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## Effect of black cumin (*Nigella sativa*) seeds extract in tris egg yolk citrate extender on viability of cryopreserved Surti buck semen

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### Abstract

The present research work was conducted to examine effect of different concentrations of Black cumin (*Nigella sativa*) seeds extract in Tris egg yolk citrate extender on sperm viability of cryopreserved Surti buck semen. Total 64 semen ejaculates were collected from four Surti buck (16 ejaculates/buck) twice a week by artificial vagina method. Semen samples were diluted with Tris egg yolk citrate extender and Black cumin (*Nigella sativa*) seeds aqueous extract was supplemented at different concentrations viz. 0% (T<sub>1</sub>), 3% (T<sub>2</sub>), 4% (T<sub>3</sub>), 5% (T<sub>4</sub>) (pH 6.5-6.8) maintaining final concentration of 100×10<sup>6</sup> sperm/ml. After making the groups, the semen samples were examined for structural plasma membrane integrity (viability) (%) and functional plasma membrane integrity (HOST) (%) for all groups. Result showed that highest mean live sperm (%) and mean host reacted sperm (%) was found at initial, pre-freeze and post-thaw stage in T<sub>3</sub> group followed by T<sub>2</sub>, T<sub>4</sub> and T<sub>1</sub> group. It was concluded that post-thawed viability and host reacted sperm (%) parameters were well maintained at 4% supplementation of Black cumin (*Nigella sativa*) seed extract in Tris egg yolk citrate extender.

**Keywords:** Cryopreserved Semen, Black cumin (*Nigella sativa*) seed aqueous extract, Viability, HOST, Surti buck semen

### Introduction

Buck semen can be preserved either at room temperature temporarily, at refrigerated temperature for 24- 48 hours (Ferdinand *et al.*, 2012) <sup>[1]</sup> or cryopreserved (Beltran *et al.*, 2013) <sup>[2]</sup> for long term storage. The viability of the preserved sperms may be affected by many factors including storage temperature, cryoprotectant concentration, cooling rate, extender composition, free radical contents, seminal plasma contents, and antiseptic factors (Hezavehei *et al.*, 2018) <sup>[3]</sup>. During the process of cryopreservation, endogenous antioxidant concentration, semen quality and fertility become compromised due to the generation of reactive oxygen species (ROS) (Holt, 2000) <sup>[4]</sup>. More number of viable spermatozoa is important to maintain quality of cryopreserved semen which can be achieved by minimizing deteriorative factors.

Researchers found that addition of exogenous plant-based extract can reduce the oxidative stress caused by ROS. Such a one plant based extract from seeds of Black cumin (*Nigella sativa*) have same potential to reduce oxidative stress originates during cryopreservation. Black cumin (*Nigella sativa*) is believed to contain essential elements such as thymoquinone, dithymoquinone, 4-terpineol, Carvacrol, Anethole, Thymol, and alpha-pinene (Awan *et al.*, 2018) <sup>[5]</sup>. Beneficial effects of various forms of Black cumin (*Nigella sativa*) seeds was reported in rats (Mansour *et al.*, 2013) <sup>[6]</sup>; in rabbits (El-Gindy, 2022) <sup>[7]</sup> and in cocks (Ezzat *et al.*, 2019) <sup>[8]</sup>. Black cumin (*Nigella sativa*) seed extract is effective for improving the quality of sperm after Vitrification (Nasiri *et al.*, 2022) <sup>[9]</sup>. Looking into the various properties of Black cumin (*Nigella sativa*) seeds extract study has been undertaken on effect of black cumin (*Nigella sativa*) seeds extract in tris egg yolk citrate extender on viability of cryopreserved Surti buck semen.

## Materials and Methods

**Semen collection, experimental group and cryopreservation:** The study was conducted on four apparently healthy mature Surti bucks above one year of age maintained under the All India Coordinated Research Project (AICRP) on Surti Goat at Livestock Research Station, Kamdhenu University Navsari. The selected bucks were housed in a common covered pen and under naturally existing photoperiod and air temperature of the South Gujarat. The selected bucks were housed in a common covered pen and separated from females. The bucks were trained to donate the semen in artificial vagina by using female (doe) as dummy. After completion of the training period of about one month, semen was collected regularly by using artificial vagina twice a week from each buck for up to 8 weeks and total 64 semen ejaculates (16 ejaculates from each buck) were collected.

