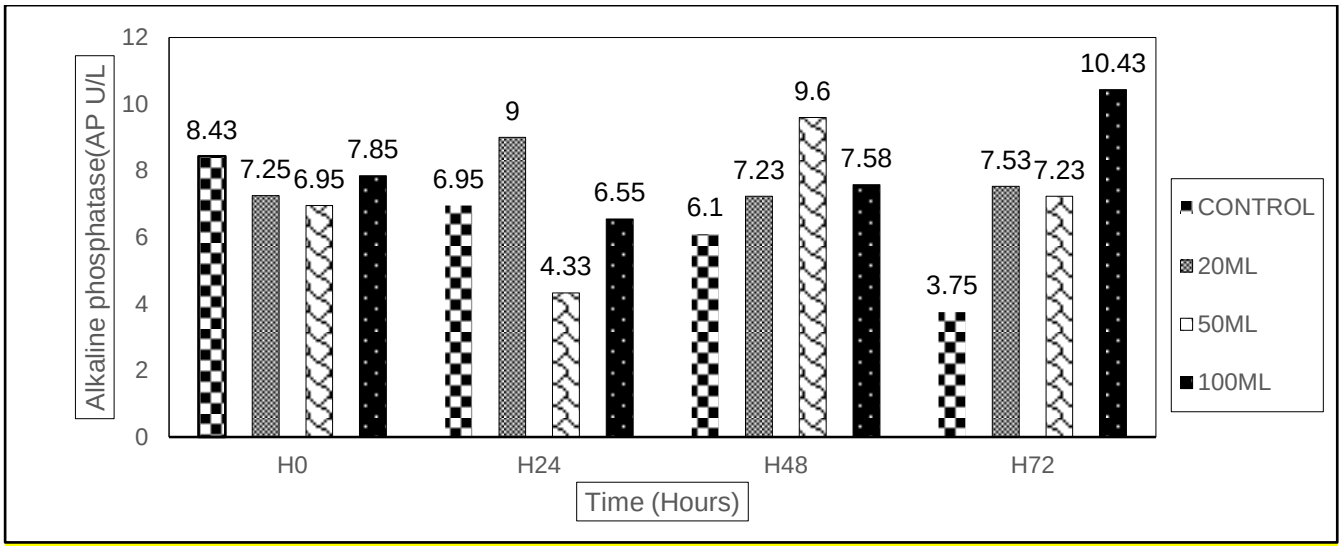



*Figure 3:Effect of adding different concentrations of Cordia myxa alcoholic extract to the Tris-egg yolk extender on alkaline phosphatase (AP U/L) in diluted and cooled semen from local male sheep over various time intervals (Mean ± Standard Error). Uppercase letters differ significantly (P ≤ 0.05) between groups within the same time. Lowercase letters differ significantly across time within the same treatment.*

**Effect of Cordia myxa alcoholic extract in skim milk extender on enzymatic leakage**

Statistical analysis revealed a significant effect (P≤0.05) of treatment with the alcoholic extract of *Cordia myxa*, diluted with a skim milk-based extender, on aspartate aminotransferase (AST) at a 50 µL concentration at 48 hours (Figure 4).

The release of the AST enzyme into the seminal plasma from the sperm head is a consequence of metabolic activity [24]. In the present study, treatment with the alcoholic *Cordia myxa* extract reduced the concentration of this enzyme; this finding aligns with results showing that adding antioxidants to ram semen extenders lowers AST levels [17].

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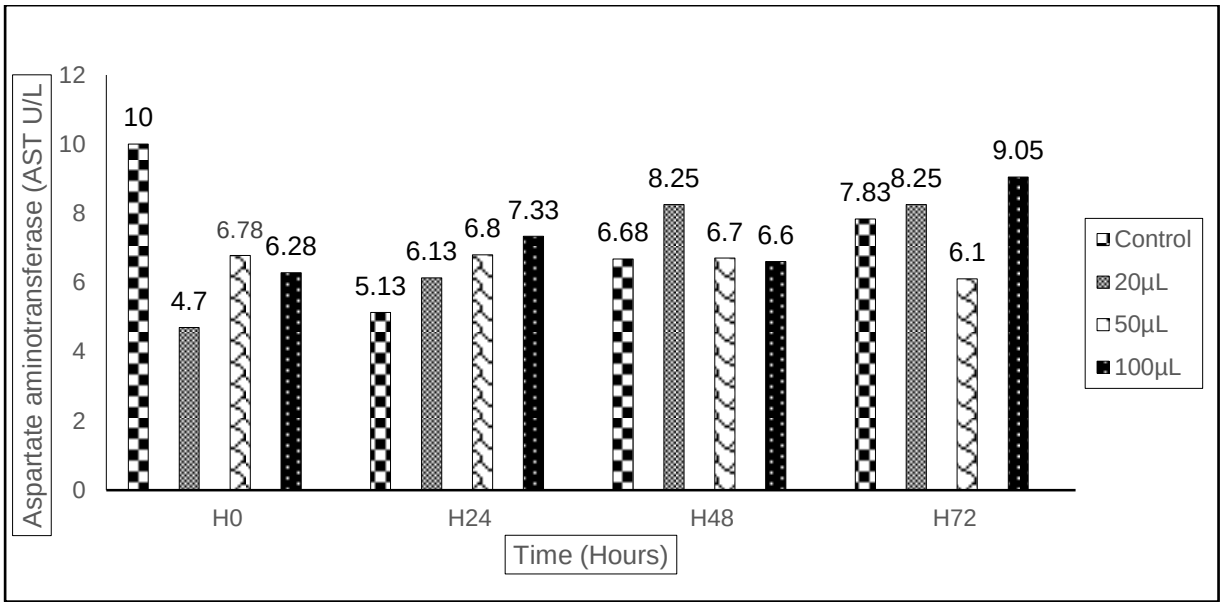



