The effect of adding aqueous and oily extracts of marjoram to Tris dilute on some epididymal sperm characteristics of bulls under cold storage conditions for different periods

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Abstract

A study was performed in the postgraduate laboratory of the Animal Production Department at Al-Muthanna University's College of Agriculture, from September 15, 2024, to February 21, 2025. Fifty-seven samples were utilized to investigate the impact of incorporating aqueous and oily extracts of marjoram at concentrations of 2% and 1% on certain epididymal sperm characteristics of local bulls under cryopreservation circumstances at 5°C for various durations (0, 24, 48, 72, and 96 hours). Testes were procured from male bulls at a local slaughterhouse in Al-Muthanna Governorate immediately post-slaughter and brought to the laboratory within one hour to extract mature sperm from the tail of the epididymis. The sperm were diluted with a pre-prepared Tris buffer. The samples were categorized into five treatments: the control treatment utilized solely Tris diluent; the second treatment incorporated a 1% aqueous extract of marjoram into the Tris diluent; the third treatment included a 2% aqueous extract of marjoram; the fourth treatment involved a 1% oily extract of marjoram added to the Tris diluent; and the fifth treatment comprised a 2% oily extract of marjoram mixed with the Tris diluent. The results indicated that the percentage of individual sperm motility in the interaction between diluents and storage periods demonstrated a significant superiority ($P<0.05$) for the T4 treatment compared to the T5 treatment and the control at the 96, 72, 48, and 24-hour intervals, with values of (79.67±2.40, 76.67±2.85, 74.33±2.67, 71.33±3.18, 64.67±2.33) for the four treatments, respectively. The plant extracts significantly influenced individual motility, including the percentages of live, dead, and deformed sperm, as well as plasma membrane integrity ($P<0.05$). The T4 treatment exhibited a marked superiority over T1, T2, and T3, with values of (61.64±2.29, 63.12±2.27, 66.5±2.07, 75.13±1.15). Additionally, the 1% oil extract treatment significantly outperformed both T1 and T3, with readings of (57.43±2.60, 59.38±4.69, 76.45±0.78). *Summary*: The results showed that adding aqueous and oily extracts of marjoram to the semen diluent had a positive effect on semen characteristics, especially at a 1% oil extract concentration, as individual sperm motility, the percentage of live sperm, and the integrity of the plasma membrane improved.

Key Words : marjoram, Tris, sperm, bulls, periods.

Introduction

Livestock is crucial for fulfilling the nutritional requirements of the population, especially in terms of dairy and meat, while also providing the industry with leather, hair, wool, fur, and other raw materials, as well as serving as a source of income [1]. This has resulted in the advancement of contemporary technology aimed at enhancing investment in livestock production through the provision of economically valuable breeds and the improvement of animal product quality [2].

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Cows hold significant economic value globally, as they contribute to livestock through several products, namely milk and meat. They are regarded as the foremost producer of milk globally [3]. The attributes and standard of animal products, especially those from cattle, have been enhanced. Dairy cows are crucial to the economic development and progress of numerous countries globally. Reproductive efficiency is a crucial economic trait in cow breeding and significantly contributes to the enhancement of dairy cattle production. This has fostered the advancement of multiple reproductive management aspects, such as enhanced artificial insemination and elevated pregnancy rates in dairy cattle herds [4]. Cryopreservation of semen and artificial insemination significantly enhance cattle output and product quality. This is accomplished by the utilization of cryopreserved semen and artificial insemination. Notwithstanding advancements in the cryopreservation of bull semen, deficiencies in the technological knowledge base persist. These represent vulnerabilities that impede the progress of both fundamental science and reproductive biotechnology. As a result, numerous diluents have been formulated and enhanced with compounds to mitigate damage from refrigeration or oxidative stress, attaining differing degrees of efficacy [5]. Reproductive issues in cows that adversely affect reproductive efficiency are either congenital or acquired. The predominant reproductive issues identified in cows encompass polycystic ovary syndrome (PCOS) and fallopian tube diseases, regarded as the most prevalent reproductive complications. Inadequate postpartum breeding practices and poor management contribute to infections in cows, as do artificial elements in many cow care techniques. Immune variables contributing to infertility have also been investigated. A preventative strategy is essential, employing contemporary reproductive technologies to accurately diagnose and enhance the diverse reproductive issues impacting cows [6.[