Semen was collected from all the selected bucks at early morning between 6.30 AM to 7.30 AM with the help of Eight-inch Artificial Vagina (AV) maintaining inner temperature of 40°C to 42°C and sufficient pressure. In order to maintain quality of semen, all the parts of artificial vagina are properly sterilized and for each buck separate Artificial vagina was used and during collection buck apron was applied to prevent further contamination. In order to increase the semen volume as well as eliminate individual buck variability the ejaculates of all four bucks were pooled. Only semen samples with initial motility  $\geq 70\%$  were considered for further processing. The pooled semen was extended with tris egg yolk citrate extender to achieve final concentration of  $100 \times 10^6$  sperm/ml.

The diluted semen was separated into four equal aliquots, and each aliquot was treated with different concentrations of Black cumin (*Nigella sativa*) seeds aqueous extract viz. 0% (control T<sub>1</sub>), 3% (T<sub>2</sub>), 4% (T<sub>3</sub>) and 5% (T<sub>4</sub>) (pH 6.5-6.8). According to different groups, extended semen was filled in previously marked 0.5ml French medium straw (IMV Technologies, France) using micropipette having final concentration of  $50 \times 10^6$  sperm/straw. At least ten straws were prepared for each group. The filled straws were sealed with the help of polyvinyl alcohol powder (HiMedia Laboratories Pvt. Ltd.) and all the loaded straws were laid on a floating rack (Minitube, Germany) and placed in a refrigerator at 4°C for equilibration about 4 hours. After equilibration, the floating rack holding the straws were placed in a manual vapour freezing unit (Minitube, Germany) for 10 minutes in such a way that the straws were remain 5 cm above the liquid nitrogen in vapor phase. After completion of freezing the straws were directly and quickly plunged into liquid nitrogen container. After making the groups as above, the semen samples were examined for

Viability (Structural plasma membrane integrity) (%) and HOST (Functional plasma membrane integrity) for all groups at initial, Pre-freeze and at post-thaw (24 hours after cryopreservation) using standard methods.

## Preparation of black cumin (*Nigella sativa*) seeds aqueous extract:

The seeds were carefully washed with clean water to get rid of dust and dirt. They were air shade dried for four days at room temperature and pulverized into a fine powder using mixer grinder. 100 gm of dried seed powder was extracted with water in glass container covered with aluminum foil and allowed to stand at room temperature for a period of 24 hours with frequent agitation until soluble matter had dissolved. The aqueous extract of seeds of Black cumin (*Nigella sativa*) was prepared through liquid partition method. The aqueous extract was stored in vial and kept in a refrigerator at -20 °C prior to sample preparation for subsequent analyses.

## Statistical analysis

Descriptive analysis was carried out and mean  $\pm$  SE was calculated for all the designated groups of neat semen parameters as well as extended semen parameters at various time intervals. The test of significance among the groups for above parameters was made by analysis of variance (ANOVA) and the mean difference between the groups were tested by using Duncan's new Multiple Range test (DNMRT) at 5 and 1 percent level of significance.

## Results and Discussions

### Viability (Structural plasma membrane integrity)

The initial mean live sperm (%) differed non-significantly among T<sub>1</sub> (80.56 $\pm$ 0.79), T<sub>2</sub> (82.50 $\pm$ 0.84), T<sub>3</sub> (82.75 $\pm$ 0.93) and T<sub>4</sub> (81.69 $\pm$ 1.21) groups (Table 1). Pre-freeze mean live sperm (%) was significantly ( $p < 0.01$ ) higher in T<sub>3</sub> (68.88 $\pm$ 1.75) group as compared to T<sub>4</sub> (62.56 $\pm$ 1.79) and T<sub>1</sub> (58.06 $\pm$ 1.56) groups while it was non-significantly higher as compared to T<sub>2</sub> (65.00 $\pm$ 1.39) group. Pre-freeze mean live sperm (%) in T<sub>2</sub> (65.00 $\pm$ 1.39) group was non-significantly higher as compared to T<sub>4</sub> (62.56 $\pm$ 1.79) group and significantly ( $p < 0.01$ ) higher as compared to T<sub>1</sub> (58.06 $\pm$ 1.56) group. Pre-freeze mean live sperm (%) in T<sub>4</sub> (62.56 $\pm$ 1.79) group was non-significantly higher as compared to T<sub>1</sub> (58.06 $\pm$ 1.56) group. Post-thaw mean live sperm (%) was significantly ( $p < 0.01$ ) higher in T<sub>3</sub> (50.50 $\pm$ 1.45) group as compared to T<sub>2</sub> (44.63 $\pm$ 1.54), T<sub>4</sub> (43.13 $\pm$ 1.12) and T<sub>1</sub> (34.44 $\pm$ 1.64) groups. Post-thaw mean live sperm (%) in T<sub>2</sub> (44.63 $\pm$ 1.54) and T<sub>4</sub> (43.13 $\pm$ 1.12) groups differed non-significantly with each other and significantly ( $p < 0.01$ ) higher as compared to T<sub>1</sub> (34.44 $\pm$ 1.64) group.

