EXCEPTIONAL ANTIOXIDANT EFFECT OF *URTICA DIOICA* EXTRACT ON MOTILITY AND SOME OTHER POST THAWED GOAT SEMEN CHARACTERISTICS

AL-JAF, K.[1*] – OMAR, C.[1] – VAZIRY, A.[2]

[1]*Department of animal science, college of agricultural engineering sciences, University of Sulaimani, Sulaymaniyah 46001, Iraq*

[2]*Department of animal science, college of agriculture, University of Kurdistan, Sanandaj 66414, Iran*

[*]*Corresponding author*
e-mail: komar.hasan@univsul.edu.iq; phone: +964-770-157-4882

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Abstract. This study was carried out to examine the effects of *Urtica dioica* extract on goat cryopreserved semen quality. The ejaculates of Markhoz goats (n = 12) were collected by an electro-ejaculator. The semen was diluted and supplemented with different concentration of aqueous or hydroalcoholic *Urtica dioica* extract of aerial or root parts as follows: aerial-aqueous ($AA1_{5μg/ml}$, $AA2_{15μg/ml}$ and $AA3_{45μg/ml}$); aerial-ethanolic ($AE1_{5μg/ml}$, $AE2_{15μg/ml}$ and $AE3_{45μg/ml}$); root aqueous ($RA1_{5μg/ml}$, $RA2_{15μg/ml}$ and $RA3_{45μg/ml}$); root-ethanolic ($RE1_{5μg/ml}$, $RE_{215μg/ml}$ and $RE3_{45μg/ml}$) and control ($CCC_{0mg/ml}$) without additives. After thawing, sperm samples were subjected to viability, acrosome integrity, plasma membrane integrity, motility, sperm chromatin dispersion (SCD) and concentration of malondialdehyde (MDA) assays. Addition of $AA1_{5μg/ml}$ to the extender significantly ($P < 0.05$) improved progressive motility, viability, plasma membrane integrity, acrosome integrity, and DNA integrity of the semen samples. Sperm viability and plasma membrane integrity in aqueous extract (both aerial & root parts)-supplemented extenders were relatively higher, as compared to the control. Moreover, a significant decrease in MDA concentration was detected in semen supplemented with *Urtica dioica* extract treatments. In conclusion, the results indicate that 5μg/ml of aqueous extract of *Urtica dioica* aerial part improved sperm quality and could be recommended as an additive to the extender for cryopreservation. This study shows a novel approach in goat semen cryopreservation due to the remarkable influence of *Urtica dioica* on post-thawed sperm parameters.
Keywords: *angora goats, cryopreservation, sperm quality, medicinal plant*

Introduction

Artificial insemination (AI) can definitely be considered as the oldest and most widely used assisted reproductive technology (ARTs) in the livestock industry (Faigl et al., 2012). It is a well-established biotechnique in small ruminant production which includes collection of semen from the male and transferring it manually into the reproductive tract of the female (Allison et al., 2014). This reproductive technology has contributed greatly to genetic improvement and better control of sexually transmitted diseases. AI is a technique that has already been used for years in goats, especially in developed countries (Arrebola et al., 2013; Bucak et al., 2020). Efforts also have been increased to improve post-thawed cryopreserved semen quality. According to the findings, ice crystal formation, osmotic stress, lipid peroxidation and alterations in the plasma membrane are the causes of the poor rate of cryo-survival (Bahmani et al., 2015).

Cryopreservation as a complex process and temperature alteration cause damaging effect on sperm cells during freeze-thawing procedure, especially goat sperm is prone to

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thermal shock that lead to reduce fertilizing capacity (Bansal and Bilaspuri, 2011). Also, as unavoidable biproduct of physiological activities, reactive oxygen species (ROS) are significant components that cause damage to the sperm cells. Over production of ROS as a result of an imbalance between ROS generation and ROS removal during freeze-thaw process bring extra damages to the sperm cell by interacting with a number of biological macromolecules, particularly lipids, proteins, and nucleic acids. Mammalian sperms are very vulnerable to lipid peroxidation (LPO) due to the high concentration of polyunsaturated fatty acids in the cell membrane. LPO decreases sperm motility, viability, membrane integrity, sperm function, and fertility by damaging the lipid matrix structure of the sperm membrane (Yüce et al., 2013). The antioxidant system available in the semen cannot counteract the oxidative stress by its own; so, in order to reduce oxidative stress during freezing, antioxidants can be added to the extender which consequently reduces ROS generation and improve sperm quality after thawing.

Markhoz goat is considered as most important genetic resources of angora breed that serves as multifunctional animal which provide milk, meat and wool. This angora goat is at the risk of extinction and genetic erosion because of disease and natural disasters (Bahmani et al., 2015). Therefore, it is necessary to focus on increase the population of this breed by improving reproductive performance and enhancing of fertilizing capability.

Several studies have explored the use of plant extracts to mitigate cryodamage and improve the viability and functionality of cryopreserved semen after thawing. These extracts contain bioactive compounds that possess cryoprotective properties, including antioxidant and membrane-stabilizing effects. While the specific mechanisms may vary among plant species, their application has shown promising results in enhancing the quality and fertility potential of cryopreserved semen samples.

Certain plant species have received particular attention in the field of semen cryopreservation due to their known cryoprotective properties. For example, *Tribulus terrestris* and *Cinnamomum zeylanicum* (Ariyan et al., 2021) have been extensively studied for their ability to improve semen cryopreservation outcomes. These plant extracts have demonstrated the ability to safeguard spermatozoa from cryoinjury and maintain post-thaw motility, membrane integrity, and DNA integrity.

Urtica dioica (UD) also known as common nettle or stinging nettle, is a herbaceous perennial flowering plant, belongs to the family Urticaceae which includes herbs, shrubs, and trees, usually with small greenish flowers (Kukrić et al., 2012; Ros-Santaella and Pintus, 2021). UD has a wide use in traditional folk medicine; its seeds have been used for cancer treatment (Kaya et al., 2013), as an anti-inflammatory agent, to treat urinary tract disorder as well as regulating factor for the operating-cycle (Baninameh et al., 2016). In some countries, leaves of UD is used for the treatment of vaginal discharge, diarrhea, anemia, internal/external bleeding and general well-being (Kukrić et al., 2012).

