International Journal of Veterinary Sciences and Animal Husbandry 2024; SP-9(2): 182-186

ISSN: 2456-2912
VET 2024; SP-9(2): 182-186

www.veterinarypaper.com
Received: 07-02-2024
Accepted: 14-03-2024

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Effect of purslane (*Portulaca oleracea*) leaves extract supplementation on sperm viability of cryopreserved surti buck semen

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Abstract
The purpose of this study was to see how the addition of Purslane (*Portulaca oleracea*) leaves extract to the Tris egg yolk citrate extender affected the sperm viability of cryopreserved Surti buck semen. A total of 64 semen samples were taken from four Surti bucks, with 16 samples collected twice a week via the artificial vagina method. To attain a final concentration of 100×10^6 sperm/ml, the pooled sperm was diluted with the tris-egg yolk citrate extender and the Purslane (*Portulaca oleracea*) leaves aqueous extract was at varied concentrations: 0% (T_1), 1% (T_2), 2% (T_3), and 3% (T_4). The initial mean live sperm (%) and host reacted sperm (%) was non-significantly differed between all the groups. Pre-freeze and post thaw mean live sperm (%) and host reacted sperm (%) was significantly higher ($p<0.01$) in T_4 group as compared to T_1, T_2 and T_3 groups. Highest mean live sperm (%) and host reacted sperm (%) was found at initial, pre-freeze and post-thaw stage in T_4 group followed by T_3, T_2 and T_1 group.

Keywords: Cryopreserved semen, purslane (*Portulaca oleracea*) leaves aqueous extract, Live, HOST, Surti buck semen

1. Introduction
Goat is an important livestock species in India and other developing countries. Because it provides a good source of meat, milk, fiber, and skin, it is popularly known as the "poor man's cow" (Machugh and Bradley 2001) [1]. Surti goat is an important milk producing breed among the different goat breeds in Gujarat, India. Given the goat's low production potential and the importance of its milk, genetic enhancement through the introduction of Artificial Insemination (AI) programmes based on semen preservation is critical. This is only achievable with the long-term storage of excellent bucks' sperm with appropriate extenders, for the success of AI programmes. In Mammals sperm protection against oxidative stress is provided mainly by seminal plasma. The protective capacity of endogenous antioxidants may be insufficient to prevent peroxidative damage during storage (Aurich *et al.* 1997) [2]. Now a day's medicinal herbs have recently attracted attention of many researchers owing to their highly antioxidative properties (Krishnaiah *et al.*, 2011) [3], having more effective bioactive compounds, and being less toxic compared to synthetic medications (Ardeshirnia *et al.* 2017) [4]. *Portulaca oleracae* (Portulacaceae), also known as Purslane, is an annual edible green-grass plant consumed by human beings, in either raw or cooked form, and used in traditional medicine in many countries (Uddin *et al.*, 2014) [5]. As one of the most commonly used medicinal herbs, this plant is named 'Global Panacea' by the World Health Organization (WHO) (Anthony, 2001) [6]. The water extract of the purslane leaf contained the highest amount of total flavonoids and ascorbic acid. The main compounds of purslane (phenols and flavonoids) might be responsible for its antioxidant effects (yang *et al*., 2009) [7]. Looking into the various properties of purslane leaves and active compound flavonoids, study has been undertaken to investigate the effect of Purslane (*Portulaca oleracea*) leaves aqueous extract in tris egg yolk citrate extender on sperm viability of cryopreserved Surti buck semen.

2. Materials and Methods

2.1 Selection and management of bucks

Total four apparently healthy Surti bucks above one years of age maintained under All India Coordinated Research Project (AICRP) on Goat at Livestock Research Station, Kamdhenu University, Navsari were selected. The selected bucks were managed under uniform managemental and feeding conditions. 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.

2.2 Preparation of Purslane (*Portulaca oleracea*) leaves aqueous extract

Purslane (*Portulaca oleracea*) plant was collected from surrounding area of Navsari. The leaves 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. 100g of dried leaves powdered 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 has dissolved. The aqueous extract of Purslane (*Portulaca oleracea*) leaves 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.