*Figure 4: Effect of adding different concentrations of the alcoholic extract of Cordia myxa to a skim-milk extender on aspartate aminotransferase (AST, U/L) activity in diluted, chilled semen from local male sheep over various time intervals (Mean ± Standard Error). Uppercase letters differ significantly ($P \leq 0.05$) between groups within the same time. Lowercase letters differ significantly across time within the same treatment.*

The results demonstrate a significant, progressive increase in ALT leakage into the extender over time across all treatments, from hour 0 (H0) to hour 72 (H72) (Figure 5). This rise is attributed to the natural degenerative breakdown of the sperm plasma membrane during prolonged liquid storage and chilling. Furthermore, the accumulation of reactive oxygen species (ROS) alongside a reduction in intracellular ATP levels increases membrane permeability, thereby accelerating enzymatic leakage [25]. Alanine Aminotransferase (ALT) is an intracellular enzyme contained within the cytoplasm and plasma membrane of spermatozoa, as well as the mitochondria. Measuring extracellular ALT levels in the extender is a crucial direct biomarker for assessing sperm plasma membrane integrity and permeability. Damage or lipid peroxidation of the membrane causes enzyme leakage from the intracellular space into the extracellular matrix [26]. Antioxidant materials reduced the ALT leakage during the first 24 hours following chilled storage. [25 and 27] concluded that the membrane-protective effect is primarily attributed to the interaction between the bioactive antioxidants (phenolics and flavonoids) present in *Cordia myxa* extract and milk proteins, which effectively scavenge reactive oxygen species (ROS) and inhibit lipid peroxidation of the sperm plasma membrane, thereby maintaining structural integrity and preventing enzyme release [25][27]