The UD root extract is broadly used for the treatment of hormonal disorders such as prostate disorder (Huang et al., 2010) as antiproliferative agent (Konrad et al., 2000). Aqueous extract of UD is also used as co-supplementation to semen extender in Holstein bulls with significant effect on post-thawed semen characteristics (Mohamed and Abdulkareem, 2020). Both aerial and root parts of UD compose of varieties of valuable chemical components, containing essential amino acids, vitamins, and flavonoids (Aksu and Kaya, 2004) with some pharmacological properties such as antiulcer (Gülçin et al., 2004), anti-bacterial, anti-fungal (Hadizadeh et al., 2009), anti-oxidant, anti-tumor and anti-mutation effects (İşler et al., 2010).

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However, further research is needed to fully understand the underlying mechanisms behind the cryoprotective effects of plant extracts and optimize their application in semen cryopreservation protocols. Therefore, this study aims to investigate the potential of *Urtica dioica* extract in enhancing the success of semen cryopreservation. Our research will assess its impact on sperm quality parameters such as motility, viability, acrosomal integrity, sperm chromatin dispersion SCD, DNA integrity and lipid peroxidation, contributing to the ongoing efforts to optimize semen cryopreservation techniques.

Materials and methods

Study Site

This study was conducted at the farm facilities and the laboratories of reproductive physiology-department of animal science-college of agriculture, University of Kurdistan-Sanandaj, main campus (35.2784° N, 46.9939° E) Islamic Republic of Iran.

Chemicals

All chemicals used in this experiment were obtained from Sigma-Aldrich (St. Louis, MO, USA) and Merck (Darmstadt, Germany).

Urtica Dioica extraction

The complete fresh plant (aerial and root parts) of *Urtica dioica* (common nettle) was collected from Tawriwar village in Sanandaj-Kurdistan, Iran at Friday 3rd September 2021. The plant was washed perfectly with fresh water to completely remove dirt and mud. The aerial part separated from root part then both parts dried in room temperature away from direct sun light. Both parts of the dried plant were crushed to fine powder using a mill.

Preparation of hydro-alcoholic extraction of UD

The dried fine powder of (aerial and root) parts of the herb were used as: 100 grams fine powder of each part macerated in 900 ml of ethanol 96% (1: 9 ratio) in dark bottle for 48 hours with regular shaking every 2 hours to prepare hydro-alcoholic extraction of both parts separately. After 48 hours of maceration, the extract was obtained by filtering the mixture using filter papers. Finally, the rotary evaporator (at 40 °C) was used to remove the ethanol part of the mixture. The extract was skipped into a petri dish and put in oven at 35 °C for 24 hours to remove the remaining ethanol and then the pure extracts were stored at 4 °C (Dhouibi et al., 2018).

Preparation of aqueous extraction of UD

Same procedure was used to prepare aqueous extraction of the plant by maceration 100 grams of the fine powder in 900 ml of distilled water (1: 9 ratio) separately for both aerial and root parts. After 48 hours of maceration, the extract was obtained by filtering the mixture using filter papers, and the water part removed using rotary evaporator (at 40 C), then the remaining part was skipped into a petri dish and kept in oven at 35 °C till the moisture dried and then stored at 4 °C (Ahmadi et al., 2014; Dhouibi et al., 2018).

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Identification of volatile compounds in UD extract

Gas chromatography device connected to mass spectrophotometry (GC / MS) (Model: Agilent cA789) was used to identify volatile chemical compounds in the UD extracts following heidarizadeh et al. protocol (Haidarizadeh, 2018).

Experimental animals

A total of twelve (12) young, mature (2-3 years of age) Kurdish Maraz (Iranian Angora-Markhoz) bucks (mean weight 37 kg ±3) were trained for semen collection using the Electro-ejaculator. The general process of the study is illustrated in *Figure 1*. The study was conducted between September (early autumn) and December (early winter), 2021. During this period, the bucks were grazed on natural pastures and provided by freshwater *ad libtum*. Additional nutrient supplementation was also provided (Al-Jaf and Del, 2019).

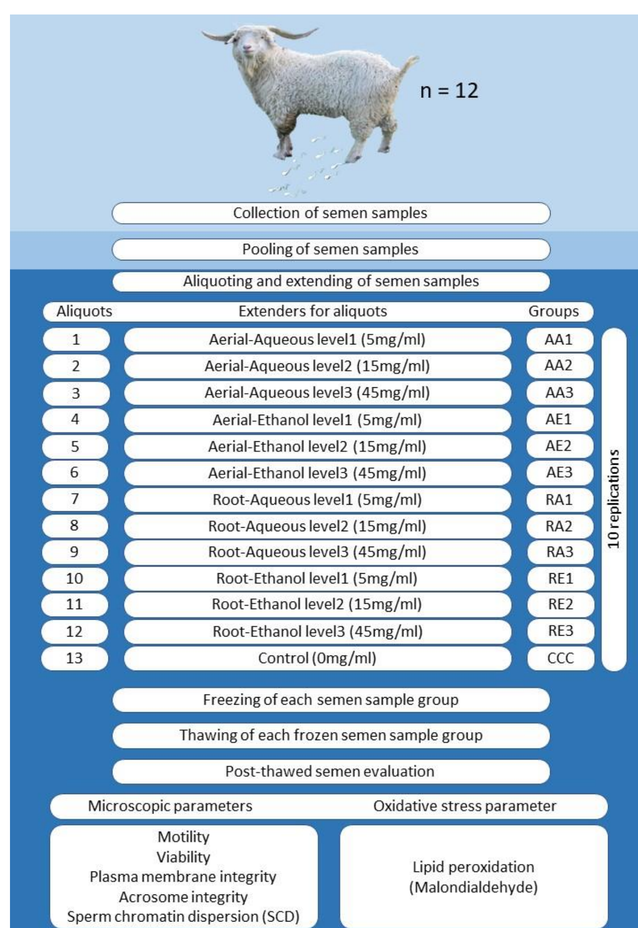

Figure 1. *Schematic illustration of the study indicating the animals, experimental design and all evaluations*

Semen preparation and extender

The semen samples were taken from each male for 10 weeks (10 replications). The selected goats were under uniform feeding and housing conditions. Immediately after collection and macroscopic evaluation (semen spermatozoa less than 500×10^6 sperm/ml

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were removed and did not pool) (Sadeghi et al., 2020), the ejaculates were placed and kept in a thermo-flask at 37 °C, until semen quality assessment. Semen samples were evaluated macroscopically and then pooled together to remove individual differences. Pooled semen allocated into Tris base extender for dilution consisting of 3.786 gr Tris, 2.172 gr citric acid and 1 gr fructose in 100 ml distilled water, containing 7% (v/v) glycerol and 5% (v/v) egg yolk (Evans and Maxwell, 1987) by dilution ratio 1:12 (1 ml semen: 12 ml extender). The osmolarity of the solution was fixed on mOsm 320 and pH = 7.2.

