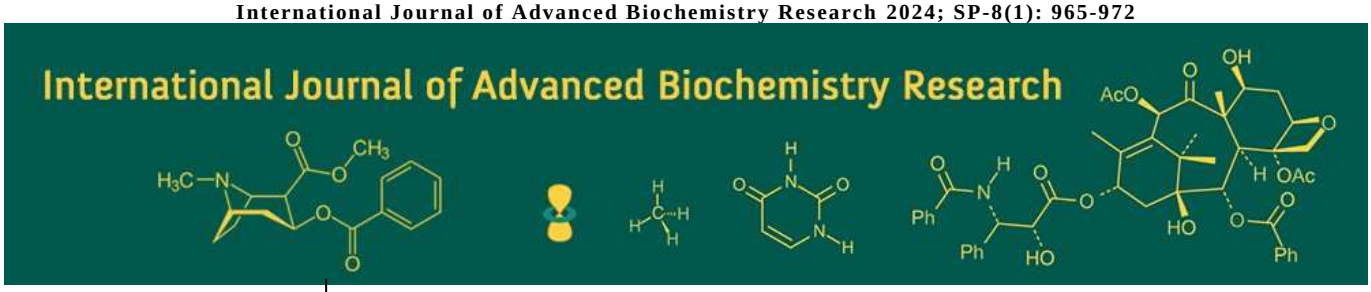

ISSN Print: 2617-4693
ISSN Online: 2617-4707
IJABR 2024; SP-8(1): 965-972
www.biochemjournal.com
Received: 18-10-2023
Accepted: 21-11-2023

Nikhil Pal Bajia
Ph.D., Scholar, Animal Reproduction Division, ICAR-IVRI, Izatnagar, Bareilly, Uttar Pradesh, India

Sumit Prakash Yadav
Assistant Professor, VGO, PGIVER, Jaipur, RAJUVAS, Bikaner, Rajasthan, India

Nirmala Saini
Principal Scientist, ICAR-CSWRI, Arid Region Campus, Bikaner, Rajasthan, India

Ashish Chopra
Sr. Scientist, ICAR-CSWRI, Arid Region Campus, Bikaner, Rajasthan, India

Amit Kumar
Assistant Professor, VGO, CVAS, RAJUVAS, Bikaner, Rajasthan, India

Krishna Kumar Sharma
VO, Government Veterinary Hospital, Bhilwari, Jhalawar, Rajasthan, India.

Shivendra Kumar Bhalothia
Ph,D. Scholar, VGO, CVAS, RAJUVAS, Bikaner, Rajasthan, India

Ashok Kumar
Scientist, ICAR-CSWRI, Arid Region Campus, Bikaner, Rajasthan, India

Corresponding Author:
Ashok Kumar
Scientist, ICAR-CSWRI, Arid Region Campus, Bikaner, Rajasthan, India

Effect of giloy (*Tinospora cordifolia*) supplementation to semen extender on selected quality parameters of magra ram semen

Nikhil Pal Bajia, Sumit Prakash Yadav, Nirmala Saini, Ashish Chopra, Amit Kumar, Krishna Kumar Sharma, Shivendra Kumar Bhalothia and Ashok Kumar

DOI: https://doi.org/10.33545/26174693.2024.v8.i1Sm.487

Abstract
To investigate the impact of antioxidant potential on several seminal parameters of Magra ram semen during chilled storage at 4 °C, ethanolic extract of *Tinospora cordifolia* (Giloy) was added at concentrations of 100 µg/ml, 300 µg/ml, 500 µg/ml, and 700 µg/ml to ovine semen extender. Tris egg yolk citrate fructose extender (TEYCF), which is aided with various quantities of *T. cordifolia* extract, served as the basic extender. Findings showed that, mean values for percentage of individual sperm motility and live sperm percentage were significantly (p<0.05) higher in all treatment groups as compared to control at 24, 48, 72 and 96 hrs of storage. At 48, 72 and 96 hrs, the mean values of hypo-osmotic swelling test positive spermatozoa percentage were noted significantly (p<0.05) higher in all treatment groups as compared to control. It was concluded that the quality of liquid semen stored at 4 °C was enhanced by the addition of *T. cordifolia* extract at varying quantities in semen extender. This enhancement may have been caused by *T. cordifolia*'s antioxidative properties. Maximum effects were obtained with 300 µg/ml of *T. cordifolia* extract concentration.

Keywords: *Tinospora cordifolia*, ram semen, individual sperm motility, live sperm percentage, HOST, antioxidant

1. Introduction
Sheep (*Ovis aries*) is an economically important livestock species which contributes significantly to the Indian agricultural economy. In arid harsh environment where crop and dairy farming is not economical, sheep are reared for their multi productive potential of meat, wool, milk, manure and skin (Pampori *et al*., 2018) [55]. The total number of sheep in India is 74.26 million enhanced by 14.13 percent over previous census (20th Livestock Census, 2019). Management of reproductive efficiency is key factor for improving farmer's income and also to maintain and propagate superior germplasm of this pure breed. In reproductive assisted techniques, Artificial insemination (AI) is one of the efficient tool to fasten the genetic improvement. Quality of semen is the main factor that contributes more in male fertility in sheep. Fresh ram semen is widely used for cervical artificial insemination in sheep around the world (Evans and Maxwell, 1987) [19]. For assessing the semen quality and fertility, physical characteristic of semen like motility, percentage of live spermatozoa etc. are mostly examined (Pal, 1957; Kumar, 2014; Allai *et al*., 2018) [54, 31, 4]. HOST is principally done for assessing the membrane-stabilizing action of antioxidants (Sariozkan *et al.*, 2015) [67]. The success of the AI technique depends on various factors where semen preservation is the most critical one. Time period of insemination (with in 10 hrs of collection) is also important. Liquid stored semen is convenient, economic and offers satisfactory results compared to frozen semen (Lopez-Saez *et al.*, 2000) [24] mostly used over cryopreserved semen. However, during longer storage period, the quality of semen is adversely affected resulting in the reduction of sperm fertilizing capacity accordingly with chilling preservation time (Alam *et al.*, 2005; Kasimanickam *et al.*, 2007; Munsi *et al.*, 2007) [3, 28, 49].