2.3 Semen collection, experimental group and cryopreservation

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 ≥ 70% were considered for further processing. The pooled semen was extended with tris egg yolk citrate extender to achieve final concentration of 100 x 10^6 sperm/ml. The diluted semen was separated into four equal aliquots, and each aliquot was treated with different concentrations of Purslane (*Portulaca oleracea*) leaves aqueous extract *viz.* 0% (control T_1), 1% (T_2), 2% (T_3) and 3% (T_4) (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 50×10^6 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 vaporous phase. After completion of freezing the straws were directly and quickly plunged into liquid nitrogen container. The structural plasma membrane integrity (%) and functional plasma membrane integrity (%) were evaluated at just after dilution (Initial), Pre-freeze and Post thaw stage (24 hours after cryopreservation) using standard methods.

2.4 Statistical analysis

Descriptive analysis was carried out and mean ± SE was calculated for all the designated groups of 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.

3. Results and Discussion

3.1 Structural Plasma Membrane Integrity (live sperm) (%)

The initial mean live sperm (%) was non-significantly differed between T_1 (71.50 ± 3.09), T_2 (73.19 ± 3.44), T_3 (76.13 ± 3.43) and T_4 (78.75 ± 1.82) groups (Table 1). Pre-freeze mean live sperm (%) was significantly higher ($p<0.01$) in T_4 (66.94 ± 1.87) group as compared to T_1 (50.19 ± 2.40), T_2 (56.69 ± 3.40) and non-significantly differed T_3 (60.94 ± 2.53) groups. Pre-freeze mean live sperm (%) was non-significantly differed among T_1 and T_2; T_2 and T_3; T_3 and T_4 group. Similarly, post-thaw mean live sperm (%) was significantly higher ($p<0.01$) in T_4 (52.94 ± 2.15) group as compared to T_1 (33.31 ± 1.83), T_2 (37.50 ± 2.50) and T_3 (46.06 ± 2.38) groups. Post-thaw mean live sperm (%) was significantly higher ($p<0.01$) in T_3 group as compared to T_1 and T_2 group, whereas T_1 and T_2 was non-significantly differed.

The corresponding overall mean live sperm (%) irrespective of time interval was significantly higher ($p<0.01$) in T_4 (66.21 ± 1.89) as compared to T_1 (51.67 ± 2.68) and T_2 (55.79 ± 2.77) group whereas, non-significantly higher as compared to T_3 (61.04 ± 2.40) group. The corresponding overall mean live sperm (%) was non-significantly differed among T_1 vs. T_2 and T_2 vs. T_3 groups.

Table 1: Effect of different concentrations of Purslane (*Portulaca oleracea*) leaves aqueous extract on Live sperm percent of Surti buck semen at various stages of cryopreservation (Mean±SE).

Groups	Live sperms (%) (n=16)			Overall (n= 48)	F value	P value
	Initial	Pre-freeze	Post-thaw			
T_1	$71.50\pm3.09_x$	$50.19\pm2.40^{c}_y$	$33.31\pm1.83^{c}_z$	51.67 ± 2.68^{c}	58.97**	0.00
T_2	$73.19\pm3.44_x$	$56.69\pm3.40^{bc}_y$	$37.50\pm2.50^{c}_z$	55.79 ± 2.77^{bc}	32.28**	0.00
T_3	$76.13\pm3.43_x$	$60.94\pm2.53^{ab}_y$	$46.06\pm2.38^{b}_z$	61.04 ± 2.40^{ab}	28.45**	0.00
T_4	$78.75\pm1.82_x$	$66.94\pm1.87^{a}_y$	$52.94\pm2.15^{a}_z$	66.21 ± 1.89^{a}	43.89**	0.00
Overall (n=64)	$74.89\pm1.51_x$	$58.69\pm1.49_y$	$42.45\pm1.45_z$	--	119.50**	0.00
F value	1.13	7.32**	15.49**	6.61**	--	--
P value	0.34	0.00	0.00	0.00	--	--