The experimental treatments of this study included tris base extender with zero additives (control-$CCC_{0µg/ml}$) and three different doses (5 µg/ml, 15 µg/ml and 45 µg/ml) of each extract part of the plant as the following: aerial part-aqueous extract ($AA1_{5µg/ml}$, $AA2_{15µg/ml}$ and $AA3_{45µg/ml}$); aerial part-ethanolic extract ($AE1_{5µg/ml}$, $AE2_{15µg/ml}$ and $AE3_{45µg/ml}$); root part-aqueous extract as ($RA1_{5µg/ml}$, $RA2_{15µg/ml}$ and $RA3_{45µg/ml}$) and root part-ethanolic extract as ($RE1_{5µg/ml}$, $RE2_{15µg/ml}$ and $RE3_{45µg/ml}$).

After being deposited into 0.25 ml straws and having their ends sealed with polyvinyl chloride powder, the diluted specimens were placed at 4 °C for three hours. Then, the straws were placed in nitrogen vapor 4 cm away from the liquid nitrogen level. The straws were placed in the liquid nitrogen after 15 minutes. Frozen straws were thawed at 37 °C for 30 seconds to assess the post thawed sperm parameters.

Sperm motility and velocity parameters

Motility and velocity parameters were assessed by computer-assisted sperm analysis system (CASA-Program: Model-: IVOS version 12; Hamilton-Thorne Biosciences, MA, USA: phase contrast; frame rate - 60 Hz; minimum contrast - 70; low and high static size gates - 0.6 to 4.32; low and high intensity gates - 0.20–1.92; low and high elongation gates 7–91; default cell size - 10 pixels; default cell intensity 80). Semen was diluted after thawing immediately with a tris-base extender (without glycerol and egg-yolk) by a ratio of (5:95) microliter extender and evaluated directly. A 7-µl of diluted semen sample was put onto a pre-warmed chamber slide (Leja 4, Leja Products, Luzernestraat B.V., Holland) and Spermatozoa motility characteristics were determined using 10X objective lens at 37 °C as following; TM (total motility %), PM (progressive motility%), NPM (Non-progressive motility %), IM (immobile %), VAP (average path velocity, µm/s), VSL (straight line velocity, µm/s), VCl (curvilinear velocity, µm/s), ALH (amplitude of lateral head displacement, µm), BCF (beat cross frequency, Hz), STR (straightness, rel. units), LIN (linearity, rel. units). For each evaluation, 22 microscopic fields were captured and analyzed to include at least 1800 cells (Bucak et al., 2010).

Assessment of sperm viability

Viability of spermatozoa was measured by eosin-nigrosine staining method. 5 µl of thawed semen was mixed with 10 µl of eosin-nigrosin solution (Buffer containing; 17.35 g sodium glutamate, 1.28 g potassium citrate, 8.51 g sodium citrate, and 0.68 g magnesium chloride in 1 L distilled water, then dissolving 5 g nigrosine with 1 g eosin in 100 ml of above buffer for eosin-nigrosine stain preparation) (Azimi et al., 2020; Cecere, 2021) and spread on a clean slide to make a smear. Viability of sperm was determined by counting 200 sperm cells in different microscopical fields (Labomed LX400; Labomed Inc., Culver City, CA, USA) (400X) (Mehdipour et al., 2020). Spermatozoa colored in pink were considered non-viable while un-stained spermatozoa were considered viable. See *Figure 2.*

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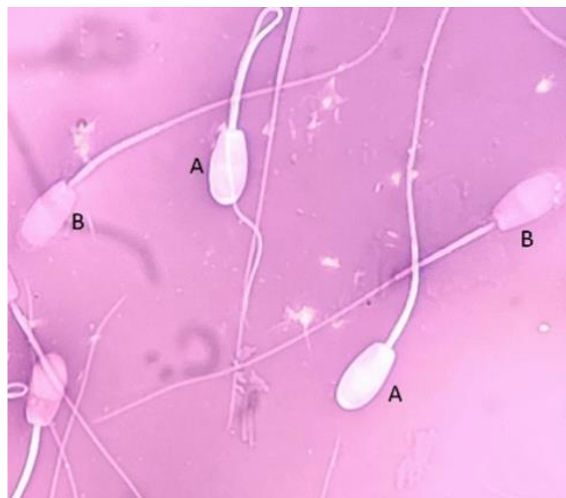

Figure 2. *Evaluation of goat sperm viability using eosin-nigrosine staining. A (unstained / white color) is alive spermatozoa, B (stained / pink color) spermatozoa is dead*

Acrosome integrity evaluation

Acrosome integrity was assessed using formaldehyde citrate solution 4% (Adding 2.9 grams tri-sodium citrate dehydrate to 100 ml of D.W to prepare 2.9% sodium citrate, and 4 ml of 2.9% sodium citrate added to 96 ml formaldehyde 37% to prepare 4% formaldehyde citrate) (Thuwanut et al., 2008). 10 µl of formaldehyde citrate solution mixed with 10 µl of eosin-nigrosin solution in a 0.5cc microtube then 5 µl of diluted semen added, after 30 seconds 25 µl of the mixture added to clean slide then making a smear by dragging the drop by another clean slide in 45° angle. The dried smear examined under the light microscope-1000X to count 10 fields of spermatozoa. Sperms with tab tip acrosome considered normal and vice versa (Bamba, 1988). See *Figure 3.*

Figure 3. *Evaluation of goat sperm acrosome integrity using Formalin citrate solution. A (sperms with normal acrosome), B (sperms with abnormal acrosome)*

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Plasma membrane integrity evaluation

Plasma membrane integrity of the sperm cell was evaluated using the hypo-osmotic solution test (HOST). 100 µl of Hypo-osmotic solution (9 grams fructose, 4.9 grams sodium citrate in 1 L distilled water) mixed carefully with 10 µl of diluted semen in a 0.5 micro-tube and kept in water bath at 37 °C for 60 minutes (Azimi et al., 2020). 10 µl of incubated sample was placed on a clean and pre-warmed slide then covered with a cover slide. Ten different microscopic fields were monitored to count at least 200 sperm cells (400X). Sperms with curled and swollen tails considered as normal. Finally the percentage of normal spermatozoa with intact plasma membrane were calculated (Revell and Mrode, 1994). See *Figure 4.*