Ovine spermatozoon is more sensitive to oxidative damage compared to other species (Coyan *et al.*, 2012) [12]. As sperm membranes are vulnerable to free radical-induced damage because of a low concentration of antioxidant enzymes (Sawyer *et al*., 2001; Maneesh and Jayalekshmi, 2006) [68, 38], an increase in reactive oxygen species (ROS) leads to lipid peroxidation of polyunsaturated fatty acid present (PUFA) in spermatozoa membrane (Fujii *et al*., 2003) [20]. Perusal of literature revealed that antioxidants inclusion in semen dilutor may be useful to reduce the impact of oxidative stress and protecting sperm during liquid storage (Michael *et al.*, 2007; Dai *et al.*, 2018) [45, 14]. Spermatogenesis in testicles is an amazingly replicate to produce sperms at a high rate. This high pace of cell multiplication is coupled with higher creation of free radicals due to increased mitochondrial oxygen utilization by germinal epithelium (Aitken and Roman, 2008) [2]. According to Bucak and Tekin (2007) [10], the storage of ram spermatozoa under refrigeration produces a significant amount of ROS that impairs sperm function and reduces fertility. Low-grade quality semen contributes to more than 80 percent of fertilization and embryogenesis failure, miscarriage and infertility in livestock (Gadea and Matas, 2000; Rabbani *et al*., 2010; Enciso *et al*., 2011) [21, 59, 18] and this can be enhanced by adding antioxidants to semen diluents (Perumal *et al*., 2011) [56] or offering antioxidants to the animals (Ziegler and Filer, 1996) [83]. Natural plant extracts and their derivatives have recently been proven to be useful antioxidants that may be applied widely in semen extenders. The most potent components that give plants their antioxidative qualities include flavonoids, terpenes, phenolic compounds, and hydrolysable tannins (Gupta and Sharma, 2006; Ogunlesi *et al*., 2009; Carlsen *et al*., 2010) [23, 52, 11].

Giloy or Guduchi (*T. cordifolia*) is considered as the herbal constituent of "soma" or "heavenly nectar" (food for immortals, cited in Rigveda) (Mishra and Kaur, 2013; Leonti and Casu, 2014) [46, 32]. Because of its more than 100 compounds, Giloy is also known as the Rasayan plant. These constituents provide Giloy its anti-inflammatory, liver-protective, antioxidant, antibacterial, and immunomodulatory qualities. The therapeutic benefits of this herb are recognized in Ayurveda, veterinary folk medicine and other therapeutic traditions (Krishna *et al*., 2009) [29]. *T. cordifolia* has a wide spectrum of antioxidant qualities and reduces ROS (Desai *et al*., 2002) [15]. Arabinogalactan polysaccharide and polyphenolic compound (epicatechin) are the reason behind the antioxidant property of this plant (Subramanian *et al*., 2002; Pushp *et al*., 2013) [74, 58].

According to Rawal *et al*. (2004) [62], *T. cordifolia* has antioxidative actions and minimizes the consequences of oxidative stress-mediated cellular damage. After analyzing the hydro-ethanolic extract of *T. cordifolia*, Kumar *et al*. (2018) [30] reported that the extract's main constituents include cordifolide A, tinocordioside, palmatine, quercetin, syringing, heptacosanol, β-sitosterol and a variety of assays for antioxidative potential reveals extract's excellent antioxidant properties. Ram semen's levels of antioxidant enzymes were enhanced by *T. cordifolia* dietary supplementation (Jayaganthan *et al*., 2013) [26]. Many investigations using *T. cordifolia* as a dietary supplement have been conducted, but there is still little information available on the impact of *T. cordifolia* stem (ethanolic extract) on the quality of ram semen during refrigeration at 4 °C. Considering the aforementioned information, the primary goal of this investigation was to assess the effects of different concentrations on *T. cordifolia* stem (ethanolic extract) on several seminal characteristics.

2. Materials and Methods

The current study was conducted in February and March of 2021 at the Indian Council of Agricultural Research's Central Sheep & Wool Research Institute, located in the Arid Region Camps in Bikaner, Rajasthan, India. Bikaner is situated 230 meters above mean sea level at longitude 73˚18'E and latitude 28˚1'N. The region experiences dry weather, with 200 to 300 mm of annual precipitation falling in irregular amounts throughout the year. The annual temperature ranges from 4 °C to 49 °C for the minimum and maximum.

2.1 Chemicals

All the chemicals used in this study were procured from Sigma Aldrich (St. Louis, MO, USA), unless otherwise indicated.

2.2 Animals, semen collection and preliminary evaluation

Healthy breeding Magra rams (n = 8), averaging 1.5-3 years old, weighing 38±5 kg, and with a strong libido were utilized as artificial vagina technique semen donors. According to the guidelines issued by the Indian Council of Agricultural Research (ICAR, 2013), rams were allowed to graze in free-land pasture for a minimum of seven hours each day and were fed dry roughage and concentrate in the form of pellets. For three weeks in a row, two ejaculations a week, or a total of 48 ejaculates, were collected from semen donor rams. Shortly following collection, the semen collection cups were marked and placed in a water bath set at 37°C and examined for color, consistency, volume, pH, mass activity, spermatozoa abnormality and viability (the percentage of living sperm). After meeting the established requirements (mass motility >3+; individual sperm motility >70%; concentration >2.5 × 109 spermatozoa/ml and total abnormalities <10%), the semen samples were subjected to further evaluation (Gil *et al*., 2004) [22].

2.3 Preparation of extract of *T. cordifolia* (ethanolic) and extender

T. cordifolia stem was bought from a local herbal medicine supplier, and a botanical specialist verified its identity. After washing, the stem was dried for four days at 40 °C in a moisture extraction oven. After that, the dried stem was ground into a rough powder. The Soxhlet apparatus (continuous hot extraction) method, as outlined by Redfern *et al*. (2014) [63], was used to extract ethanol from the dried powder of *T. cordifolia* stem. In current investigation, TEYCF (Tris 3.63 g, egg yolk 14% (v/v), citric acid 1.99 g, fructose 0.5 g, 100,000 i.u. penicillin, 100 mg streptomycin and distilled water up to 100 ml) was prepared and used for all the ejaculates (Salamon and Maxwell, 2000) [65]. Additionally, various experimental doses of *T. cordifolia* extract (ethanolic) were added to the extender.

2.4 Semen processing and cooling

All of the fresh semen ejaculates were combined to lessen individual variance. The pooled semen sample was divided

into five identical fractions and placed into five separate test tubes that had been cleaned and sterilized. Pre-warmed (37 °C) extender was added to extend these fractions (1:10). The first fraction was used as a control and was extended using TEYCF extender devoid of any antioxidants. Extended semen was added in the second, third, fourth, and fifth aliquots with *T. cordifolia* extract at a rate of 100, 300, 500, and 700 µg per ml, respectively. At a rate of 0.2–0.3°C/min, these extended semen samples were cooled from 37°C to 4°C, and they were kept there for the whole 96 hours of liquid storage. The time was recorded as 0 hours when the extended semen samples' temperature reached 4°C. Using established protocols, the microscopic characteristics of semen (such as the percentage of sperm motility, viability and plasma membrane integrity) were evaluated.