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

[x-z] Means with different subscript between a column (between various stages) differs significantly at $p<0.01$. **$p<0.01$

T_1 – control, T_2 – 1% Purslane (*Portulaca oleracea*) leaves aqueous extract, T_3 - 2% Purslane (*Portulaca oleracea*) leaves aqueous extract, T_4 - 3% Purslane (*Portulaca oleracea*) leaves aqueous extract

Moreover, mean live sperm (%) in T_1, T_2, T_3 and T_4 groups were significantly higher ($p<0.01$) at initial (71.50 ± 3.09, 73.19 ± 3.44, 76.13 ± 3.43 and 78.75 ± 1.82) stage as compared to pre-freeze (50.19 ± 2.40, 56.69 ± 3.40, 60.94 ± 2.53 and 66.94 ± 1.87) stage and post-thaw stage (33.31 ± 1.83, 37.50 ± 2.50, 46.06 ± 2.38 and 52.94 ± 2.15) stage. Furthermore, mean live sperm (%) among initial, pre-freeze and post-thaw stage were differed significantly ($p<0.01$) in all the groups.

The corresponding overall mean live sperm (%) irrespective of treatment groups were reduced with increasing preservation time at initial (74.89 ± 1.51), pre-freeze (58.69 ± 1.49) and post-thaw (42.45 ± 1.45) stage. The overall mean live sperm (%) irrespective of different treatment groups were significantly ($p<0.01$) differed among various stages of cryopreservation.

Highest mean live sperm (%) was found at initial, pre-freezing and post thaw stage in T_4 (78.75 ± 1.82, 66.94 ± 1.87 and 52.94 ± 2.15) group followed by T_3 (76.13 ± 3.43, 60.94 ± 2.53 and46.06 ± 2.38) and T_2 (73.19 ± 3.44, 56.69 ± 3.40 and37.50 ± 2.50) groups. While lowest mean live sperm (%) was found in T_1 group at initial (71.50 ± 3.09), pre-freeze (50.19 ± 2.40) and post-thaw (33.31 ± 1.83) stage.

Scanning through the available literature, it was found that, few studies was carried out regarding use of Purslane (*Portulaca oleracea*) leaves aqueous extract in tris egg yolk citrate extender for cryopreservation of Surti buck semen. The main compound of Purslane (*Portulaca oleracea*) leaves is also contained higher amount of flavonoids and ascorbic acid. However, many researchers studied the effect of various medicinal plant (Turraeafischeri, Nigella sativa, cucumber etc) extract and as such quercetin, catechin having flavonoids compound. Hence, the discussion was made on that basis.

In the present study the highest sperm viability was observed in all stages of preservation (initial, pre-freeze and post thaw) in Purslane (*Portulaca oleracea*) leaves aqueous extract added in tris-based extender group as compare to control group.

Similarly, Awan *et al*. (2018) [8] who also found significantly higher ($p<0.05$) sperm viability in post-cooling and post-thaw semen in *Nigella sativa* extract added (0%, 1%, 2%, 3%, 4% and 5%)group as compared to control group in buffalo semen. Ahmed *et al.* (2019) [9] also reported higher sperm viability in post-cooling and post-thaw semen in different concentration of quercetin (T_1-50 mM, T_2-100 mM, T_3-150 mM, T_4-200 mM) added group as compared to control group in buffalo bull semen. Similarly, Azimi *et al*. (2020) [10] observed significantly ($p<0.05$) higher post thaw viability in Markhoze goat semen in Purslane (*Portulaca oleracea*) leaves extract added group than control group.