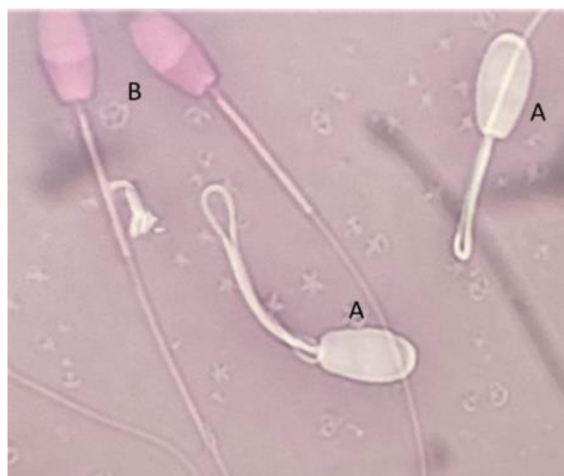

Figure 4. *Evaluation of goat sperm plasma membrane integrity using hypo-osmotic swelling test (HOST). A (sperms with intact plasma membrane), B (dead sperms)*

Sperm chromatin dispersion assay

Sperm chromatin dispersion (SCD) test was used to determine the intact and fragmented DNA in sperm cells. To perform this assay, a pre-coat slide was prepared by adding 150 µl of 65% high melting point agarose, covered by cover slide and left in 4 °C for 5 minutes till became solid. After solidifying the agarose and formation of agarose solid coat, the coverslip removed carefully. Then a mixture of 30 µl of diluted sperm with 70 µl of 0.7% low melting point agarose was transferred to the pre-coated slide and covered with a coverslip directly. After drying in air temperature, the coverslip removed slowly then the slide horizontally put in acid denaturing solution (0.08 N HCl) at 37 °C in the dark for 7 minutes. after that, the slide put in lysing solution (0.4 M Tris base, 0.8 M DTT, 1% SDS, 50 mM EDTA, and 2 M NaCl, pH ¼ 7.5) at room temperature for 25 minutes; then, the slide rinsed by distilled water carefully and dehydrated with 70%, 90% and 100% each for 2 minutes respectively and dried in the air. Finally, dried slide stained by ethidium bromide solution then examined by fluorescence microscopy. After examining and capturing photo of the sperm cells, the ratio between the halo size and the whole nucleus size was calculated. See *Figure 5.*

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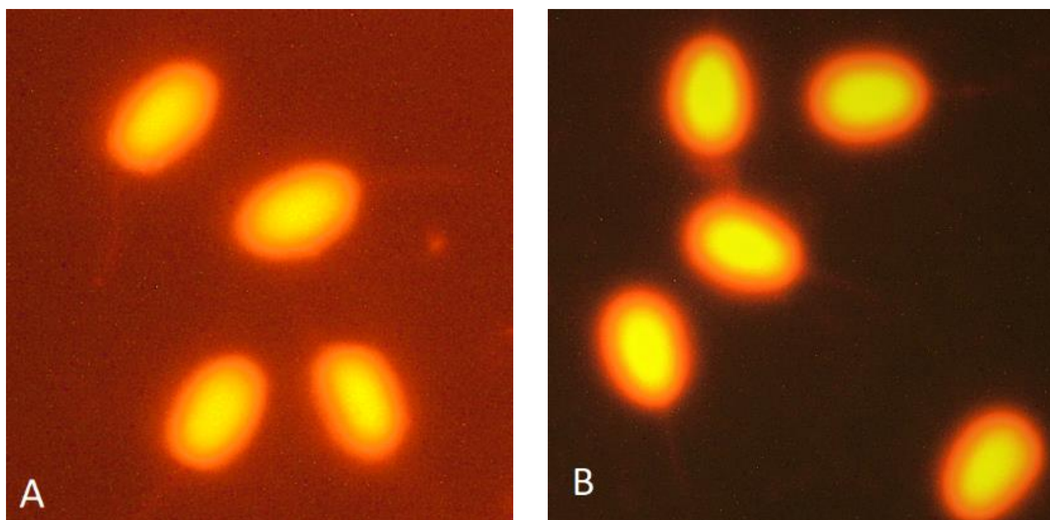

Figure 5. *Sperm chromatin dispersion in A: (Control) and B: (AA1)*

Lipid peroxidation assay

Lipid peroxidation (LPO) was assessed by measuring the concentration of malondialdehyde (MDA) in semen samples using the thiobarbituric acid (TBA) reagent method as explained by Esterbauer and Cheeseman (Esterbauer and Cheeseman, 1990). For this test, semen samples were thawed and immediately centrifuged at 1500 RPM for 5 minutes, then supernatant was isolated.

A mixture from 1 ml supernatant with 1 ml of EDTA (0.037 g EDTA in 10 ml distilled water), 1 ml BHT (0.2 g BHT in 10 ml ethanol) and 2 ml TCA (3 g TCA in 30 ml distilled water), was prepared and then centrifuged at 1200 g for 15 min. 1 ml of this supernatant mixed with 1 ml of TBA (0.134 g TBA in 20 ml distilled water) and incubated in a water bath at 90 °C for 20 min and cooled in normal temperature. the absorbance was read on a spectrophotometer at 532 nm. The MDA concentrations were expressed as nmol/ml.

Statistical analysis

All collected data were subjected to analysis of variance as a completely random design trial using the general linear model procedures of SAS software (2001) (SAS 9.2, Carey, NC). Tukey's test was used as a post hoc test to compare and describe differences between means when the model was declared significant ($P < 0.05$).

Results

The chemical composition of UD extracts (aerial part/aqueous extract) is presented in *Table 1*, (aerial part/ethanolic extract) in *Table 2.* and (root part/aqueous & ethanolic extracts) in *Table 3*.

Motility and velocity parameters of frozen-thawed goat sperm supplemented with different concentrations of UD extract are presented in *Table 4*. The data showed that AA1 (5 µg/ml) was improved significantly ($P < 0.05$) total motility TM ($P = 0.0004$) and progressive motility PM ($P = 0.0001$) as compared to all other treatment groups including control. Also, VCL and ALH in AA1 treatment were significantly ($P < 0.05$) higher than other groups and control.