In males, infertility constitutes a significant issue, resulting in economic losses in livestock and subsequently diminished reproduction attributed to subpar semen quality [7[

Artificial insemination has been established for cattle, rabbits, and numerous other species, with initiatives undertaken to create solutions and semen diluents. Artificial insemination is seen as a strategy for addressing reproductive issues, hence facilitating genetic enhancement initiatives [8]. Cryopreservation of semen facilitates genetic enhancement via artificial insemination. Sperm preservation induces functional alterations in sperm, necessitating the employment of artificial insemination technology to aid and conserve endangered species [9.[

Extracts from medicinal plants have been utilized as diluents, included into semen diluents. Recent studies indicate that extracts aid in semen preservation, through freezing or cooling, due to their active and beneficial compounds that may function as antioxidants for various medical applications [10.[

Medicinal and aromatic plants are regarded as non-conventional crops with diverse applications. Active compounds are extracted directly or indirectly for application in the pharmaceutical, fragrance, and food sectors. Medicinal plants encompass either plants, medicinal and aromatic herbs, or pharmaceuticals [11]. The efficacy of

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numerous medicinal plants is ascribed to their volatile essential oils, along with the observation that certain species possess a greater oil content than others within the same genus [12.[

Recent research have validated the application of essential oils from several plants, including marjoram, in the pharmaceutical sector [13.[

In recent years, there has been a growing need for the transformation of medicinal and aromatic plants into significant goods for the pharmaceutical sector. Marjoram is a perennial herb indigenous to the Mediterranean, extensively utilized in traditional medicine for its antioxidant and antibacterial qualities to address many ailments[14.[

Marjoram possesses numerous applications beyond its utilization in industries such as cosmetics, essential oils, and soap. It may decrease cholesterol levels, enhance immunological function, and mitigate vascular calcification due to the presence of the rosemary component. It diminishes the likelihood of memory impairment and possesses antiseptic qualities for disinfecting areas contaminated with germs [15]. Marjoram is regarded as an aromatic herb that possesses metabolites advantageous to animal reproduction and growth. It also include phenolic compounds and oils distinguished by their antioxidant qualities, as well as thymol, which functions as an antibacterial agent. It additionally functions as a nutritional adjunct for sexual performance and semen [16]. This study seeks to examine the potential enhancement of some epididymal sperm attributes in bulls and to preserve their viability for extended durations following refrigeration at 5°C at intervals of 96, 72, 48, and 24 hours post-dilution. This research employs aqueous and oily extracts of marjoram, incorporating them into a diluted Tris solution.

Materials and Methods

Research Location

The present study was carried out in the graduate laboratories of the Animal Production Department at the College of Agriculture, Al-Muthanna University, throughout the designated timeframe (September 15, 2024, to February 13, 2025). Fifty-seven testicular samples were utilized to assess the epididymal sperm of bulls subjected to cryopreservation at 5°C for varying durations: 24, 0, 96, 72, and 48 hours. This encompassed the manufacture and preservation of botanical extracts. Sperm were subsequently harvested from the testes of bulls executed at the abattoir in Al-Muthanna Governorate. Immediately post-slaughter, the specimen was conveyed to the laboratory for requisite procedures. The plant extracts were subsequently frozen after application and incorporated into the prepared medium. The primary extracts were the aqueous and oily extracts of marjoram, each incorporated at two distinct strengths.

Preparation of the Aqueous Extract of Origanum majorana

The aqueous extraction of marjoram was performed in the central laboratories of the Animal Production Department at Al-Muthanna University, as documented by [17]. The marjoram herbs were preserved until required for utilization. Fifty grams of marjoram were combined with 300 milliliters of distilled water to generate the aqueous extract. The extract was thereafter placed in hermetically sealed containers, agitated and

shook. The containers were thereafter immersed in a water bath at 60°C for one hour and then allowed to rest for 24 hours. The extract was subsequently filtered in two phases: initially employing layers of medical gauze, followed by the use of filter paper. The extract was further subjected to centrifugation to eliminate contaminants and evaporate the water, yielding a concentrated aqueous extract. The extract was subsequently partitioned and allocated into tubes for utilization.