In accordance to present study Ismail *et al*. (2020) [11] reported significantly ($p<0.05$) higher viability in 50 and 100µg/ml of mint, thyme, or curcumin extract supplemented group as compared to control group in basic extender on equilibrate and post thaw periods of Baladi bucks semen. Moreover, Hassan *et al*. (2021) [12] reported the addition of *Turraeafischeri* leaf extracted @ 125, 250, and 375 µg/ml to Baladi buck semen in extender showed significantly ($p<0.05$) higher average mean of post thaw viability as compared to control group. Altyeb *et al*. (2022) [13] also reported higher post thaw sperm viability in higher concentration of cysteine and L-carnitine supplemented group as compared to other and control groups in Zaraibi buck semen. Similarly, Khalil *et al*. (2023) [14] also reported significantly ($p<0.05$) higher post thaw sperm viability in Damascus bucks semen in higher concentration of ethanolic purslane (*Portulaca oleracea*) leaf extract (100µg/ml) supplemented group as compared to other and control group.

In the present study the significantly ($p<0.01$) higher post thaw viability of spermatozoa in Surti buck semen was found in T_4 (3% purslane (*Portulaca oleracea*) leaves aqueous extract) group as compare to T_2 (1%), T_3 (2%) and control group. It's showed that percent of sperm viability depend on concentration purslane (*Portulaca oleracea*) leaves aqueous extract added in the extender. Similarly, Awan *et al*. (2018) [8] reported significantly ($p<0.05$) higher post thawed sperm viability in 3% Nigella sativa extract added group than control and other groups.

Moreover, Ahmed *et al.* (2019) [9]; Ismail *et al.* (2020) [11]; Hassan *et al*. (2021) [12] and Altyeb *et al*. (2022) [13] reported higher concentration of different antioxidant additives (quercetin; mint, thyme, or curcumin; *Turraeafischeri;* and cysteine) supplemented in tris-based extender showed significantly ($p<0.05$) higher post thaw sperm viability as compared to others and control group of buffalo bull; Baladi buck; and Zaraibi buck semen, respectively. Moreover, Khalil *et al*. (2023) [14] also reported significantly ($p<0.05$) higher post thaw sperm viability in Damascus bucks semen in higher concentration of ethanolic purslane (*Portulaca oleracea*) leaf extract (100µg) supplemented group as compared to other and control group.

Contrary to present findings Azimi *et al*. (2020) [15] reported non-significantly lower post thaw sperm viability in Markhoze buck semen at higher concentration of Purslane (*Portulaca oleracea*) leaves extract added group (PAE100µg/ml) as compared to control groups. They concluded such negative effect of antioxidants can be attributed to over cleaning of free radicles owing to using higher doses of antioxidants, which thereby can change the levels of ROS needed for physiological actions of sperm (Mata-Campuzano *et al*. 2015) [16].

3.2 Functional Plasma Membrane Integrity (HOST) (%):

The mean initial host reacted sperm (%) was differed non-significantly between T_1 (67.13 ± 2.12), T_2 (69.50 ± 2.36), T_3 (71.50 ± 1.64) and T_4 (71.88 ± 1.62) groups (Table 2). Pre-freeze mean host reacted sperm (%) was significantly higher ($p<0.01$) in T_4 (63.13 ± 1.59) group as compared to T_1 (47.5 ± 2.11) and T_2 (53.94 ± 2.67) groups, whereas it was non-significantly differed among the group T_1 vs. T_2; T_2 vs. T_3 and T_3 vs. T_4. Post-thaw mean host reacted sperm (%) was significantly higher ($p<0.01$) in T_4 (48.38 ± 1.98) group as compared to T_1 (31.88 ± 1.19), T_2 (36.38 ± 1.71) and T_3 (42.00 ± 1.51) groups, whereas it was non-significantly differed between T_1 vs. T_2 groups and significantly ($p<0.01$) differed among T_2, T_3 and T_4 groups.