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Table 1. *Different chemical compositions of aerial part/aqueous extract of UD*

Aerial / Aqueous (AA) extract of UD Plant							
s	**Retention time (mints.)**	**Compound name**	**% Of total**	**s**	**Retention time (Mints.)**	**Compound name**	**% Of total**
1	3.685	Acetamide	0.27	16	9.605	phenylhydrazone	16.04
2	3.847	Pyrazole	0.269	17	10.282	Ethanone	2.613
3	5.702	Phenylmethyl	1.248	18	10.867	2-Propena	3.099
4	5.816	Benzhydrazide	1.165	19	11.76	Benzeneethanamine	0.354
5	6.248	Acetic acid	6.155	20	12.102	Phthalic acid	0.813
6	6.358	Benzamide	3.816	21	12.445	Cinnamaldehyde	0.304
7	6.591	benzoic acid	0.81	22	12.662	Phenylpropanamide	0.223
8	6.66	1-Butanone	1.842	23	15.314	Phenanthrene	1.13
9	7.152	7-Hexadecenal	0.612	24	15.804	enzofuran-2-one	1.328
10	7.5	1-Butanon	0.234	25	16.433	Ethanone	1.564
11	7.647	3,5-Dihydroxytoluene	2.883	26	17.182	Naphthalene	3.933
12	8.149	1,5-Pentanedio	0.285	27	17.777	Benzene	0.282
13	8.589	2-Phenylpropenal	34.01	28	19.059	alpha. -Bromoacetophenone	2.146
14	8.932	Amino-3-bromo	2.197	29	22.965	Naphthalene	3.379
15	9.016	Heptane diamide	2.091	-	-	-	-

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Table 2. *Different chemical compositions of aerial part/ethanolic extract of UD*

Aerial / Ethanolic (AE) extract of UD plant							
s	**Retention time (mints.)**	**Compound name**	**% Of total**	**s**	**Retention time (Mints.)**	**Compound name**	**% Of total**
1	3.296	Tetrazole	0.998	33	9.369	Glycidyl ether	1.908
2	3.464	Acetic acid	3.153	34	9.688	Octadecanoic acid	0.867
3	3.517	Propanoic acid	1.273	35	10.015	Isobarbituric acid	0.731
4	3.607	Formamide	2.692	36	10.745	Pyrimidine-4,6-diol, 5-methyl	0.212
5	3.706	Butanenitrile	0.386	37	10.828	Benzoic acid	0.505
6	3.772	Imidazole	0.603	38	11.267	Alpha-D-galactopyranoside	3.821
7	4.131	Acetamide	1.19	39	11.305	1,2,4,5-Tetrazine	2.728
8	4.227	Formic acid	0.8126	40	12.166	d-Glycero-d-ido-heptose	0.861
9	4.31	1-Aminocyclopentane hydroxamic acid	0.369	41	12.326	Carbonic acid	0.62
10	4.566	Furfural	1.789	42	12.636	1H-Indole	0.593
11	4.654	2-Furanmethanol	1.747	43	13.153	tau-Muurolol	0.529
12	4.725	6-Nonena	0.507	44	13.409	Copaene	5.787
13	5.246	Cyclohexanone	0.19	45	13.698	3-Methyl-2-furoic acid	0.459
14	5.418	Cyclobutanol	0.109	46	13.767	Azulene	0.281
15	5.519	Trimethylene oxide	0.077	47	13.897	Cedrene	0.269
16	5.845	Pyridazinone	0.606	48	14.314	Cinnamal	5.308
17	5.946	2-Hexenoic acid	0.145	49	14.426	CARBAMULT	2.292
18	6.362	Phospholane	0.385	50	14.475	6-Acetyl-. beta. -d-mannose	0.628
19	6.462	Glutamine	1.459	51	14.526	2,4-Pentadien-1-ol, 3-propyl	1.689
20	6.704	3,4-Altrosan	1.783	52	14.759	Phenol, 2-(butylthio)	5.362
21	6.769	2-n-Propylthiane	2.157	53	14.868	4-Mercaptopheno	1.25
22	6.883	d-Mannose	1.719	54	15.028	Maltol	5.077
23	6.971	DL-Arabinose	1.216	55	15.129	Thiophene, 2-propyl	2.565

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Aerial / Ethanolic (AE) extract of UD plant							
s	**Retention time (mints.)**	**Compound name**	**% Of total**	**s**	**Retention time (Mints.)**	**Compound name**	**% Of total**
24	7.505	1,2,4-Triazine	0.907	56	15.337	4-Octen-3-one	5.172
25	7.124	dl-Glyceraldehyde dimer	0.865	57	15.404	Phloroglucinol	6.231
26	7.261	Butyrolactone	2.644	58	15.553	4-Fluorobenzyl alcohol	3.543
27	7.337	n-Hexyl acrylate	1.489	59	15.764	Cedrene	1.267
28	7.479	Isosorbide Dinitrate	1.725	60	16.25	alpha. -Murolene	1.271
29	7.681	1,3-Propanediol	2.963	61	16.681	alpha-Longipinene	0.959
30	8.11	3-Octanol	0.469	62	17.013	10,12-Docasadiyndioic acid	0.292
31	8.3	Methamphetamine	0.229	63	17.165	Calamenene	0.682
32	8.973	Benzenemethanamine	0.271	-	-	-	-

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Table 3. *Different chemical compositions of root part both aqueous and ethanolic extract of Urtica dioica*

	Root / Aqueous (RA) extract of UD plant			**Root / Ethanolic (RE) extract of UD plant**		
s	**Retention time (mints.)**	**Compound name**	**% Of total**	**Retention time (mints.)**	**Compound name**	**% Of total**
1	5.836	Acetic acid	6.256	3.276	Acethydrazide	1.662
2	7.502	phenyl ethynyl	8.136	3.571	Propenoic acid	44.626
3	8.473	Amino-6-methoxypyridine	1.572	4.09	1,2-Hydrazinedicarboxamide	7.464
4	9.677	benzoic acid	25.634	7.2	Acetic acid	4.975
5	11.708	Benzeneethanamine	14.447	8.976	benzoic acid	5.728
6	13.809	Benzene acetic acid	4.538	10.964	Acetamide	18.97
7	15.7	Acethydrazide	9.818	12.274	Phenylmethyl	5.759
8	16.963	Propoxyphene	13.496	14.352	Ethanone	4.531
9	17.59	Acetamide	7.717	15.041	Benzeneethanamine	6.286
10	22.972	Quinoline	2.328	-	-	-

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Table 4. *Effects of different concentrations of Urtica dioica extract on motility parameters of post-thawed goat spermatozoa analyzed by CASA*