2.5 Semen evaluation

2.5.1 Sperm motility percentage

Sperm motility was assessed subjectively in five distinct microscopic fields for all extended samples using a Dewinter Binocular Microscope (Italy) at 40X magnification. The samples were placed on a glass slide that had been pre-warmed to 37°C and covered with a pre-warmed cover slip. The average of these various microscopic fields was expressed as a percentage of progressive motile spermatozoa (Gil *et al*., 2003) [22]. The temperature of the preserved semen samples was adjusted to 37°C in a water bath for five minutes prior to monitoring progressive motility.

2.5.2 Sperm viability percentage

The eosin–nigrosin staining method was used to assess sperm viability (the percentage of living sperm) (Swanson & Bearden, 1951) [75]. Eosin (1 gm) and nigrosin (5 gm) were combined with 100 ml of buffer solution (2.94% sodium citrate dihydrate solution in double glass distilled water) to make the stain. After 30 minutes of heating in a water bath at 37°C, the mixture was cooled and filtered using Whatman's filter paper No. 40 before being kept at room temperature for later use. To prepare the slide, a small drop (30 µl) of semen was placed on a grease-free, clean slide, and the same amount of eosin–nigrosin dye was added, thoroughly mixed with a blunt end fine glass rod. After a minute, the mixture was thinly smeared onto a glass slide, allowed to air dry, and 300 spermatozoa were counted at 40X and 100X magnifications, respectively, to determine the viability of the sperm. The appearance of total stain exclusion was used to assess the vitality of spermatozoa (Evans & Maxwell, 1987) [19].

2.5.3 Plasma membrane integrity

The hypo-osmotic swelling test (HOST) was used to evaluate the functional integrity of the sperm plasma membrane (Jayendran *et al*., 1984). In summary, 0.1 ml of semen was combined with 1.0 ml of a 150 mOsm/l hypo-osmotic solution (1.351 g fructose + 0.735 g sodium citrate dehydrate / 100 ml of distilled water) for the experiment, which was then incubated for 60 minutes at 37°C in a water bath. Better visibility was achieved by adding 0.2 ml of eosin solution (0.5% w/v in sodium citrate 2.9% +10% formalin) to the test tube after incubation. Under a 40X microscope magnification, the wet preparation was inspected by placing a drop of a well-mixed semen sample on a glass slide and covering it with a cover slip. Spermatozoa with inflated and coiled tails were counted as HOS positive and a total of 200 spermatozoa were evaluated in five distinct fields (Mosaferi *et al*., 2005) [47].

2.6 Statistical Analysis

Statistical analysis of the study's data was conducted using SPSS version-20 and the one-way analysis of variance test (ANOVA) (Snedecor and Cochran, 2004) [72].

3. Results

3.1 Individual sperm motility percentage

In the present experiment, the mean values of individual sperm motility percentages in ram semen at different hours of preservation in different treatment groups are presented in table 1. At 0 hr no significant difference was found for the mean values of individual sperm motility percentages among the all groups. After 24 hrs of preservation the individual sperm motility percentage was significantly (p<0.05) higher in all the treatment groups as compared to control. The mean individual sperm motility percentage was significantly (p<0.05) higher in all the treatment groups as compared to control and treatment group after 48 hrs and 72 hrs. Statistical analysis revealed no significant difference between TC100 and TC300 as well as TC100 and TC500 groups. At 96 hrs, the mean individual sperm motility percentage didn't differ significantly between TC100 and TC300 as well as TC100 and TC500 groups.

Table 1: Mean (±S.E) individual sperm motility (percentage) in different groups during different hrs of preservation at refrigeration temperature (4 °C)

Duration of Preservation	Groups				
	Control	TC100	TC300	TC500	TC700
0 Hr	74.67±1.26	76.67±1.26	77.67±1.61	77.34±1.50	76.00±1.77
24 Hr	60.67±1.38^{a}	69.50±1.29^{b}	71.34±1.52^{b}	68.84±1.82^{b}	67.5±1.95^{b}
48 Hr	47.34±1.54^{a}	59.34±1.50^{cd}	63.67±1.33^{d}	57.67±2.49^{bc}	52.84±1.78^{b}
72 Hr	35.17±1.01^{a}	50.17±1.45^{cd}	53.67±1.17^{d}	48.67±2.50^{c}	41.67±1.54^{b}
96 Hr	23.67±1.28^{a}	40.17±1.14^{cd}	42.17±1.08^{d}	37.34±1.78^{c}	31.34±1.71^{b}

Means having different superscript in a row differ significantly (p<0.05)

3.2 Live sperm percentage

In the present study, the mean values of live sperm percentage in ram semen at different hours of preservation are furnished in table 2. At 0 hour, the mean live sperm percentage didn't differ significantly among all the groups. At 24 hrs, the mean live sperm percentage was significantly (p<0.05) higher in all the treatment groups compared to control but didn't differ significantly among the treatment groups. Afterv48 hrs, the mean live sperm percentage was significantly (p<0.05) higher in all the treatment groups as compared to control. At 72 hrs and 96 hrs the mean live sperm percentage was differed significantly (p<0.05) among

all groups. Statistical analysis revealed no significant difference between TC100 and TC500 groups after 96 hrs preservation. Values for mean individual sperm motility was highest in TC300 group as compared to rest of groups after 48 and 72 hrs of preservation at 4 °C.