**Table 1:** Effect of different concentrations of Black cumin (*Nigella sativa*) seeds aqueous extract on live sperm percent of Surti buck semen at various stages of cryopreservation (Mean $\pm$ SE)

Groups	Live sperm (%) (n=16)			Overall (n= 48)	F value	P value
	Initial	Pre-freeze	Post-thaw			
T <sub>1</sub>	80.56 $\pm$ 0.79 <sub>x</sub>	58.06 $\pm$ 1.56 <sub>y</sub>	34.44 $\pm$ 1.64 <sub>z</sub>	57.69 $\pm$ 2.86 <sup>b</sup>	277.79**	0.00
T <sub>2</sub>	82.50 $\pm$ 0.84 <sub>x</sub>	65.00 $\pm$ 1.39 <sup>ab</sup> <sub>y</sub>	44.63 $\pm$ 1.54 <sup>b</sup> <sub>z</sub>	64.04 $\pm$ 2.37 <sup>ab</sup>	215.63**	0.00
T <sub>3</sub>	82.75 $\pm$ 0.93 <sub>x</sub>	68.88 $\pm$ 1.75 <sup>a</sup> <sub>y</sub>	50.50 $\pm$ 1.45 <sup>a</sup> <sub>z</sub>	67.38 $\pm$ 2.09 <sup>a</sup>	130.42**	0.00
T <sub>4</sub>	81.69 $\pm$ 1.21 <sub>x</sub>	62.56 $\pm$ 1.79 <sup>bc</sup> <sub>y</sub>	43.13 $\pm$ 1.12 <sup>b</sup> <sub>z</sub>	62.46 $\pm$ 2.43 <sup>ab</sup>	188.35**	0.00
Overall (n=64)	81.88 $\pm$ 0.48 <sub>x</sub>	63.63 $\pm$ 0.94 <sub>y</sub>	43.17 $\pm$ 1.01 <sub>z</sub>	--	2.70*	0.04
F value	1.06	7.70**	20.99**	527.95**	--	--
P value	0.37	0.00	0.00	0.00	--	--

<sup>a-c</sup> Means with different superscript within a column (between the groups) differs significantly at  $p < 0.01$ .

<sub>x-z</sub> Means with different subscript between a column (between various stages) differs significantly at  $p < 0.01$ . \*\* $p < 0.01$

T<sub>1</sub> – control, T<sub>2</sub> – 3% Black cumin (*Nigella sativa*) seeds aqueous extract, T<sub>3</sub> – 4% Black cumin (*Nigella sativa*) seeds aqueous extract, T<sub>4</sub> – 5% Black cumin (*Nigella sativa*) seeds aqueous extract.

The corresponding overall mean live sperm (%) irrespective of treatment groups were reduced with increasing preservation time at initial ( $81.88 \pm 0.48$ ), pre-freeze ( $63.63 \pm 0.94$ ) and post-thaw ( $43.17 \pm 1.01$ ) stage. The overall mean live sperm (%) irrespective of different treatment groups differed significantly ( $p < 0.01$ ) among various stages of cryopreservation.