Treat	PM	TM	STR %	VCL	ALH	LIN %	VSL	VAP	MAD
Control	24.73 ^{b}	61.17 ^{b}	75	50.97 ^{b}	0.90 ^{b}	28 ^{ab}	17.58 ^{ab}	21.56 ^{b}	53.75
AA1	37.47 ^{a}	76.97 ^{a}	76	58.06 ^{a}	1.25 ^{a}	35 ^{a}	23.33 ^{a}	23.98 ^{a}	53.91
AA2	25.33 ^{b}	62 ^{b}	74	53.09 ^{b}	0.91 ^{b}	28 ^{ab}	18.69 ^{ab}	22.67 ^{ab}	53.43
AA3	13.1 ^{cdef}	41.9 ^{de}	75	30.8 ^{cde}	0.62 ^{cdefg}	27 ^{ab}	10.04 ^{bcdef}	11.9 ^{bcde}	54.18
AE1	12.3 ^{defg}	38.93 ^{de}	72	29.6 ^{de}	0.59 ^{defg}	25 ^{ab}	9.41 ^{cdef}	11.36 ^{bcdef}	55.37
AE2	10.23 ^{efg}	34.57 ^{ef}	77	26.31 ^{def}	0.54 ^{efgh}	27 ^{ab}	8.19 ^{efg}	9.65 ^{def}	57.14
AE3	1.63 ^{g}	11.67 ^{g}	68	13.57 ^{f}	0.25 ^{h}	19 ^{b}	2.88 ^{g}	3.5 ^{f}	57.89
RA1	28.6 ^{ab}	62.15 ^{abc}	71	49.97 ^{ab}	0.92 ^{bc}	26 ^{ab}	16.41 ^{ab}	20.43 ^{a}	54.72
RA2	24.47 ^{abc}	59.17 ^{bc}	77	46.30 ^{ab}	0.90 ^{bcd}	31 ^{a}	15.75 ^{abc}	19.13 ^{ab}	53.52
RA3	20.63	46.03 ^{cde}	72	40.29 ^{bcd}	0.76 ^{bcdef}	26 ^{ab}	13.46 ^{abcde}	16.19 ^{abcd}	54.36
RE1	23.5 ^{abcd}	52.4 ^{bcd}	72	45.18 ^{abc}	0.86 ^{bcde}	27 ^{ab}	15.47 ^{abcd}	18.56 ^{abc}	53.96
RE2	10.83 ^{efg}	29.9 ^{ef}	73	26.65 ^{def}	0.51 ^{fgh}	27 ^{ab}	9.07 ^{defg}	10.65 ^{cdef}	52.42
RE3	4.83 ^{fg}	18.93 ^{fg}	69	18.72 ^{ef}	0.35 ^{gh}	22 ^{ab}	5.34 ^{fg}	6.47 ^{ef}	57.01
P value	0.0001	0.0001	0.1618	0.0001	0.0001	0.0091	0.0001	0.0001	0.2273

Progressive motility (PM); Total motility (TM); straightness (STR = VSL/VAP); curvilinear velocity (VCL, µm/s); amplitude of lateral head displacement (ALH, µm); linearity (LIN% = VSL/VCL); straight-line velocity (VSL, µm/s); average path velocity (VAP, µm/s); mean angular deviation (MAD). a, b, c, d, e... etc.: Different superscripts within the same column indicate significant differences ($P < 0.05$), ($n = 12$)

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Data presented in *Figure 6.* showed the viability of spermatozoa. The viability in AA1 was significantly ($P < 0.05$ / $P = 0.0001$) improved compared to the other treatments and control. After the treatment AA1, AA2 was significantly ($P < 0.05$) higher than control and other treatments except RA1; while AE3 recorded lowest percentage of viable sperms. Other treatments were not registered any significancy among themselves.

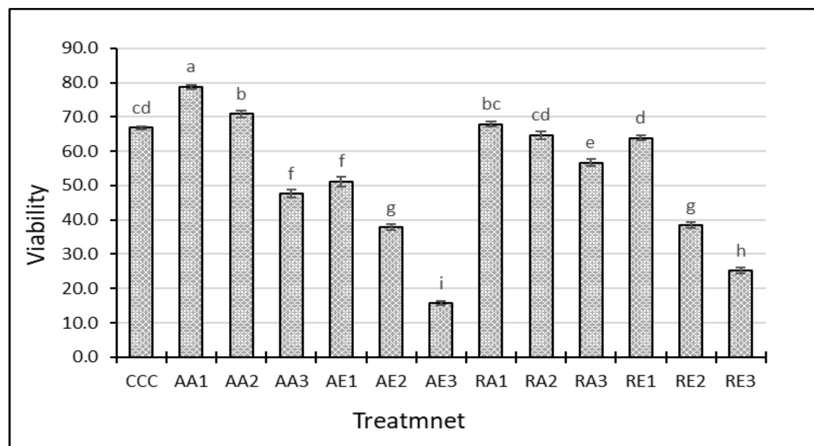

Figure 6. *Viability of the spermatozoa (%). Error bars were expressed as mean ± SE. Means with different letters in each graph are significantly different from each other (P < 0.05)*

While data presented in *Figure 7.* reveals that plasma membrane integrity in AA1 was significantly ($P < 0.05$ / $P = 0.0002$) higher than control and other treatment groups. though some treatments were slightly higher than control, but they did not show any significancy in term of plasma membrane integrity compared to control. Treatment AE3 and RE3 recorded lowest percentage of plasma membrane integrity respectively.

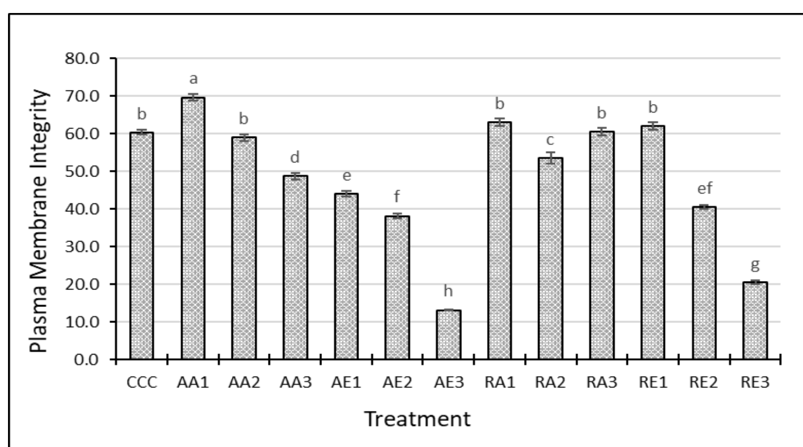

Figure 7. *Plasma membrane integrity of the spermatozoa (%). Error bars were expressed as mean ± SE. Means with different letters in each graph are significantly different from each other (P < 0.05)*

Moreover, as data demonstrate in *Figure 8.*, the acrosomal integrity in AA1 is significantly ($P < 0.05$) higher than other treatments and control. AA2 and RE1 were slightly higher than control but not significant, other treatments did not show any

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remarkable improvement of this parameter while AE3 and RE3 showed lowest percentage of acrosomal integrity.