Table 2: Mean (±S.E) Live sperm (percentage) in different groups during different hrs of preservation at refrigeration temperature (4 °C)

Duration of Preservation	Groups				
	Control	TC100	TC300	TC500	TC700
0 Hr	78.5±2.20	81±1.79	82.67±1.38	81.17±1.36	79.92±1.43
24 Hr	65.84±2.18^{a}	77.17±2.32^{b}	77.00±1.63^{b}	74.57±2.00^{b}	71.93±1.73^{b}
48 Hr	57.84±1.49^{a}	67.84±0.95^{c}	72.34±0.67^{d}	65.17±2.21^{bc}	63.09±1.67^{b}
72 Hr	42.34±1.12^{a}	54.84±1.58^{c}	61.34±0.72^{d}	56.25±1.93^{c}	50.44±0.66^{b}
96 Hr	33.17±1.25^{a}	46.34±1.65^{cd}	49.84±0.95^{d}	44.69±1.69^{c}	40.22±1.08^{b}

Means having different superscript in a row differ significantly (p<0.05)

3.3 Hypo-osmotic Swelling (HOS) Test

In the present study, the mean values of percentage HOS-test positive sperm in ram semen at different hours of preservation are presented in table 3. No significant difference was observed among all the groups at 0 and 24 hrs of preservation at 4 °C for the values of mean HOST positive spermatozoa percentage. At 48 hrs, the mean HOST positive spermatozoa percentage was significantly (p<0.05) higher in all the treatment groups as compared to control. The highest values of the mean HOST positive spermatozoa percentage found in TC300 treatment group which significantly (p<0.05) differ from control and TC700 treatment groups. The mean HOST positive spermatozoa percentage was significantly (p<0.05) higher in all the treatment groups as compared to control at 72 and 96 hrs of preservation at 4 °C. No significant difference was revealed among TC100, TC300 and TC500 treatment groups.

Table 3: Mean (±S.E) HOST positive sperm (percentage) in different groups during different hrs of preservation at refrigeration temperature (4 °C)

Duration of Preservation	Groups				
	Control	TC100	TC300	TC500	TC700
0 Hr	66.63±1.66	69.93±2.11	70.85±2.45	68.54±2.43	67.00±2.24
24 Hr	57.84±1.38	62.98±2.55	64.89±2.19	62.47±2.38	60.97±3.08
48 Hr	47.07±1.07^{a}	57.43±2.04^{bc}	60.95±1.74^{c}	57.64±1.63^{bc}	53.03±2.31^{b}
72 Hr	37.17±1.31^{a}	51.41±2.08^{c}	55.33±1.46^{c}	50.87±1.82^{c}	45.29±1.27^{b}
96 Hr	27.87±1.37^{a}	42.52±1.94^{c}	46.08±1.37^{c}	42.29±1.94^{c}	35.57±1.11^{b}

Means having different superscript in a row differ significantly (p<0.05)

4. Discussion

The cellular constituents of semen produced reactive oxygen species (ROS) that led to oxidative damage to sperm during liquid storage. One potential primary reason for the reduction in sperm motility and fertility during storage could be this; another possibility is the effect of low temperature on the instability of sperm membrane structure (Bucak and Tekin 2007) [10]. The modification of extenders (Marti *et al*., 2003) [39] and the addition of particular components to sustain structural integrity of membrane, avoid oxidative stress, or maintain spermatozoa motility in rams (Watson and Anderson, 1983; Maxwell and Stojanov, 1996; Upreti *et al*., 1998; Sanchez-partida *et al*., 1997) [80, 41, 76, 66] have been the recent focus of efforts to improve the preservation of cooled ram semen quality over the storage period. To reduce the harm that cooling and freezing-thawing can cause to ram spermatozoa, a variety of antioxidants and additions have been tried (Spalekova *et al*., 2011; Rather *et al*., 2016) [73, 61]. Positive effects of *T. cordifolia* on semen production as well as quality profiles were documented by several authors (Prashant *et al*., 2023; Jayaganthan *et al*., 2013; Rawal *et al*., 2004; Sharma *et al*., 2011) [57, 26, 62, 70]. But this was the first study on supplementing *T. cordifolia* stem extract at refrigeration temperature (4 °C) as a semen additive for Magra ram semen perseveration. With the exception of one study done on bovine species by Prashant *et al*. (2023) [57], almost no research has been done on the supplementation of *T. cordifolia* stem extract in semen extender on the seminal parameters in livestock species. Consequently, it was not possible to evaluate the data or compare it with the use of *T. cordifolia* stem ethanolic extract in ram semen extender.

The results of mean individual sperm motility percentage were found significantly (p<0.05) higher in all treatment groups as compared to control during 24, 48, 72 and 96 hours of preservation at refrigerator temperature (4°C) in this study. Similar results were also recorded by Mahdi (2010) [36] in Awassi rams after addition of licorice (*Glycyrrhiza glabra L.*) extract into semen diluters and Allai *et al.* (2016) [5] in Boujaad rams after addition of *Opuntia ficus-indica* cladodes extract (@ 1 per cent) into semen diluters. El-Harairy *et al.* (2018) [17] also observed similar findings after supplementation of Propolis ethanolic extract (@ 0.5 and 1.0 mg/ml) in Rahmani ram semen extender for 48 hrs. In contrast to results of the present study, El-Harairy *et al.* (2016) [16] observed that supplementation of *Moringa oleifera* leaves or *Arctium lappa* roots aqueous extracts had non-significant (@ 1000 µg MOL, 100 and 1000 µg ALR) or poorer effect (@ 100, 500 µg MOL and 500 µg ALR) on progressive motility in Rahmani rams. Similarly, Habeeb *et al.* (2020) [24] observed that the higher levels (0.1 and 0.05 gm) of Lycopene powder decrease the individual motility (0%) in diluted chilled ram semen.

The results of mean live sperm percentage was significantly (P<0.05) higher in all treatment groups as compared to control during 24, 48, 72 and 96 hours of preservation at refrigerator temperature (4°C) in this study. The present findings are in agreement with the observation made by Zaenuri *et al.* (2014) [82] who supplemented Fig fruit (4-7%

crude extract) to the semen extender in Boer cross buck preserved at 5°C for 6 day. The findings are in agreement with previous report that live sperm percentage improved after addition of argan oil from *Argania spinosa* seed (1%) in Tris diluter preserved upto 48 hrs (Allai *et al.*, 2015) [6] whereas similar findings were also observed by Allai *et al.* (2016) [5] after supplementation of *Opuntia ficus indica* cladodes extract (1%) into semen extender stored upto 72 hrs in Boujaad rams. In contrast to results of the present study, El-Harairy *et al.* (2018) [17] observed no significant difference in live sperm percentage after incorporation of Propolis ethanolic extract to the ram extenders. Contrary to the present study, Sharawy *et al.* (2015) [69] didn't find any effect on percentage of live spermatozoa after addition of ascorbic acid and trehalose as antioxidant to chilled ram semen. It is commonly recognized that adding antioxidant supplements increases the viability and motility of cryopreserved or liquid-stored ram spermatozoa (Maxwell and Stojanov, 1996; Sanchez-Partida *et al*., 1997) [41, 66]. Ethanolic extract of *T. cordifolia* has excellent antioxidant activities because it contains many antioxidant compounds like quercetin (Kumar *et al*., 2018) [30], epicatechin (Pushp *et al*., 2013) [58] and these compounds reduce ROS levels (Desai *et al*., 2002) [15]. Quercetin is a flavonoid that neutralizes ROS (Boots *et al*., 2008). This compound serves as a major component in ethanolic extract of *T. cordifolia* (Kumar *et al*., 2018) [30] and it is previously known that the addition of Quercetin improved post-thaw progressive motility, membrane integrity of buffalo bull spermatozoa (Ahmed *et al*., 2019) [1].