Extraction of Marjoram Oil

The oil extraction procedure was performed in the central laboratories of the Animal Production Department of the College of Agriculture, Al-Muthanna University, as described by [18]. The marjoram herbs were preserved until needed. To create the marjoram oil extract, 100 grams of the plant were combined with 500 milliliters of olive oil. The extract was thereafter placed in hermetically sealed containers, agitated and shook. The containers were thereafter immersed in a water bath at 60°C for one hour and then allowed to rest for 24 hours. The extract was subsequently filtered in two phases: initially with layers of medical gauze, followed by filter paper. The extract was further subjected to centrifugation to eliminate contaminants and evaporate the oil, yielding a concentrated oil extract. The extract was subsequently partitioned and allocated into tubes for application.

Collection of sperm

The accessible samples (testes) in the laboratory were positioned at ambient temperature. The epididymis was subsequently detached from the testicular body with scissors. The tail of the epididymis was subsequently sectioned into small fragments and placed in specialized 10 ml tubes containing a pre-prepared solution: diluted Tris with egg yolk for the first treatment, aqueous marjoram extract for the second, and oily marjoram extract for the third, aimed at diluting the elevated sperm concentration and preserving the sperm. The diluted solution was well mixed, and the sperm-containing solution was extracted using a BioPet device and deposited on a glass slide for preliminary examination under a light microscope at 40x magnification

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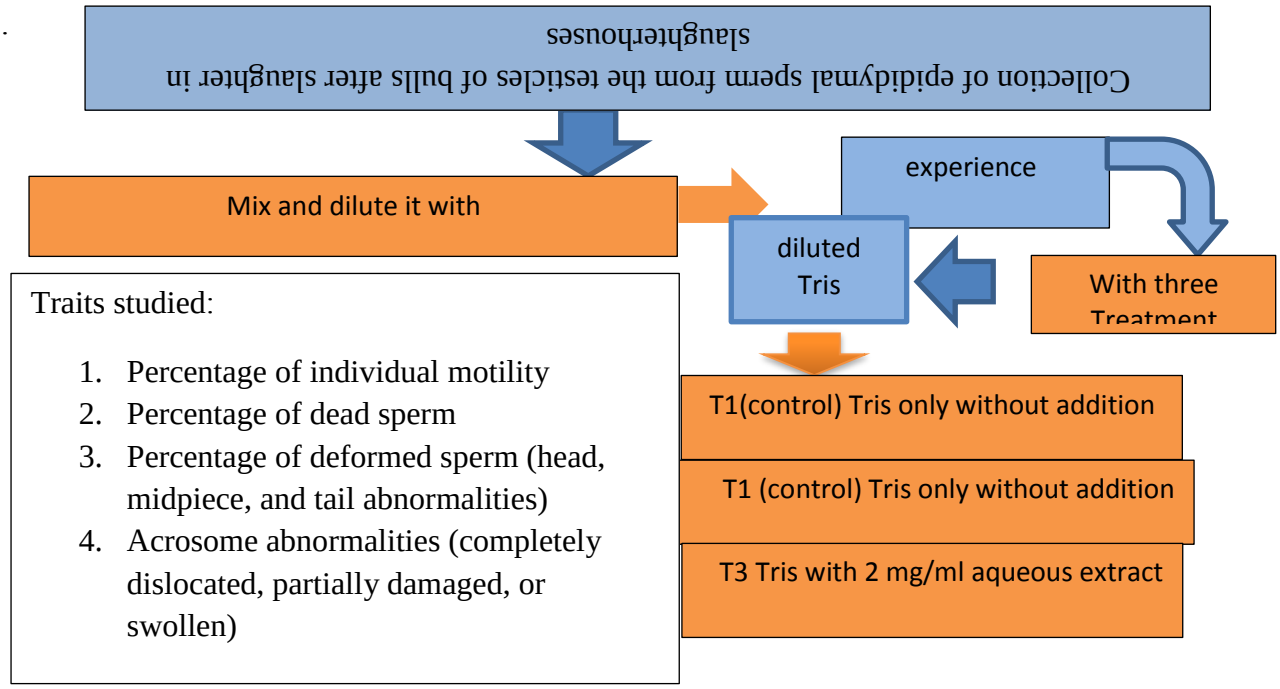

Figure (1) Experimental diagram

Semen Evaluation.