Moreover, mean live sperm (%) in T<sub>1</sub>, T<sub>2</sub>, T<sub>3</sub> and T<sub>4</sub> groups were significantly ( $p < 0.01$ ) higher at initial ( $80.56 \pm 0.79$ ,  $82.50 \pm 0.84$ ,  $82.75 \pm 0.93$  and  $81.69 \pm 1.21$ ) stage as compared to pre-freeze ( $58.06 \pm 1.56$ ,  $65.00 \pm 1.39$ ,  $68.88 \pm 1.75$  and  $62.56 \pm 1.79$ ) stage and post-thaw stage ( $34.44 \pm 1.64$ ,  $44.63 \pm 1.54$ ,  $50.50 \pm 1.45$  and  $43.13 \pm 1.12$ ) stage. Furthermore, mean live sperm (%) among initial, pre-freeze and post-thaw stage differed significantly ( $p < 0.01$ ) in all the groups. The corresponding overall mean live sperm (%) irrespective of time interval was significantly ( $p < 0.01$ ) higher in T<sub>3</sub> ( $67.38 \pm 2.09$ ) as compared to T<sub>1</sub> ( $57.69 \pm 2.86$ ) group and non-significantly higher as compared to T<sub>2</sub> ( $64.04 \pm 2.37$ ) and T<sub>4</sub> ( $62.46 \pm 2.43$ ) groups. Overall mean live sperm (%) in T<sub>2</sub> ( $64.04 \pm 2.37$ ) and T<sub>4</sub> ( $62.46 \pm 2.43$ ) groups differed non-significantly with each other and also non-significantly higher as compared to T<sub>1</sub> ( $57.69 \pm 2.86$ ) group. Highest mean live sperm (%) was found at initial, pre-freeze and post-thaw stage in T<sub>3</sub> ( $82.75 \pm 0.93$ ,  $68.88 \pm 1.75$  and  $50.50 \pm 1.45$ ) group followed by T<sub>2</sub> ( $82.50 \pm 0.84$ ,  $65.00 \pm 1.39$  and  $44.63 \pm 1.54$ ) group and T<sub>4</sub> ( $81.69 \pm 1.21$ ,  $62.56 \pm 1.79$  and  $43.13 \pm 1.12$ ) group. While lowest mean live sperm (%) was found in T<sub>1</sub> group at initial ( $80.56 \pm 0.79$ ), pre-freeze ( $58.06 \pm 1.56$ ) and post-thaw ( $34.44 \pm 1.64$ ) stage. The main compound of Black cumin (*Nigella sativa*) seeds contained higher amount of flavonoids and ascorbic acid. However, many researchers studied the effect of various medicinal plant (coconut water, green tea, honey, Entada abyssinica, moringa leaf etc.) extract and as such quercetin, thymoquinone having flavonoids compound. Hence, the discussion was made on that basis.

Result from present study showed that percentage of live sperm was significantly ( $p < 0.01$ ) higher in 4% Black cumin (*Nigella sativa*) seeds aqueous extract supplemented group in pre-freeze and post-thawed semen followed by in 3% Black cumin (*Nigella sativa*) seeds aqueous extract at same respective stages as compared to control group. While in 5% Black cumin (*Nigella sativa*) seeds aqueous extract supplemented group live sperm count was non-significantly decreased as compared to 3% Black cumin (*Nigella sativa*) seeds aqueous extract supplemented group. In accordance to the present findings, Awan *et al.* (2018) [5] in buffalo, Inanc *et al.* (2021) [10] in ram, Miah *et al.* (2018) [11] in ram, EL-Gindy (2022) [7] in rabbit buck, El-Nattat *et al.* (2019) [12] in rabbit buck, EL-Tohamy *et al.* (2010) [13] in rabbit buck, AL-Saaidi *et al.* (2009) [14] in male rat also reported higher live sperm count as in *Nigella sativa* supplemented groups as compared to control group. Similar trend has also been reported by Khan *et al.* (2017) [15] and Ahmed *et al.* (2019) [16] in bull, Sobeh *et al.* (2020) [17] in ram in different antioxidants additives. Whereas, El-Seadawy *et al.* (2022) [18] reported significantly ( $p < 0.05$ ) higher sperm viability (%) in 0.64 mg/ml group ( $60.01 \pm 1.05$ ), 0.56 mg/ml group ( $49.22 \pm 0.71$ ) and 0.48 mg/ml ( $38.44 \pm 0.69$ ) moringa leaf supplemented group as compare to control ( $43.25 \pm 0.76$ ) group of Awassi rams semen.