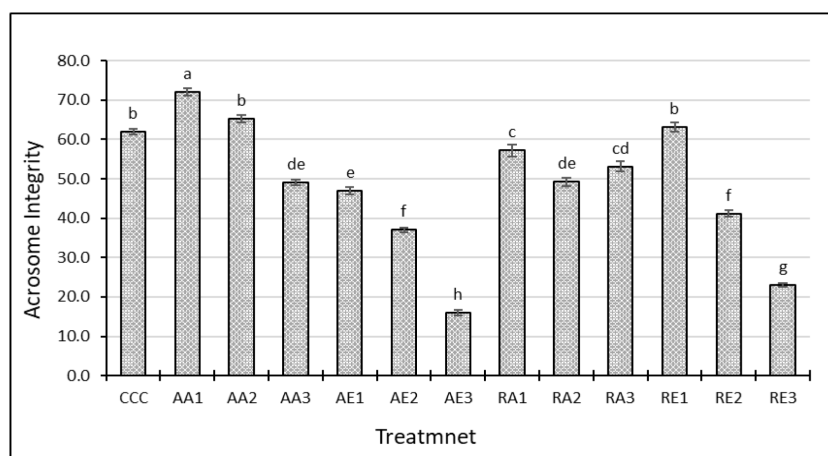

Figure 8. *Acrosomal integrity of the spermatozoa (%). Error bars were expressed as mean ± SE. Means with different letters in each graph are significantly different from each other (P < 0.05)*

In addition, *Figure 9.* presents the DNA integrity (SCD) which statistically proves that AA1 is significantly ($P < 0.05$ / $P = 0.0004$) higher than other groups and control. RA1 higher remarkably than control and other treatments except AA1 while RA3 recorded lowest ratio of DNA integrity.

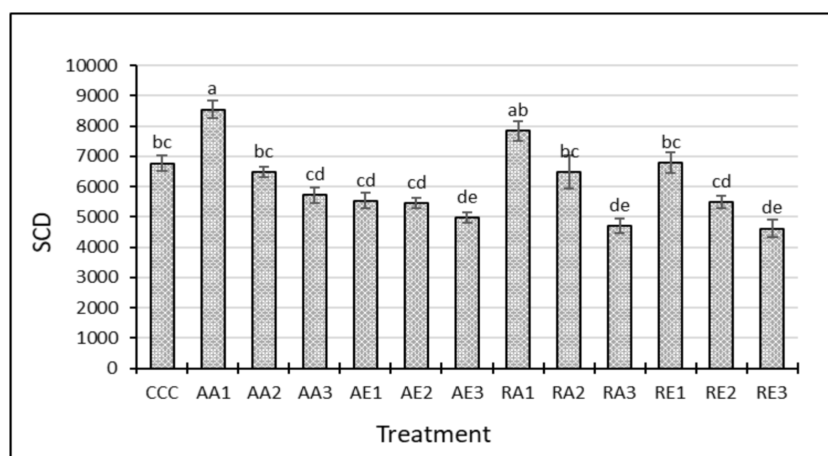

Figure 9. *Sperm chromatin dispersion (SCD) of the spermatozoa (%). Error bars were expressed as mean ± SE. Means with different letters in each graph are significantly different from each other (P < 0.05)*

Finally, the data of malondialdehyde concentration (MDA), as lipid peroxidation from *Figure 10.* reveals that the treatment AA1 is significantly ($P < 0.05$) lower than other treatments and control. AA2 and RA1 showed nearly about same amount of MDA concentration while AE3 showed highest concentration of MDA.

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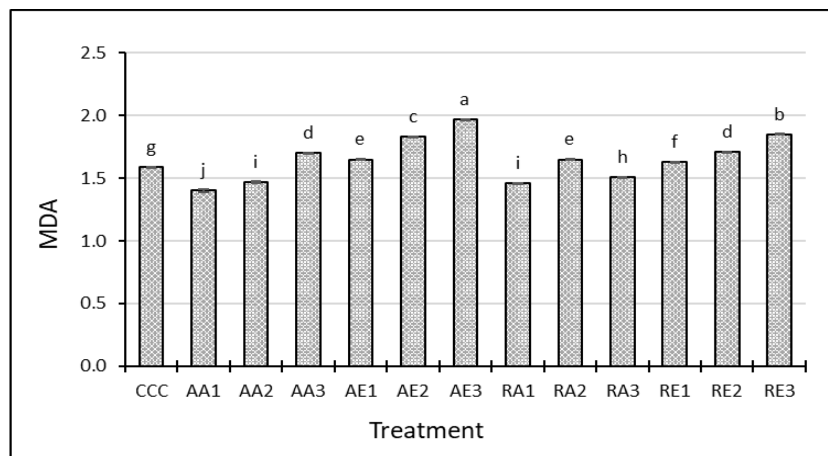

Figure 10. *Malondialdehyde concentration (nmol/ml). Error bars were expressed as mean ± SE. Means with different letters in each graph are significantly different from each other (P < 0.05)*

Discussion

Recently, researchers have focused their concerns on the antioxidant effect of different plants on post-thawed cryopreserved semen quality, especially biomedical plants, due to valuable compounds available in their structure, such as phenols, flavonoids (Nikavar et al., 2008; Ariyan et al., 2021) and capability of reducing free radicals by its antioxidative characteristics (Farshad et al., 2012). In this study, the antioxidant effect of the plant *Urtica dioica* (common nettle) was investigated by adding different doses of both aqueous and ethanolic extract obtained from aerial and root parts separately to semen dilutants then post-thawed semen parameters were measured. The exact effect of the *Urtica dioica* extract on cryopreserved semen quality is not fully addressed previously. However, several studies had revealed that the *Urtica dioica* shows a remarkable influence on strengthening the sperm motility, count and normal morphology while used in large doses in mice intravenously (Jalili et al., 2014) and improved many post-thawed sperm parameters, such as viability, normal sperms and motility when aqueous extract of the plant was used as an additive to Holstein bull semen extender (Mohamed and Abdulkareem, 2020). The benefits of utilizing various antioxidants available in many medicinal plants to minimize oxidative damage and maintain the quality of goat spermatozoa have been investigated in the past. However, knowledge about the plant *Urtica dioica* is very little and it has not been studied in detail. Therefore, it is necessary to identify the exact compounds that increase the effectiveness of this herb in goat semen that reduces oxidative stress and cell damage.