In this study the results of mean percentage of HOST positive spermatozoa was significantly (p<0.05) higher in treatment groups compared to control after 48 hrs of preservation stored at 4 °C. The observations in current study are in accordance with the studies of Zaenuri *et al.* (2014) [82] in Boer cross buck (plasma membrane integrity at day 6 was significantly higher in crude extract of Fig fruit added extender group @ 6 percent), Allai *et al.* (2015) [6] and Allai *et al.* (2016) [5] in Boujaad rams (addition of argan oil from *Argania spinosa* seed @ 1% in Tris for 24 and 48 hrs storage and addition of *Opuntia ficus-indica* cladodes extract @ 1% in extenders increase the HOST positive spermatozoa from 8 to 72 hrs of storage, respectively). Effect of various plant extracts as antioxidant on integrity of plasma membrane also studied after cryopreservation of extended ram semen and results raveled that various plant extracts had significant positive effect at certain concentrations on plasma membrane integrity like Clove bud extract @ 75 µg/ml (Baghshahi *et al.*, 2014) [8], Rosemary aqueous extract @ 4 and 6% (Motlagh *et al.*, 2014) [48], Green tea extract @ 5 and 10 mg/L (Mehdipour *et al.*, 2016) [42], Curcumin @ 1, 2, mM (Omur and Coyan 2016) [53], Pomegranate extract @ 5 mg/L (Mehdipour *et al.*, 2017) [43], *Thymus vulgaris* extract @ 4 and 8 ml/dl (Vahedi *et al.*, 2018) [78], Echinacea extract @ 0.5 mg/ml and 1 mg/ml (Yavas and Yavas, 2018) [81], Fennel extract @ 10 mg/L (Najafi *et al.*, 2019) [50] and Costmary extract @ 8 and 12 ml/dl (Vahedi, 2012) [77]. Contradict to present study, El-Harairy *et al.* (2016) [16] reported that the supplementation of different concentration of *Moringa oleifera* leaves or *Arctium lappa* roots aqueous extracts did not showed higher values of HOST positive percentage compared to control in Rahmani ram semen. Similarly, supplementation of Propolis ethanolic extract (El-Harairy *et al.*, 2018) [17] and Echinacea/Ginger extracts (Merati and Farshad, 2020) [44] to the semen extender also didn't reveal significant effect on plasma membrane integrity of ram spermatozoa. Polyunsaturated fatty acids rich plasma membrane of ram spermatozoa is highly vulnerable to lipid peroxidation due to attacks from ROS (Hinkovska *et al.*, 1986) [25]. Because of their interaction with lipid bi-layers, which altered the characteristics of the membrane, polyphenolic molecules may have a protective impact on the plasma membrane of spermatozoa (Mandic *et al*., 2019) [37]. *T. cordifolia* contains polyphenols (epicatechin) and arabinogalactan polysaccharide which have antioxidant property (Pushp *et al*., 2013) [58] and these antioxidants minimize ROS levels (Desai *et al*., 2002) [15]. HOST is principally useful when assessing the membrane protecting effects of antioxidants (Sariozkan *et al.*, 2015) [67]. Flavonoids increase the resilience of membranes by limiting damaging agents from entering the hydrophobic portion of the cellular membrane (Daghigh-Kia *et al*., 2016) [13]. During cold preservation, *T. cordifolia*'s alkaloids like choline (V), tinosporin, isocolumbin, palmatine, tetrahydropalmatine (VI), and magnoflorine display synergistic antioxidant activity to lessen the stress on the spermatozoa that is induced by ROS (Ng *et al*., 2000) [51]. The stem extract of *T. cordifolia* contains significant levels of vitamin C (41.36 mg/g of extract) and glutathione (6.86 mg/g of extract), which effectively scavenge a broad range of free radicals and function as strong inhibitors of lipid peroxidation (Maslova & Boboriko, 1990) [40]. The quality of semen is impacted dose-dependently by *T. cordifolia* stem extract (Sharma *et al*., 2011) [70]. According to research by Shoae and Zamiri (2008) [71], an overabundance of antioxidant in the extender increased plasma membrane fluidity over the desired level, increasing the spermatozoa's vulnerability to both plasma membrane and acrosomal damage. Additionally, the amount of antioxidants given to the extender should be assessed because high concentrations of antioxidants may be harmful to the sperm due to modifications in the physiological state of the extender. The possible alteration in the physiological state of the extender are increased diluent viscosity, increased debris in the diluent (van Wagtendonk-de Leeuw *et al*., 2000) [79], lower diluent osmotic pressure (Salmani *et al*., 2014) [64], decreased acrosome and plasma membrane functional integrity (Atessahin *et al*., 2008) [7], higher antioxidant incorporation does not prevent the creation of ROS and consequently increases cryo-damage to the spermatozoa (Lv *et al*., 2019) [35], and excessive antioxidants upset the equilibrium between ROS and antioxidants in the cells (Rahal *et al*., 2014) [60].

5. Conclusion

In conclusion addition of various concentrations of *T. cordifolia* extract (ethanolic) to tris extender improved percentage of individual sperm motility, percentage of live spermatozoa and spermsma membrane integrity at different hours of preservation at refrigeration temperature (4 °C). The 300 µg/ml concentration of *T. cordifolia* extract was found to be better additive than the 100, 500 and 700 µg/ml concentration of *T. cordifolia* extract for liquid storage of semen at 4 °C upto 96 hours.

6. Acknowledgement

The authors express their gratitude to Dean PGIVER, Jaipur, Dean PGS, CVAS, Bikaner and Head/Incharge

VGO, PGIVER, Jaipur for their technical and financial assistance. For granting animals and laboratory resources during the study, the authors are also grateful to the Head, ICAR-CSWRI, ARC, Bikaner, Project Coordinator, NWPSI and Project incharge on the Magra Field Unit, as well as the Director, ICAR-CSWRI, Avikanagar. A particular thank you to Dr. Amit Kumar, assistant professor, VGO, CVAS, Bikaner and Dr. Ashok Kumar, scientist, CSWRI-ICAR, ARC, Bikaner for their insightful counsel, ongoing inspiration, and useful tangible ideas during the study.