In support to present findings, Awan *et al.* (2018) [5] reported significantly ( $p < 0.05$ ) higher post thaw live sperm count in 4% *Nigella sativa* extract supplemented extender in buffalo

semen followed by in 5% *Nigella sativa* extract supplemented group as compared to control group. Moreover, Inanc *et al.* (2021) [10] also reported addition of thymoquinone in tris-based extender at 50 µg/mL showed significantly ( $p < 0.05$ ) higher sperm viability followed by addition at 100 µg/ml as compared to control group. Similar trend in sperm viability was also reported by Miah *et al.* (2018) [11] in cryopreserved ram semen. Result showed sperm viability was significantly ( $p < 0.05$ ) higher in 100 g/ml addition of *Nigella sativa* oil in tris-based extender at 0 and 2 hours of post-thawed incubation. While higher concentration of *Nigella sativa* oil at 1000 g/ml could not preserve sperm viability. Similar result of *Nigella sativa* aqueous extract addition in tris-based extender was reported by El-Battaway and Riad (2011) [19]. They observed significantly ( $p < 0.05$ ) decrease dead sperm count at 2<sup>nd</sup> day of incubation in *Nigella sativa* aqueous extract at 200 µl/ml as compared to control group.

Sobeh *et al.* (2020) [17] reported similar findings from addition of polyphenol rich *Entada Abyssinica* bark extract in extended ram semen. They reported highest post-equilibrated and post-thawed live sperm count in 375 µg/ml concentration of *Entada Abyssinica* bark extract supplemented group in ram semen. They concluded that live sperm count was decreased with increasing of *Entada Abyssinica* bark extract in extender. Likewise, Khan *et al.* (2017) [15] reported significantly ( $p < 0.05$ ) higher post thaw live sperm count at 0.75% green tea extract supplemented group as compared to control group in bull semen. While addition of green tea extract at 1% could not preserve sperm viability greater than that at 0.75%. Ahmed *et al.* (2019) [16] observed that, addition of quercetin at 200 mM showed live sperm count (%) was  $72.33 \pm 0.83$  and  $26.56 \pm 0.90$  as compared to control group  $26.56 \pm 0.90$  and  $18.11 \pm 0.82$  in post-dilution and post-thawed bull semen, respectively. This beneficial result of quercetin is due to dose dependent increase in antioxidant capacity which protects spermatozoa from cryo-damage that justifies lower live sperm count in lower concentrations of quercetin (*viz.* 50, 100, 150), which supports results from present study as dose dependent increase in live sperm count up to 4% *Nigella sativa* aqueous extract. Similar trends in both extracts might be due to presence of polyphenolic compounds. In accordance to present findings AL-Saaidi *et al.* (2009) [14] reported oral administration of *Nigella sativa* alcoholic extract at 0.5 g/kg and 1.5 g/kg in male rates significantly ( $p < 0.01$ ) improved sperm viability at both doses as compared to control group. Likewise, EL-Tohamy *et al.* (2010) [13], EL-Nattat *et al.* (2019) [12], EL-Gindy (2022) [7] showed similar results in various doses through oral administration of *Nigella sativa* in rabbits.

#### Functional Plasma Membrane Integrity (%)

The mean initial host reacted sperm (%) differed non-significantly among T<sub>1</sub> ( $70.44 \pm 1.15$ ), T<sub>2</sub> ( $71.00 \pm 2.42$ ), T<sub>3</sub> ( $74.25 \pm 1.77$ ) and T<sub>4</sub> ( $70.19 \pm 2.06$ ) groups (Table 2). Pre-freeze mean host reacted sperm (%) was significantly ( $p < 0.01$ ) higher in T<sub>3</sub> ( $63.94 \pm 2.04$ ) group as compared to T<sub>4</sub> ( $57.38 \pm 2.23$ ) and T<sub>1</sub> ( $52.50 \pm 1.72$ ) group and non-significantly higher as compared to T<sub>2</sub> ( $59.69 \pm 2.07$ ) group. Pre-freeze mean host reacted sperm (%) was non-significantly higher in T<sub>2</sub> ( $59.69 \pm 2.07$ ) group as compared to T<sub>4</sub> ( $57.38 \pm 2.23$ ) group and significantly ( $p < 0.01$ ) higher as compared to T<sub>1</sub> ( $52.50 \pm 1.72$ ) group. Pre-freeze mean host reacted sperm (%) was non-significantly higher in T<sub>4</sub>



(57.38±2.23) group as compared to T<sub>1</sub> (52.50±1.72) group. Post-thaw mean host reacted sperm (%) was significantly ( $p<0.01$ ) higher in T<sub>3</sub> (49.19±2.06) group as compared to T<sub>2</sub> (43.88±1.69), T<sub>4</sub> (42.5±1.52) and T<sub>1</sub> (34.13±1.77) groups. Post-thaw mean host reacted sperm (%) in T<sub>2</sub> (43.88±1.69) and T<sub>4</sub> (42.5±1.52) groups differed non-significantly with each other and significantly ( $p<0.01$ ) higher as compared to T<sub>1</sub> (34.13±1.77) group.