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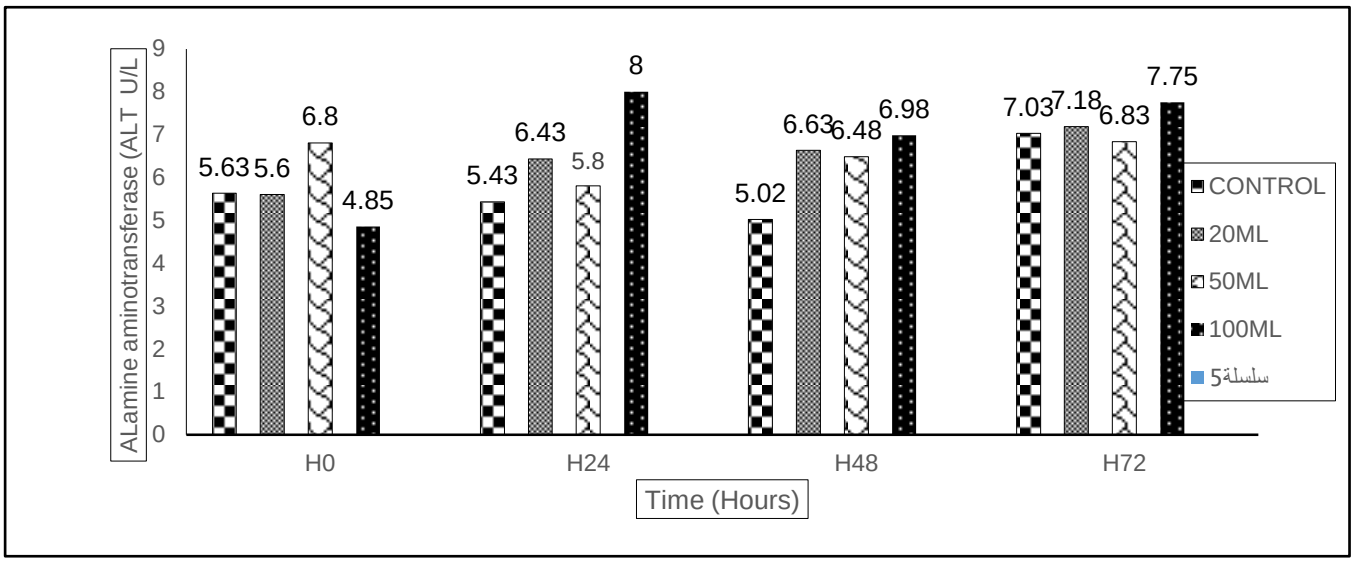
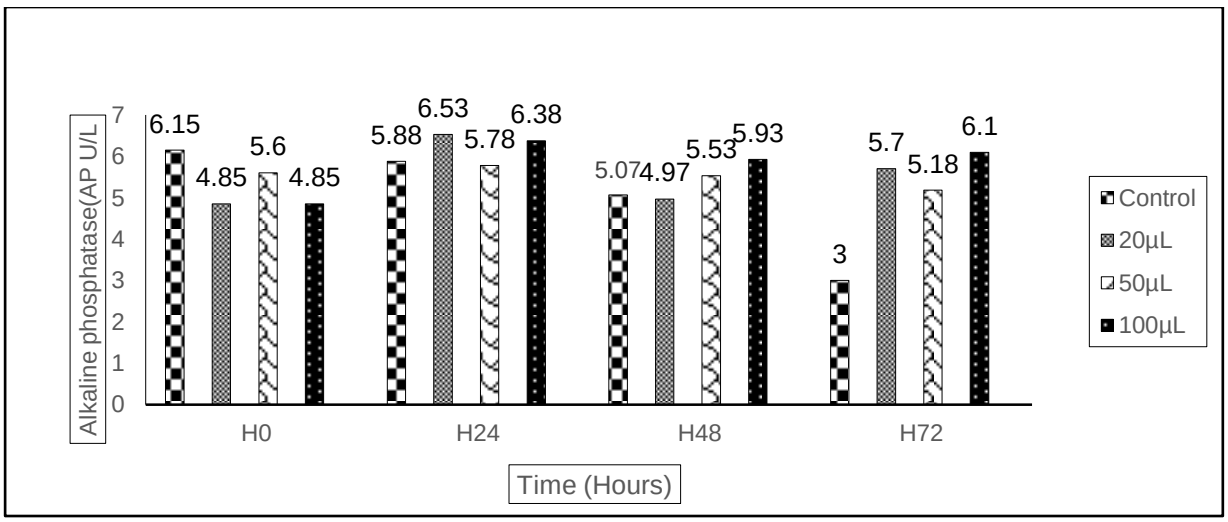



*Figure 5: Effect of adding different concentrations of the alcoholic extract of Cordia myxa to a skim-milk extender on alanine aminotransferase (ALT, U/L) activity in diluted, chilled semen from local male sheep over various time intervals (Mean ± Standard Error). Uppercase letters differ significantly (P ≤ 0.05) between groups within the same time. Lowercase letters differ significantly across time within the same treatment.*

Regarding the AP, the statistical analysis showed no significant difference in AP activity (Figure 6). The absence of statistically significant differences ($P > 0.05$) in AP activity among the treatment groups indicates that Cordia myxa extract exerted a membrane-stabilizing effect without causing cytotoxic or osmotic damage to spermatozoa. The natural antioxidants present in the extract effectively scavenged reactive oxygen species (ROS) and inhibited lipid peroxidation, thereby preserving plasma membrane integrity and preventing the abnormal leakage of intracellular AP into the extender during chilled storage [25][26].




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*Figure 6: Effect of adding different concentrations of the alcoholic extract of Cordia myxa to a skim-milk extender on Alkaline phosphatase (AP U/L) activity in diluted, chilled semen from local male sheep over various time intervals (Mean ± Standard Error). Lowercase letters differ significantly across time within the same treatment.*

## CONCLUSION

In conclusion, the current results for Awassi semen preservation at 5 °C revealed that reduced sperm membrane integrity increased over time, as evidenced by AST, ALT, and AP leakage in seminal plasma. However, adding Cordia myxa to diluted semen in both extenders mitigated the leakage as a result of cold shock and oxidative stress. Low Cordia myxa concentrations (20 and 50 µL), compared with the higher dose, revealed the sperm membrane's protective ability against leakage of seminal plasma enzymes, providing promising results for chilled storage of sheep sperm. Future research is warranted to evaluate semen Kinematics and antioxidant parameters using i

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