Individual Motility

Sperm motility was assessed using the methodology of [19] by applying a drop of diluted semen onto a glass slide at 37°C, covering it with a slip, and observing it under a microscope at 40x magnification. Individual motility was assessed by evaluating the percentage of progressive motility, the vigor of movement, and the velocity. Subsequently, sperm exhibiting aberrant motility were quantified, their movement assessed, and transformed into percentages for statistical analysis and result presentation.

Sperm Viability

The proportion of viable sperm was determined according to the findings presented in [20]. A drop of diluted semen was placed on a glass slide at a temperature of 37°C. A single drop of a dye combination composed of eosin (1.67), necrosin (5g), and sodium esters (2.9g) was subsequently added. Subsequently, the two droplets were thoroughly combined utilizing an additional glass slide. A smear was prepared on a separate glass slide at a 45-degree angle and thereafter inspected under a light microscope at a magnification of 40x. Live sperm exhibited transparency as they did not absorb the dye, whereas dead sperm displayed a pink hue. Subsequently, 200 spermatozoa were enumerated from various regions of the slide using a microscope. Subsequently, the proportion of viable sperm in the ejaculate was assessed. This is in accordance with the subsequent equation:

percentage of live sperm= Number of live, uncolored sperm/ Total sperm count*100

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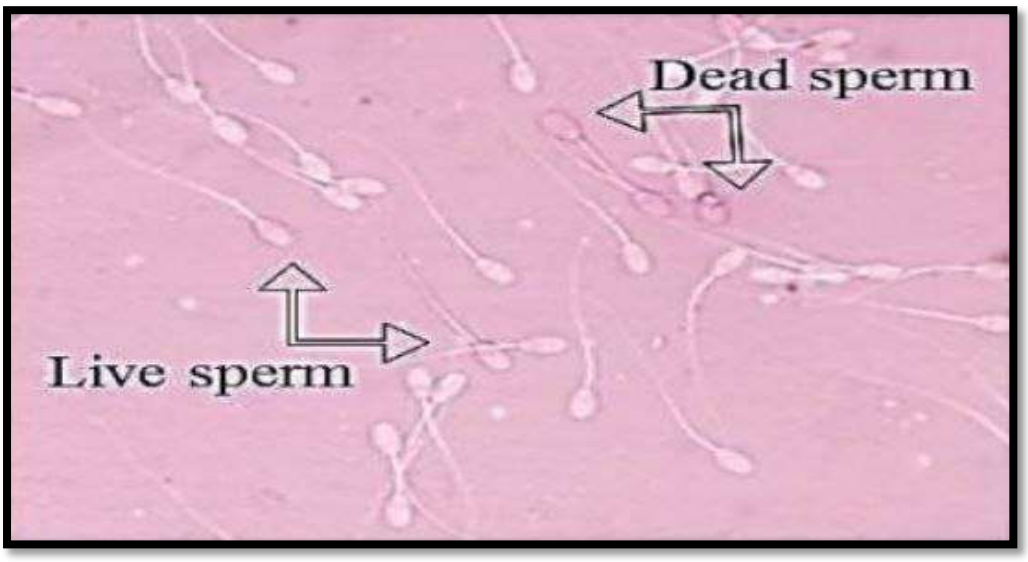

Figure (2) Live and dead sperm

Abnormal sperms percentage

The proportion of aberrant sperm was determined using the methodology of [21], utilizing the same slide employed for assessing the percentage of viable and non-viable sperm. Eosin and necrocin stains were utilized, and 200 spermatozoa were analyzed across several microscopic fields. As to [22], the anomalies were characterized as irregularities in the sperm head, which may present as pear-shaped, diminutive, thin, expanded, bifurcated, or detached. The anomalies of the sperm midpiece may include swelling, duplication, or protoplasmic characteristics. Additional sperm abnormalities include irregularities in the principal and terminal segments of the sperm tail, which may be coiled, bifurcated, or fractured. The proportion of abnormal sperm was determined for each segment of the total aberrant sperm using the following equation:

Abnormal sperms percentage= Number of deformed sperm/ Total number of sperm *100

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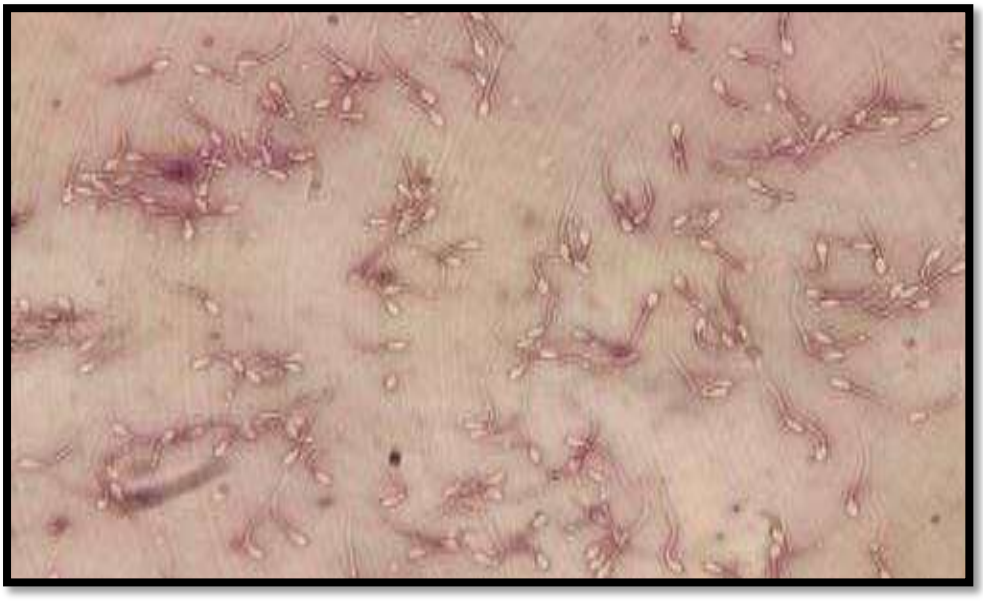

Figure 3: Abnormal sperms

Statistical Examination.

A completely randomized design (CRD) is employed to examine the impact of various treatments on the qualities under investigation. Substantial variations between means are assessed via the [23] multinomial test. The pre-existing statistical software SAS (2001) is employed for statistical analysis based on the subsequent mathematical model:

Yij=M+Ti+eij

Yij= The value of the trait under study.

M= Average value.

Ti= Effect of treatment.

Eij= The value of a random experimental error that is normally distributed with an overall mean of zero and a variance equal to the sum of the squares of the experimental error.

Table (1) Tools and devices used in the study and their details

No.	Device	Country of manufacture	Equipment and instruments
1	CO2 incubator	Germany	Co2 incubator
2	Centrifuge	Germany	Centrifuge
3	Sensitive balance	England	Sensitive Balance
4	Refrigerator	France	Refrigerator
5	pH meter	China	pH-meter

Result and Desiccation

Effect of extracts and storage periods on the percentage of individual sperm motility

Table (2) illustrates the impact of extracts and storage durations on the percentage of individual sperm motility during cryopreservation at 5°C. The findings demonstrate that there are no significant disparities in individual sperm motility percentages between the oily and aqueous dilutions of marjoram and Tris dilution during the initial hour of cryopreservation at 5°C. The

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results demonstrated a statistically significant superiority ($P<0.05$) of the T4 treatment compared to the control treatment at 24, 48, 72, and 96 hours, with values of (71.67±3.76, 68.00±5.20, 60.33±5.17, 52.67±3.28). No significant changes were seen between the T4 treatment and the T2 treatment at the 24 and 48-hour intervals. The results from Table (2) indicated that the T4 treatment exhibited a markedly significant superiority compared to the T3, T2, and T5 treatments. And T0 over the 96-hour storage duration.