**Table 2:** Effect of different concentrations of Black cumin (*Nigella sativa*) seeds aqueous extract on HOST reacted spermatozoa percent of Surti buck semen at various stages of cryopreservation (Mean±SE)

Groups	HOST reacted sperm (%) (n=16)			Overall (n= 48)	F value	P value
	Initial	Pre-freeze	Post-thaw			
T <sub>1</sub>	70.44±1.15 <sub>x</sub>	52.50±1.72 <sub>y</sub>	34.13±1.77 <sub>z</sub>	52.35±2.34 <sup>b</sup>	133.07**	0.00
T <sub>2</sub>	71.00±2.42 <sub>x</sub>	59.69±2.07 <sup>ab</sup> <sub>y</sub>	43.88±1.69 <sup>b</sup> <sub>z</sub>	58.19±2.00 <sup>ab</sup>	42.90**	0.00
T <sub>3</sub>	74.25±1.77 <sub>x</sub>	63.94±2.04 <sup>a</sup> <sub>y</sub>	49.19±2.06 <sup>a</sup> <sub>z</sub>	62.46±1.87 <sup>a</sup>	41.17**	0.00
T <sub>4</sub>	70.19±2.06 <sub>x</sub>	57.38±2.23 <sup>bc</sup> <sub>y</sub>	42.50±1.52 <sup>b</sup> <sub>z</sub>	56.69±1.99 <sup>ab</sup>	49.90**	0.00
Overall (n=64)	71.47±0.95 <sub>x</sub>	58.38±1.12 <sub>y</sub>	42.42±1.10 <sub>z</sub>	--	4.11**	0.00
F value	1.28	0.97	5.54**	12.37**	188.59**	--
P value	0.28	0.41	0.00	0.00	0.00	--

<sup>a-c</sup> Means with different superscript within a column (between the groups) differs significantly at  $p<0.01$ .

<sub>x-z</sub> Means with different subscript between a column (between various stages) differs significantly at  $p<0.01$ . \*\* $p<0.01$ ; HR- HOST reacted sperm.

T<sub>1</sub> – control, T<sub>2</sub> – 3% Black cumin (*Nigella sativa*) seeds aqueous extract, T<sub>3</sub> - 4% Black cumin(*Nigella sativa*) seeds aqueous extract, T<sub>4</sub> - 5% Black cumin(*Nigella sativa*)seeds aqueous extract.

Moreover, mean host reacted sperm (%) in T<sub>1</sub>, T<sub>2</sub>, T<sub>3</sub> and T<sub>4</sub> groups were significantly higher ( $p<0.01$ ) at initial (70.44±1.15, 71.00±2.42, 74.25±1.77 and 70.19±2.06) stage as compared to pre-freeze (52.50±1.72, (59.69±2.07, 63.94±2.04 and 57.38±2.23) stage and post-thaw (34.13±1.77, 43.88±1.69, 49.19±2.06 and 42.50±1.52) stage. Furthermore, mean host reacted sperm (%) among initial, pre-freeze and post-thaw stage differed significantly ( $p<0.01$ ) in all the groups. The corresponding overall mean host reacted sperm (%) irrespective of time interval was significantly ( $p<0.01$ ) higher in T<sub>3</sub> (62.46±1.87), T<sub>2</sub> (58.19±2.00) and T<sub>4</sub> (56.69±1.99) groups as compared to T<sub>1</sub> (52.35±2.34) group and non-significantly differed with each other.