7. References

1. Ahmed H, Jahan S, Salman MM, Ullah F. Stimulating effects of quercetin (QUE) in tris citric acid extender on post thaw quality and *in vivo* fertility of buffalo (*Bubalus bubalis*) bull spermatozoa. Theriogenology. 2019;134:18-23.
2. Aitken AJ, Roman SD. Antioxidant systems and oxidative stress in the testes. Oxidative Medicine and Cell Longevity. 2008;1:15-24.
3. Alam MGS, Yeasmin S, Bari FY, Mishra B. The effect of duration of preservation on the quality of chilled bull semen. The Bangladesh Veterinarian. 2005;22:16-22.
4. Allai L, Benmoula A, da-Silva MM, Nasser B, El-Amiri B. Supplementation of ram semen extender to improve seminal quality and fertility rate. Animal Reproduction Science. 2018;192:6-17.
5. Allai L, Druart X, Ozturk M, Ben-Moula A, Nasser B, El-Amiri B. Protective effects of *Opuntia ficus-indica* extract on ram sperm quality, lipid peroxidation and DNA fragmentation during liquid storage. Animal Reproduction Science. 2016;175:1-9.
6. Allai L, Druart X, Contell J, Louanjli N, Moula AB, Badi A, *et al*. Effect of argan oil on liquid storage of ram semen in Tris or skim milk based extenders. Animal Reproduction Science. 2015;160:57-67.
7. Atessahin A, Bucak MN, Tuncer PB, Kizil M. Effects of antioxidant additives on microscopic and oxidative parameters of *Angora goat* semen following the freeze-thawing process. Small Ruminant Research. 2008;77(1):38-44.
8. Baghshahi H, Riasi A, Mahdavi AH, Shirazi A. Antioxidant effects of clove bud (*Syzygium aromaticum*) extract used with different extenders on ram spermatozoa during cryopreservation. Cryobiology. 2014;69(3):482-487.
9. Boots AW, Haenen GR, Bast A. Health effects of *quercetin*: from antioxidant to nutraceutical. European Journal of Pharmacology. 2008;585(2-3):325-337.
10. Bucak MN, Tekin N. Protective effect of *taurine*, *glutathione* and *trehalose* on the liquid storage of ram semen. Small Ruminant Research. 2007;73(1-3):103-108.
11. Carlsen MH, Halvorsen BL, Holte K, Bohn SK, Dragland S, Sampson L *et al*. The total antioxidant content of more than 3100 foods, beverages, spices, herbs and supplements used worldwide. Nutrition Journal. 2010;9(1):1-11.
12. Coşan K, Bucak MN, Başpınar N, Taşpinar M, Aydos S. Ergothioneine attenuates the DNA damage of post-thawed Merino ram sperm. Small Ruminant Research. 2012;106(2-3):165-167.
13. Daghigh-Kia H, Farhadi R, Ashrafi I, Mehdipour M. Anti-oxidative effects of ethanol extract of *Origanum vulgare* on kinetics, microscopic and oxidative parameters of cryopreserved Holstein bull spermatozoa. Iranian Journal of Applied Animal Science. 2016;6(4):783-789.
14. Dai GC, Meng Y, Zhang LK, Du YQ, Wen F, Feng TY, *et al*. Effect of addition of *melatonin* on liquid storage of ram semen at 4 °C. Andrologia. 2018;51(5):132-136.
15. Desai VR, Kamat JP, Sainis KB. An immunomodulator from *T. cordifolia* with antioxidant activity in cell-free systems. Journal of Chemical Sciences. 2002;114(6):713-719.
16. El-Harairy MA, Abdel-Khalek AE, Khalil WA, Khalifa EI, El-Khateeb A, Abdulrhmn AM. Effect of Aqueous Extracts of *Moringa oleifera* leaves or *Arctium lappa* Roots on Lipid Peroxidation and Membrane Integrity of Ram Sperm Preserved at Cool Temperature. Journal of Animal and Poultry Production. 2016;7(12):467-473.
17. El-Harairy MA, Khalil WA, Khalifa EI, Saber AA. Effect of *Propolis* Ethanolic Extract Supplementation to Ram Semen Extenders on Sperm Characteristics, Lipid Peroxidation and some Enzymatic Activities in Seminal Plasma in Chilled Semen. Journal of Animal and Poultry Production. 2018;9(4):235-243.
18. Enciso M, Cisale H, Johnston SD, Sarasa J, Fernandez JL Gosalvez *et al*. Major morphological sperm abnormalities in the bull are related to sperm DNA damage. *Theriogenology*. 2011;76:23-32.
19. Evans G, Maxwell WC. Salamons' artificial insemination of sheep and goats. 1987;(No.Ed. 2), Butterworths.
20. Fujii J, Iuchi Y, Matsuki S, Ishii T. Cooperative function of antioxidant and redox systems against oxidative stress in male reproductive tissues. Asian Journal of Andrology. 2003;5:231-242.
21. Gadea J, Matas C. Sperm factors related to *in vitro* penetration of porcine oocytes. Theriogenology. 2000;54:1343-1357.
22. Gil J, Lundeheim N, Soderquist L, Rodriguez-Martinez H. Influence of extender, temperature, and addition of glycerol on post-thaw sperm parameters in ram semen. Theriogenology. 2003;59(5-6):1241-1255.
23. Gupta VK, Sharma SK. Plants as natural antioxidants. Natural Product Radiance. 2006;5:326-334.
24. Habeeb HMH, Imran HM, Albudakheel HAS. Effect of adding the different concentration of *lycopene* powder on cooled local Awassi ram diluted semen. Plant Archives. 2020;20(2):6233-6236.
25. Hinkovska VT, Dimitrov GP, Koumanov KS. Phospholipid composition and phospholipid asymmetry of ram spermatozoa plasma membranes. The International Journal of Biochemistry. 1986;18(12):1115-1121.
26. Jayaganthan P, Perumal P, Balamurugan TC, Verma RP, Singh LP, Pattanaik AK. Effects of *Tinospora cordifolia* supplementation on semen quality and hormonal profile in rams. Animal Reproduction Science. 2013;140(1-2):47-53.
27. Jeyendran RS, Van-der-Ven HH, Perez-Pelaez M, Crabo BG, Zaneveld LJD. Development of an assay to assess the functional integrity of the human sperm membrane and its relationship to other semen