The notable efficacy of the 0.01% oil extract of marjoram is ascribed to its active constituents, such as flavonoids and phenols, together with its antioxidant and antibacterial attributes [24]. The efficacy of the T4 treatment surpasses that of both alternative therapies and the control after refrigeration at 5°C for 96 hours, attributable to the presence of antioxidants in the marjoram extract that mitigate the effects of cold shock. It also comprises several phenolic derivatives and ketones that mitigate the detrimental effects of free radicals on sperm. Moreover, elevated concentrations of reactive oxygen species (ROS) adversely impact sperm motility through oxidative damage, resulting in diminished motility during fertilization. This adverse effect arises from the abundance of polyunsaturated fatty acids in sperm, which are susceptible to reactive oxygen species [25]. A notable disparity was also detected among T5, T4, and T3 in comparison to treatment T1. A greater proportion of immobile sperm was noted across all treatments in comparison to treatment T1. Cryopreservation induces considerable stress to sperm [26]. Research has demonstrated distinct variations across animals regarding the cryopreservation resistance of their sperm. This results from the varying compositions of the sperm's plasma membrane and their susceptibility to cold shock. The primary characteristic of sperm is the ratio of phospholipids to cholesterol. Consequently, an increased ratio of cholesterol and phospholipids enhances the flexibility of the plasma membrane and its resistance to cold shock [27]. During cryopreservation, the elevation of ribonucleotide synthesis (ROS) results in diminished antioxidant levels, consequently impairing individual motility [28.[

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Table (2) Effect of extracts and storage periods on the percentage of individual sperm motility (mean ± standard error(

Treatments	storage periods) hours(					significant level
	0	24	48	72	96	
T1	2.85± 75.67 A	3.76± 71.67 AB	5.2± 68.00 AB	5.1± 60.33 BC	3.28± 52.67 C	*
T2	2.23± 76.43 A	4.04± 73.00 A	4.91± 69.67 A	2.85± 65.67 AB	1.73± 58.00 B	*
T3	0.67± 77.67 A	4.63± 72.67 A	4.36± 68.00 AB	1.76± 61.67 B	2.08± 55.00 B	*
T4	2.40±79.67 A	2.85±76.67 A	2.67±74.33 A	3.18±71.33 AB	2.33±64.67 B	*
T5	1.86±77.67 A	2.85±75.67 AB	3.21±71.00 AB	3.38±67.33 BC	0.33±59.33 C	*
significant level	*	*	*	*	*	*

Different capital letters within a column indicate significant differences between storage periods. * means presence of significant differences between treatments (P<0.05).
NS indicates no significant differences between treatments (P<0.05).
T1: Control treatment with Tris dilute.
T2: Treatment using a 1% aqueous extract of marjoram with Tris dilute.
T3: Treatment using a 2% aqueous extract of marjoram with Tris dilute.
T4: Treatment using a 1% oily extract of marjoram with Tris dilute.
T5: Treatment using a 2% oily extract of marjoram with Tris dilute.

Effect of extracts on individual motility, percentage of live, dead and deformed sperm, plasma membrane integrity and acrosome integrity

Table (3) presents the impact of the extracts on individual motility, the proportions of viable, non-viable, and malformed sperm, as well as the integrity of the plasma membrane and acrosome. The findings of the present study, as illustrated in Table (2), indicate that the plant extracts exerted a significant influence (P<0.05) on the individual motility trait. Specifically, the T4 treatment demonstrated a marked superiority over treatments T1, T2, and T3, with values of (61.64±2.29, 63.12±2.27, 66.5±2.07, 75.13±1.15). Conversely, no significant differences were observed among treatments T1, T2, and T3, nor between treatments T4 and T5. The results indicated no significant changes in live sperm among treatments T1, T2, and T3, nor between treatments T4 and T5. The 1% oil extract therapy was

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substantially more effective (P<0.05) than The T1 and T3 treatments yielded measurements of (57.43±2.60, 59.38±4.69, 76.45±0.78). Table 1 indicates a notable reduction in the percentage of dead sperm between the T4 and T3 therapies compared to the control treatment, with values of (27.49±2.76, 18.53±1.94).. No significant differences were seen between the control treatment and the T2, T1, and T5 treatments. The findings indicated no significant variations in the percentage of malformed sperm among the T1, T2, T3, and T5 treatments. Nonetheless, there was a notable reduction (P<0.05) in the T4 treatment relative to all other therapies.