Highest mean host reacted sperm (%) was found at initial, pre-freeze and post-thaw stage in T<sub>3</sub> group (74.25±1.77, 63.94±2.04 and 49.19±2.06) followed by T<sub>2</sub> (71.00±2.42, 59.69±2.07 and 43.88±1.69) and T<sub>4</sub> group (70.19±2.06, 57.38±2.23 and 42.50 ±1.52). While lowest mean host reacted sperm (%) was found in T<sub>1</sub> group at initial (70.44±1.15), pre-freeze (52.50±1.72) and post-thaw (34.13±1.77) stage. Results from present study revealed that 4% Black cumin (*Nigella sativa*) seeds aqueous extract supplementation in tris egg yolk citrate extender showed significantly ( $p<0.01$ ) higher functional plasma membrane integrity in pre-freeze and post-thawed Surti buck semen as compared to control and other groups, while 3% Black cumin (*Nigella sativa*) seeds aqueous extract supplementation gave better results for functional plasma membrane integrity as compared to 2% Black cumin (*Nigella sativa*) seeds aqueous extract supplemented and control group. Similar, type of results were also observed by Nasiri *et al.* (2022) <sup>[9]</sup> in human spermatozoa; Awan *et al.* (2018) <sup>[5]</sup> in buffalo spermatozoa; EL-Seadawy *et al.* (2022) <sup>[18]</sup> and Sobeh *et al.* (2020) <sup>[17]</sup> in ram; Ahmed *et al.* (2019) <sup>[16]</sup> in bull spermatozoa, Khan *et al.* (2017) <sup>[15]</sup> in bull semen and Silva *et al.* (2016) <sup>[20]</sup> in buck semen by using different antioxidant additives.

The corresponding overall mean host reacted sperm (%) irrespective of treatment groups were decreased with increasing preservation time at initial (71.47±0.95), pre-freeze (58.38±1.12) and post-thaw (42.42±1.10) stages. The overall mean host reacted sperm (%) irrespective of different treatment groups differed significantly ( $p<0.01$ ) among various stages of cryopreservation.

Awan *et al.* (2018) <sup>[5]</sup> reported post thaw functional plasma membrane integrity (%) was significantly ( $p<0.05$ ) higher in 4% *Nigella sativa* extract supplemented group (63.00 ± 6.05) as compared to control (50.80 ± 4.57) group in Nili-Ravi buffalo bull semen. They also found similar results in post-dilution stage. While addition of 5% *Nigella sativa* extract in extender could not preserve functional plasma membrane integrity as compared to that at 4% addition of extract at various stages of preservation. Moreover, Nasiri *et al.* (2022) <sup>[9]</sup> observed significantly ( $p<0.05$ ) higher functional plasma membrane integrity (%) in 4% *Nigella sativa* aqueous extract supplemented (70.9 ± 2.8) group as compared to control (33.6 ± 2.8) group during vitrification of human spermatozoa. While addition of 5% *Nigella sativa* aqueous extract could not maintain functional plasma membrane integrity efficiently as compared to that at 4% *Nigella sativa* aqueous extract. EL-Seadawy *et al.* (2022) <sup>[18]</sup>, Khan *et al.* (2017) <sup>[15]</sup>; Sobeh *et al.* (2020) <sup>[17]</sup> and Silva *et al.* (2016) <sup>[20]</sup> reported similar trends in group of methanolic moringa leaves extract; green tea (*Camellia sinensis*); *Entada abyssinica* bark extract and quercetin supplemented in tris-based extender in ram; bull; ram and buck semen. They found post-thaw plasma membrane integrity of spermatozoa was lower in higher concentration of additives group as compared to other groups. Whereas, Ahmed *et al.* (2019) <sup>[16]</sup> observed significantly higher post thaw functional plasma membrane integrity (%) in 200mM quercetin supplemented in tris-based extender (42.89 ± 0.89) as compared to 150 mM concentration of quercetin (41.11 ± 1.24) and control (34.89 ± 1.18) group in bull semen.

Methanolic extract of moringa leaves has strong electron donor capacity which convert free radicals in stable product ultimately protecting membrane integrity from ROS damage (Lobo *et al.*, 2010) <sup>[21]</sup>. At low concentration of green tea extract exerts antioxidant property that protect membrane phospholipid by ROS damage. While green tea extract contain ample amount of Catechin polyphenol that strongly

fighters against oxidative stress (Chyu *et al.* 2004) [22]. Similar antioxidant property is exerted by thymoquinone and other compounds present in *Nigella sativa* that protects plasma membrane integrity. At higher doses it gives strong antioxidant property and results in decreased significant ROS production, lead to alter physiological process and lead to altered functional plasma membrane integrity (Alenzi *et al.*, 2010) [23].

### Conclusion

Addition of 4% Black cumin (*Nigella sativa*) seeds aqueous extract in tris egg yolk citrate extender maintained Post thaw semen parameters like structural membrane integrity and functional membrane integrity were well maintained in Surti buck semen signify its favourable effect on cryopreservation of Surti buck semen.

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