Table 2: Effect of different concentrations of Purslane (*Portulaca oleracea*) leaves 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_1	$67.13\pm2.12_x$	$47.50\pm2.11^{c}_y$	$31.88\pm1.19^{c}_z$	48.83 ± 2.35^{c}	90.08**	0.00
T_2	$69.50\pm2.36_x$	$53.94\pm2.67^{bc}_y$	$36.38\pm1.71^{c}_z$	53.27 ± 2.36^{bc}	52.86**	0.00
T_3	$71.50\pm1.64_x$	$57.81\pm2.64^{ab}_y$	$42.00\pm1.51^{b}_z$	57.10 ± 2.09^{ab}	54.78**	0.00
T_4	$71.88\pm1.62_x$	$63.13\pm1.59^{a}_y$	$48.38\pm1.98^{a}_z$	61.13 ± 1.72^{a}	46.61**	0.00
Overall (n=64)	$70.00\pm0.99_x$	$55.59\pm1.33_y$	$39.66\pm1.11_z$	--	173.84**	0.00
F value	1.24	8.22**	19.31**	6.01**	--	--
P value	0.30	0.00	0.00	0.00	--	--

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

[x-z] Means with different subscript between a column (between various stages) differs significantly at $p<0.01$. **$p<0.01$.

T_1 – control, T_2 – 1% Purslane (*Portulaca oleracea*) leaves aqueous extract, T_3 - 2% Purslane (*Portulaca oleracea*) leaves aqueous extract, T_4 - 3% Purslane (*Portulaca oleracea*) leaves aqueous extract.

The corresponding overall mean host reacted sperm (%) irrespective of time interval was significantly higher ($p<0.01$) in T_4 (61.13 ± 1.72) group as compared to T_1 (48.83 ± 2.35) and T_2 (53.27 ± 2.36) groups, whereas it was non-significantly differed among T_1 vs. T_2; T_2 vs. T_3 and T_3 vs. T_4 groups.

Moreover, mean host reacted sperm (%) in T_1, T_2, T_3 and T_4 groups were significantly higher ($p<0.01$) at initial (67.13 ± 2.12, 69.50 ± 2.36, 71.50 ± 1.64 and 71.88 ± 1.62) stage as compared to pre-freeze (47.50 ± 2.11, 53.94 ± 2.67, 57.81 ± 2.64 and 63.13 ± 1.59) stage and post-thaw (31.88 ± 1.19, 36.38 ± 1.71, 42.00 ± 1.51 and 48.38 ± 1.98) stage. Furthermore, mean host reacted sperm (%) among initial, pre-freeze and post-thaw stage were differed significantly ($p<0.01$) in all the groups.

The corresponding overall mean host reacted sperm (%) irrespective of treatment groups were decreased with increasing preservation time at initial (70.00 ± 0.99), pre-freeze (55.59 ± 1.33) and post-thaw (39.66 ± 1.11) stages. The overall mean host reacted sperm (%) irrespective of different treatment groups were significantly ($p<0.01$) differed among various stages of cryopreservation.

Highest mean host reacted sperm (%) was found at initial, pre-freeze and post-thaw stage in T_4 group (71.88 ± 1.62, 63.13 ± 1.59 and 48.38 ± 1.98) followed by T_3 (71.5 ± 1.64, 57.81 ± 2.64 and42 ± 1.51) and T_2 (69.5 ± 2.36, 53.94 ± 2.67 and 36.38 ± 1.71) group. While lowest mean host reacted sperm (%) was found in T_1 group at initial (67.13 ± 2.12), pre-freeze (47.5 ± 2.11) and post-thaw (31.88 ± 1.19) stage.

In the present study the highest HOST reacted sperm was observed in all stages of preservation (initial, pre-freeze and post thaw) in Purslane (*Portulaca oleracea*) leaves aqueous extract added in tris-based extender group as compare to control group. In accordance to the present findings, Ahmed *et al.* (2019) [9] also reported higher HOST reacted sperm in post-cooling and post-thaw semen in different concentration of quercetin (T_1-50 mM, T_2-100 mM, T_3-150 mM, T_4-200 mM) added group as compared to control group in buffalo bull semen. Azimi *et al*. (2020) [10] observed significantly ($p<0.05$) higher post thaw HOST reacted sperm in Markhoze goat semen in Purslane (*Portulaca oleracea*) leaves extract added group than control group.