The study results demonstrated a marked advantage of the T4 and T5 therapies compared to the control, T2, and T3 treatments, with measurements of (55.88±2.45, The integrity of the plasma membrane was quantified (65.67±2.09, 63.44±2.03, 76.09±1.73, 72.02±1.28), revealing a substantial reduction in the control treatment relative to all other treatments. No significant differences were observed between the T2 and T3 therapies, as well as between the T4 and T5 treatments. The results demonstrated a substantial (P<0.05) advantage in the integrity of the peripheral body for the T4 and T5 treatments compared to the T2, T3, and control treatments. The measurements were (54.84±2.44, 63.24±1.92, 64.14±1.87, 74.32±0.86, 65.24±1.30). No substantial differences were observed between the T5 and T4 therapies and the T2 and T3 treatments. There was a substantial decrease (P<0.05) in the T1 therapy compared to T2, T3, T4, and T5.

The efficacy of marjoram leaf oil extract over specific durations is attributed to the plant's flavonoids, terpenes, and other antioxidants, which are crucial for the repair, restoration, and preservation of sperm testicular cells, safeguarding them from oxidative damage.

[16]confirmed that marjoram oil possesses antioxidants that mitigate oxidative stress, which impacts fertility in sheep.

Research indicates that sperm are perpetually subjected to oxidative stress owing to the limited antioxidant capacity of the cytoplasm and the abundance of unsaturated fatty acids in the sperm membrane. This results in free radical assaults on these acids, modifying the enzymatic system within the cell and adversely affecting sperm quality and its fertilization capability. The incorporation of marjoram in enzymatic antioxidants elevated their concentrations in seminal plasma. These antioxidants disrupt the thermal oxidation chain of lipids and diminish the release of harmful aldehydes, which impair sperm motility and fertilization capacity, resulting in abnormalities in the sperm head and acrosome nucleus. Conversely, elevated quantities of reactive oxygen species in semen may alter sperm motility, consequently hindering their movement.

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Table (3) Effect of extracts on individual motility, percentage of live, dead and deformed sperm, plasma membrane integrity and acrosome integrity (mean ± standard error.(

Treatments	Traits studied					
	Individual motility	**Percentage of live sperm**	**Percentage of dead sperm**	**Percentage of deformed sperm**	**Percentage of plasma membrane integrity**	**Percentage of acrosome integrity**
T1	2.29±61.64 C	2.60±57.43 C	2.76±27.49 A	2.79±31.33 A	2.45±55.88 C	2.44±54.84 C
T2	2.37±63.12 C	2.19±64.16 Bc	2.01±24.76 Ab	2.22±27.31 Ab	2.09±65.67 B	1.92±63.24 B
T3	2.07±66.05 Bc	4.69±59.38 C	2.05±27.25 A	2.22±27.29 Ab	2.03±63.44 B	1.87±64.14 B
T4	1.15±75.13 A	0.78±76.45 A	1.94±18.53 B	2.07±21.29 B	1.73±76.09 A	0.86±74.32 A
T5	1.18±71.27 Ab	1.67±69.75 Ab	1.80±22.00 Ab	1.85±24.73 Ab	1.29±72.02 A	1.30±65.24 A
significant level	*	*	*	*	*	*

Effect of extracts on individual motility, percentage of live, dead and deformed sperm, plasma membrane integrity and acrosome integrity (mean ± standard error(

The experimental results in Table (4) illustrate the impact of storage durations on individual motility, the proportions of live, dead, and malformed sperm, as well as the integrity of the plasma membrane and acrosome. The results indicate significant differences ($P<0.05$) in sperm motility across various storage durations. Notably, individual motility was markedly superior ($P<0.05$) during the initial hour of storage, averaging 75.02, and at the 24-hour mark, averaging 72.02, in contrast to the 48, 72, and 96-hour storage periods, which averaged 66.24, 64.48, and 59.45, respectively. No substantial differences were observed in individual motility between the storage durations of 72.96 hours and 48.72 hours; however, there was a significant reduction ($P<0.05$) in the percentage of viable sperm during the 96-hour storage period when compared to the 0.24-hour storage period, with values recorded as (73.27±1.44, 68.59±3.49, 58.23±3.29). No significant variations were seen in the proportion of live sperm between the 47.72-hour and 0.24-hour storage durations. The results indicated a statistically significant superiority ($P<0.05$) in dead sperm after a 96-hour storage period

compared to the 48, 72, and 24-hour storage periods, with measurements of (13.30±1.26, 18.94±0.95, 23.89±1.18, 29.49±1.35, and 34.41±1.18.(