characteristics. Journal of Reproduction and Fertility. 1984;70(1):219-228.
28. Kasimanickam R, Kasimanickam V, Pelzer KD, Dascanio JJ. Effect of breed and sperm concentration on the changes in structural, functional and motility parameters of ram-lamb spermatozoa during storage at 4 °C. Animal Reproduction Science. 2007;101(1-2):60-73.
29. Krishna K, Jigar B, Jagruti P. Guduchi (*Tinospora cordifolia*): biological and medicinal properties, a review. The Internet Journal of Alternative Medicine. 2009;6(2):1-12.
30. Kumar V, Singh S, Singh A, Dixit AK, Srivastava B, Sidhu GK. Phytochemical, antioxidant, antimicrobial, and protein binding qualities of hydro-ethanolic extract of *T. cordifolia*. Journal of Biologically Active Products from Nature. 2018;8(3):192-200.
31. Kumar PV. Evaluation of semen characteristics and fertility in Nellore sheep (Jodipi). M.V.Sc thesis Sri Venkateswra Veterinary University, Tirupati, Hydrabad, Andhra Pradesh; 2014.
32. Leonti M, Casu L. Soma, food of the immortals according to the Bower Manuscript (Kashmir, 6th century AD). Journal of Ethnopharmacology. 2014;155(1):373-386.
33. Livestock census. 20th Live Stock Census, Department of Animal Husbandry, Dairying and Fisheries, Ministry of Agriculture 2019; GoI. New Delhi.
34. Lopez Saez A, Ortiz N, Gallego L, Garde JJ. Liquid storage (5 °C) of ram semen in different diluents. Archives of Andrology. 2000;44(2):155-164.
35. Lv C, Larbi A, Wu G, Hong Q, Quan G. Improving the quality of cryopreserved goat semen with a commercial bull extender supplemented with resveratrol. Animal Reproduction Science. 2019;208:106–127.
36. Mahdi AK. Effects of Licorice Extract on Sperm Motility of Chilled Stored Ram Semen. Ibn AL-Haitham Journal for Pure and Applied Science. 2010;23(1):34-40.
37. Mandic L, Sadzak A, Strasser V, Baranovic G, Domazet-Jurasin D, Dutour-Sikiric M, *et al*. Enhanced protection of biological membranes during lipid peroxidation: Study of the interactions between flavonoid loaded mesoporous silica nanoparticles and model cell membranes. International Journal of Molecular Sciences. 2019;20(11):2709.
38. Maneesh M, Jayalekshmi H. Role of reactive oxygen species and antioxidants on the pathophysiology of male reproduction. Indian Journal of Clinical Biochemistry. 2006;21:80-89.
39. Marti JI, Martı E, Cebrian-Perez JA, Muino-Blanco T. Survival rate and antioxidant enzyme activity of ram spermatozoa after dilution with different extenders or selection by a dextran swim-up procedure. Theriogenology. 2003;60(6):1025-1037.
40. Maslova GT, Boboriko TL. Effect of antioxidants on the status of the antioxidant system in cerebral ischemia and reperfusion injury. Ukrainskii Fizicheskii Zhurnal. 1990;62:101–105.
41. Maxwell WM, Stojanov T. Liquid storage of ram semen in the absence or presence of some antioxidants. Reproduction, Fertility and Development. 1996;8(6):1013-1020.
42. Mehdipour M, Kia HD, Najafi A, Dodaran HV, Garcia-Alvarez O. Effect of green tea (*Camellia sinensis*) extract and pre-freezing equilibration time on the post-thawing quality of ram semen cryopreserved in a soybean lecithin-based extender. Cryobiology. 2016;73(3):297-303.
43. Mehdipour M, Kia HD, Nazari M, Najafi A. Effect of lecithin nanoliposome or soybean lecithin supplemented by pomegranate extract on post-thaw flow cytometric, microscopic and oxidative parameters in ram semen. Cryobiology. 2017;78:34-40.
44. Merati Z, Farshad A. Ginger and echinacea extracts improve the quality and fertility potential of frozen-thawed ram epididymal spermatozoa. Cryobiology. 2020;92:138-145.
45. Michael A, Alexopoulos C, Pontiki E, Hadjipavlou-Litina D, Saratsis P, Boscos C. Effect of antioxidant supplementation on semen quality and reactive oxygen species of frozen-thawed canine spermatozoa. *Theriogenology*. 2007;68(2):204-212.
46. Mishra R, Kaur G. Aqueous ethanolic extract of *Tinospora cordifolia* as a potential candidate for differentiation based therapy of glioblastomas. PLoS One. 2013;8(10):e78764.
47. Mosaferi S, Niasari-Naslaji A, Abarghani A, Gharahdaghi AA, Gerami A. Biophysical and biochemical characteristics of Bactrian camel semen collected by artificial vagina. *Theriogenology*. 2005;63:92–101.
48. Motlagh MK, Sharafi M, Zhandi M, Mohammadi-Sangcheshmeh A, Shakeri M, Soleimani M, *et al*. Antioxidant effect of rosemary (*Rosmarinus officinalis* L.) extract in soybean lecithin-based semen extender following freeze–thawing process of ram sperm. Cryobiology. 2014;69(2):217-222.
49. Munsi MN, Bhuiyan MMU, Majumder S, Alam MGS. Effects of exogenous glutathione on the quality of chilled bull semen. Reproduction in Domestic Animals. 2007;42(4):358-362.
50. Najafi A, Kia HD, Mehdipour M, Shamsollahi M, Miller DJ. Does fennel extract ameliorate oxidative stress frozen-thawed ram sperm? *Cryobiology*. 2019;87:47-51.
51. Ng TB, Liu F, Wang ZT. Antioxidant activity of natural products from plants. Life Science. 2000;66:709-723.
52. Ogunlesi M, Okiei W, Ofor E, Awonuga O. Determination of the concentrations of zinc and vitamin C in oysters and some medicinal plants used to correct male factor infertility. Journal of Natural Products. 2009;2:89-97.
53. Omur AD, Coyan K. Protective effects of the antioxidants curcumin, ellagic acid and methionine on motility, mitochondrial transmembrane potential, plasma membrane and acrosome integrity in freeze-thawed Merino ram sperm. Veterinarni Medicina. 2016;61(1):10-16.
54. Pal K. Biochemical studies on buffalo bull semen. Current Science. 1957; 26(7): 212-213.
55. Pampori ZA, Sheikh AA, Aarif O, Hasin D, Bhat IA. Physiology of reproductive seasonality in sheep–an update. Biological Rhythm Research. 2018;51(4):586-598.
56. Perumal P, Selvaraju S, Selvakumar S, Barik AK, Mohanty DN, Das S *et al*. Effect of pre-freeze addition