The result in *Table 1* shows the composition of the plant *Urtica dioica* aerial part-aqueous extract (AA) identified by GC-MS, which is composed of 29 different chemical compounds with its retention time and total percentage, while the result in *Table 2.* shows the composition of aerial part-ethanolic extract (AE) that include 63 different chemical compounds. Results in *Table 3* show the composition of root part-aqueous extract (RA) of *Urtica dioica* composed of 9 different chemical compounds and root part-ethanolic extract (RE) composed of 10 different chemical compounds. The GC-MS results reveal that the yield of ethanolic extract of *Urtica dioica* is higher as compared with aqueous extract which the total amount of chemical compounds extracted by ethanol is 72

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compounds. In comparison, the total amount of aqueous extract is 39 compounds. Although, water has been considered as an excellent polar solvent through decades but it can dissolve only polar compounds, while hydro-alcoholic solution (ethanol 96%) is polar as well as semi non-polar solvent which can dissolve a variety of compounds (polar and non-polar) (Jiang et al., 2014).

Data from *Table 4* demonstrates that adding 5 µg/ml of aqueous extract-aerial part of *Urtica dioica* (AA15µg/ml) was improved sperm motility and progressive motility significantly as compared to other groups and control; as well as velocity parameters such as sperm ALH and VSL were significantly higher in same treatment (AA15µg/ml) than all other groups and control. Our results were in agreement with those obtained by Mohamed and Abdulkareem (2020) and using extracts of other plants by Motlagh et al. (2014) and Najafi et al. (2019). The lipid structures on sperm cell membranes are destroyed by lipid peroxidation, which results in decreased motility and damaged membrane integrity (Ariyan et al., 2021). Moreover, sperm motility considered as most crucial parameter of semen quality (Wang et al., 2012); so that, lower motility or immobile sperm cells in some mammals are sometimes sterile (Turner, 2005).

In the biological system, malondialdehyde (MDA) is a standard indicator of lipid peroxidation (LPO), which is a byproduct of mitochondria, plasma membrane, and endoplasmic reticulum activity (Moldovan et al., 2004). Excessive oxidative stress caused by free radicals in term of reactive oxygen species (ROS) significantly affect sperm cell parameters (Ayala et al., 2014). *Urtica dioica* (Stinging nettle) acts as an effective antioxidant which leads to minimizing the concentration of MDA (Kanter et al., 2003). Such antioxidant capabilities of the plant related to a variety of phenolic compounds available in their structure (Al-Bishri et al., 2017). In our study, the result in *Figure 10. Malondialdehyde concentration (nmol/ml).* shows that the mean level of MDA was significantly lower for diluted goat sperms containing 5 µg/ml of aerial part-aqueous extracts (AA15µg/ml) than other treatments and control, which reveals the inverse relationship between sperm motility and concentration of MDA as reported by Aitken and his colleagues (Aitken et al., 1993) that consistent with our findings, which demonstrate a rise in sperm motility and a fall in MDA levels. Extra potential LPO inhibition and positive effect of aqueous extract than ethanolic extract have been noticed also in other medical plants such as *N. nucefera* (Laoung-On et al., 2021). The exact mechanism through which *Urtica dioica* extracts improve sperm quality is still poorly understood and needs further investigations.

Our results showed that the addition of 5 µg/ml aerial part-aqueous extracts of *Urtica dioica* to the goat freezing extenders caused a higher quality of sperm parameters than control and other groups after thawing such as viability *Figure 6.*, plasma membrane integrity *Figure 7.* and acrosome integrity *Figure 8.*. Similar properties have been exhibited from extracts of other medicinal herbs such as extract of green tea improved the quality of bull sperms (Ali et al., 2014), extracts of clove bud in extenders enhanced ram sperm viability (Soejima et al., 2012). Based on our study, *Urtica dioica* has incredible influences on many post-thawed sperm parameters, which are easily obtainable at a cheap cost, and extract process is much accessible.

In the present study, results from *Figure 9.* shows the addition of 5 µg/ml aerial part-aqueous extracts (AA15µg/ml) to the freezing dilutants of goat semen resulted in higher DNA integrity significantly in comparison with other treatments and control. Similar to our results, many extracts of other plants have had the same effect of increased DNA integrity after thawing, such as curcumin on cryopreserved bull semen (Shah et al., 2016),

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Tribulus terrestris and *Cinnamomum zeylanicumon* on post-thawed goat semen (Ariyan et al., 2021). Although the precise process by which *Urtica dioica* extracts lessen sperm DNA damage is unknown, investigations suggest that flavonoids can stop DNA from being broken and damaged via electron transfer (Zribi et al., 2012). Based on the results of this study, different doses of aqueous extract of both aerial and root parts of *Urtica dioica* have desired concentrations added to dilutants to protect goat sperms from cryopreservation techniques. Additionally, it can be suggested that adding 5 µg/ml of aqueous extracts-aerial part of *Urtica dioica* to goat sperm freezing extenders can dramatically enhance the quality of goat sperm parameters after thawing.

Direct comparisons among different studies can be challenging due to various factors. However, our method showed superior post-thaw viability and improved functional outcomes compared to conventional techniques. To fully assess its advantages and limitations, further comparative studies are necessary, focusing on parameters such as recovery rates, DNA integrity, functional assays, and long-term storage viability. It is crucial for future research to explore direct comparisons and potential synergies between different approaches to advance cryopreservation techniques.

Conclusion

Based on our statistical data obtained from this study, the co-supplementation of *Urtica dioica* extract (AA15µg/ml) to the semen extender resulted in the highest post-thawed sperm microscopic parameters such as motility, viability, plasma membrane integrity, acrosome integrity and DNA integrity as well as lowest MDA concentration. In general, all doses of aqueous extract acted as more potent antioxidants and showed relatively better results than ethanolic extract in terms of microscopic parameters and MDA concentration. Though the exact mechanism of action of ethanol on sperm quality is not fully addressed, research has shown that ethanol causes sperm parameter abnormalities, several changes relating the reproductive tract inhibition, and reduced in vitro fertilization rate in some species (La Vignera et al., 2013). It should be mentioned that further research is recommended to determine the exact effects of different biomolecules compound available in *Urtica dioica* extract.

Ethical approval. This study was approved by the University of Kurdistan (UOK) research ethics committee under the approval ID (IR.UOK.REC.1401.26).

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Conflict of interest. All authors declare that they have no conflict of interests.

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