A notable reduction (P<0.05) in the proportion of dead sperm was seen across all storage durations: 72, 48, and 24 hours. Regarding malformed sperm, we noted a substantial superiority (P<0.05) for the 96-hour storage duration in comparison to the initial hour of storage, with averages of 15.59 and 37.92, respectively. A substantial decrease (P<0.05) was seen between the 0, 24, and 48-hour storage intervals, with values of (15.59±2.79, 20.46±2.22, 26.27±2.22), respectively.

The results indicated substantial differences (P<0.05) in the percentage of malformed sperm across all storage durations: 48, 72, 24, 0, and 96 hours. The statistical analysis findings from the cryopreservation of bull semen revealed substantial variations (P<0.05) in sperm plasma membrane integrity between the 96-hour storage period and the first hour of storage, with values of (71.76±1.75, 61.80±3.41). No significant differences (P<0.05) were seen across the time intervals of 0, 24, and 48 hours, as well as the storage durations of 48, 72, and 96 hours.

The results indicated no variations in the integrity of the acrosome. Notable (P<0.05) changes were detected between storage durations of 72 and 96 hours, in addition to those between 0, 24, and 48 hours. Notable variations (P<0.05) were seen between storage durations of 24 and 72 hours, in contrast to storage durations of 96 and 72 hours, with measurements of (70.92±1.61, 69.64±1.55, 61.00±2.52, 59.24±2.38), respectively.

The substantial reduction (P<0.05) observed during the 96-hour storage period, in contrast to the initial hour, is attributable to refrigeration's detrimental effect on the function and composition of the sperm's mitochondrial membrane. Alterations in the mitochondrial membrane signify malfunction, diminishing the mitochondria's efficacy in ATP production, which is crucial for sperm motility and metabolic processes [29]. The findings indicated a notable rise (P<0.05) in the proportion of deceased sperm over the 96-hour storage duration. This is ascribed to the utilization of marjoram plant extract, which comprises stored carbohydrates. Marjoram extract additionally include sugars present in the inner leaf tissue [15]. The absence of notable variations (P<0.05) in acrosome integrity during 72 and 96 hours of storage is ascribed to the antioxidants in marjoram leaves, including vitamin E. A single molecule of vitamin E can safeguard 1,000 molecules of unsaturated fatty acids, abundant in the plasma membrane and acrosome of sperm [30]. Vitamin E contributes to safeguarding sperm against oxidative damage induced by increased free radical levels. Vitamin E is significant in averting anomalies in the sperm tail [31.[

Table (4) Effect of extracts on individual motility, percentage of live and dead sperm, deformed sperm, integrity of plasma membrane and integrity of acrosome (mean ± standard error(

Storage time (hour)	Traits studied					
	Individual motility	Percentage of live sperm	Percentage of dead sperm	Percentage of deformed sperm	Percentage of plasma membrane integrity	Percentage of acrosome integrity
1	1.32±75.02 A	1.44±73.27 A	1.26±13.30 E	2.79±15.59 E	1.75±71.76 A	1.61±70.92 A
24	1.29±72.02 A	3.49±68.59 Ab	0.95±18.94 D	2.22±20.46 D	1.57±70.33 A	1.55±69.64 A
48	1.94±66.24 B	3.14±64.00 Bc	1.18±23.89 C	2.22±26.27 C	2.52±66.96 ab	2.53±65.40 ab
72	1.89±64.48 Bc	3.16±63.07 Bc	1.35±29.49 B	2.07±31.80 B	2.69±62.24 b	2.52±61.00 B
96	2.31±59.45 C	3.29±58.23 C	1.18±34.41 A	1.64±37.92 A	3.41±61.80 b	2.38±59.24 B
significant level	*	*	*	*	*	*

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