of cysteine hydrochloride and reduced glutathione in semen of crossbred Jersey bulls on sperm parameters and conception rates. Reproduction in Domestic Animals. 2011;46(4): 636-641.
57. Prashant, Tomar P, Rajoriya JS, Ojha BK, Singh AK, Deshpande D, *et al*. *Tinospora cordifolia* modulates the seminal parameters, leakage of intracellular enzymes and seminal antioxidants in equilibrated and cryopreserved semen of Sahiwal bulls. Reproduction in Domestic Animals. 2023;58(6):793-801.
58. Pushp P, Sharma N, Joseph GS, Singh RP. Antioxidant activity and detection of (−) epicatechin in the methanolic extract of stem of *T. cordifolia*. Journal of Food Science and Technology. 2013;50(3):567-572.
59. Rabbani SI, Devi K, Khanam S. Effect of rosiglitazone on the nicotinamide-streptozotocin induced type-2 diabetes mellitus mediated defects in sperm abnormalities and oxidative defense system in male Wistar rats. Acta Pharmaceutica Sciencia. 2010;52:121-128.
60. Rahal A, Kumar A, Singh V, Yadav B, Tiwari R, Chakraborty S, *et al*. Oxidative stress, prooxidants and antioxidants: The interplay. BioMed Research International. 2014;19:761264.
61. Rather HA, Islam R, Malik AA, Lone FA. Addition of antioxidants improves quality of ram spermatozoa during preservation at 4 °C. Small Ruminant Research. 2016;141:24-28.
62. Rawal AK, Muddeshwar MG, Biswas SK. *Rubia cordifolia*, *Fagonia cretica* Linn and *T. cordifolia* exert neuroprotection by modulating the antioxidant system in rat hippocampal slices subjected to oxygen glucose deprivation. BMC Complementary and Alternative Medicine. 2004;4(1):1-9.
63. Redfern J, Kinninmonth M, Burdass D, Verran J. Using soxhlet ethanol extraction to produce and test plant material (essential oils) for their antimicrobial properties. Journal of Microbiology & Biology Education. 2014;15(1):45-46.
64. Salmani H, Towhidi A, Zhandi M, Bahreini M, Sharafi M. *In vitro* assessment of soybean lecithin and egg yolk based diluents for cryopreservation of goat semen. Cryobiology. 2014;68:276-280.
65. Salomon S, Maxwell WMC. Storage of ram semen. Animal Reproduction Science. 2000;62:77-111.
66. Sanchez-Partida LG, Setchell BP, Maxwell WMC. Epididymal compounds and antioxidants in diluents for the frozen storage of ram spermatozoa. Reproduction, Fertility and Development. 1997;9(7):689-696.
67. Sariozkan S, Bucak MN, Tuncer PB, Buyukleblebici S, Eken A, Akay C. Influence of fetuin and hyaluronan on the post-thaw quality and fertilizing ability of Holstein bull semen. Cryobiology. 2015;71(1):119-124.
68. Sawyer DE, Roman SD, Aitken RJ. Relative susceptibilities of mitochondrial and nuclear DNA to damage induced by hydrogen peroxide in two mouse germ cell lines. Redox Report. 2001;6:182-194.
69. Sharawy SM, Saleh NH, Attallah SAE, Hashem GA, Khedre DH. Studies on the effect of added ascorbic acid and trehalose to tris buffered egg yolk extender on chilled ram semen. MENA Science Journal. 2015;1(1):1-4.
70. Sharma P, Parmar J, Sharma P, Verma P, Goyal PK. Radiation-induced testicular injury and its amelioration by *Tinospora cordifolia* (an Indian medicinal plant) extract. Evidence-Based Complementary and Alternative Medicine. 2011; 643847.
71. Shoae A, Zamiri MJ. Effect of butylated hydroxytoluene on bull spermatozoa frozen in egg yolk-citrate extender. Animal Reproduction Science. 2008;104(2-4):414-418.
72. Snedecor GW, Cochran WG. Statistical Methods (eighth edition). Kalcutta, India: Oxford and IBH Publishing Company; c1994.
73. Spalekova E, Makarevich A, Pivko J. Effect of caffeine on parameters of ram sperm motility. Slovak Journal of Animal Science. 2011;44(2):78-83.
74. Subramanian M, Chintalwar GJ, Chattopadhyay S. Antioxidant properties of a *T. cordifolia* polysaccharide against iron-mediated lipid damage and γ-ray induced protein damage. Redox Report. 2002;7(3):137-143.
75. Swanson EW, Bearden HJ. An eosin-nigrosin stain for differentiating live and dead spermatozoa. Journal of Animal Science. 1951;10(4):981-987.
76. Upreti GC, Jensen K, Munday R, Duganzich DM, Vishwanath R, Smith JF. Studies on aromatic amino acid oxidase activity in ram spermatozoa: role of pyruvate as an antioxidant. Animal Reproduction Science. 1998;51(4):275-287.
77. Vahedi V. Effects of Costmary (*Tanacetum balsamita* L.) extract on selected quality parameters of Moghani ram sperm after cryopreservation. Journal of Animal Science. 2012;30(4):71-82.
78. Vahedi V, Hedayat-Evrigh N, Behroozlak M, Dirandeh E. Antioxidant Effects of Thyme (*Thymus vulgaris*) Extract on Ram Sperm Quality during Cryopreservation. Iranian Journal of Applied Animal Science. 2018;8(2):263-269.
79. Leeuw VWAM, Haring RM, Kaal-Lansbergen LMTE, Daas DJHG. Fertility results using bovine semen cryopreserved with extenders based on egg yolk and soy bean extract. Theriogenology. 2000;54:57–67.
80. Watson PF, Anderson WJ. Influence of butylated hydroxytoluene (BHT) on the viability of ram spermatozoa undergoing cold shock. Reproduction. 1983;69(1):229-235.
81. Yavas I, Yavas TK. Effects of Different Plant Extracts on Post-Thaw some Spermatological Parameters of Cryopreserved Awassi Rams Spermatozoa. Bulletin UASVM Animal Science and Biotechnologies. 2018;75(1):49-52.
82. Zaenuri LA, Susilawati T, Sumitro SB, Wahyuningsih S. Effects of Additional Crude Extract of Fig Fruit (*Ficus carica* L) into Tris Egg Yolk Based Extender on Quality of Buck Semen. Journal of Biology, Agriculture and Healthcare. 2014;4(9):21-27.
83. Ziegler EE, Filer LJ. Present Knowledge in Nutrition. ILSI Press, International Life Washington, DC; c1996.