Likewise, Ismail *et al.* (2020) [11] reported significantly ($p<0.05$) higher HOST reacted sperm in 50 and 100µg/ml of mint, thyme, or curcumin extract supplemented group as compared to control group in basic extender on equilibrate and post thaw periods of Baladi bucks semen. Moreover, Hassan *et al*. (2021) [12] reported the addition of *Turraeafischeri*leaf extracted @ 125, 250, and 375 µg/ml to Baladi buck semen in extender showed significantly ($p<0.05$) higher average mean of post thaw HOST reacted sperm as compared to control group. Zhang *et al*. (2022) [17] also reported the addition of proline in Loshan buck semen significantly ($p<0.05$) higher post thaw functional membrane integrity of sperm as compared to control group. Similar result was also observed by Altyeb *et al*. (2022) [13], who reported significantly ($p<0.001$) higher post thawed functional plasma membrane integrity in Zairabi buck semen in cysteine and L-carnitine added group as compared to control group.

Moreover, Khalil *et al*. (2023) [14] reported functional membrane integrity was significantly ($p<0.05$) higher in Damascus buck semen in Ethanolic purslane leaf extract added @ POLE 50µg/mL, POLE 100µg/ml, POLENF 50µg/ml and POLENF 100µg/ml group than control group at post thaw stage. Torkamanpari *et al*. (2023) [18] reported significantly ($p<0.05$) higher functional membrane integrity in twenty human sperm sample in purslane hydroalcoholic extract (25, 50, and 100 mg/l) added group as compared to control group.

In the present study the significantly ($p<0.01$) higher post thaw HOST reacted sperms in Surti buck semen was found in T_4 (3% purslane (*Portulaca oleracea*) leaves aqueous extract) group as compare to T_2 (1%), T_3 (2%) and control group. It's showed that percent of HOST reacted sperm depend on concentration Purslane (*Portulaca oleracea*) leaves aqueous extract added in the extender. Similarly, Ahmed *et al.* (2019) [19]; Ismail *et al.* (2020) [11]; Hassan *et al*. (2021) [12] and Altyeb *et al*. (2022) [13] reported higher concentration of different antioxidant additives (quercetin; mint, thyme, or curcumin; *Turraeafischeri* and cysteine) supplemented in tris-based extender showed significantly ($p<0.05$) higher post thaw HOST reacted sperm as compared to others and control group of buffalo bull; Baladi buck; and Zaraibi buck semen, respectively. Likewise, Khalil *et al*. (2023) [14] reported post thawed HOST reacted sperm was significantly ($p<0.05$) higher in Purslane leaf extract (100 µg/ml) supplemented in tris-based extender group than control and other supplemented group. Similarly, Torkmanpari *et al*. (2023) [18] also observed functional membrane integrity was significantly ($p<0.05$) higher in Purslane 50 mg/ladded group as compared to other supplemented and control groups of human spermatozoa after vitrification.

Contrasting to present findings Azimi *et al*. (2020) [15] reported significantly (p>0.05) higher host reacted sperm in lower concentration of Purslane extracts 50µg/ml(53.29 ± 3.21) added in tris-based extender group as compared to control group in Markhoze goat semen. They concluded such negative effect of antioxidants can be attributed to over cleaning of free radicles owing to using higher doses of

antioxidants, which thereby can change the levels of ROS needed for physiological actions of sperm (Mata-Campuzano *et al*. 2015) [16].

4. Conclusion

Post thaw semen parameters like structural membrane integrity and functional membrane integrity were well maintained in Surti buck semen supplemented with 3% Purslane (*Portulaca oleracea*) leaves aqueous extract in tris egg yolk citrate extender.

5. Acknowledgement

Authors are grateful to principal, College of Veterinary Science & AH., Kamdhenu University, Navsari and all the staff of Department of Gynaecology & Obstetrics for providing facilities and support to complete the present investigation.

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