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Effect of Purslane (*Portulaca oleracea*) leaves extract supplementation on sperm motility 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 motility 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 individual sperm motility percent was differed non-significantly between all the groups. Pre-freeze and post thaw mean individual sperm motility (%) was significantly ($p<0.01$) higher in T_4 group as compared to T_1, T_2 and T_3 group. Highest mean motility degeneration rate (%) was found at 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, Motility, motility degeneration rate, surti buck semen

1. Introduction
Goats are a major livestock animal in India and other developing countries. It is commonly referred to as the "poor man's cow" due to its high content of meat, milk, fiber, and skin. Surti goats are prominent milk producers among the several goat breeds in Gujarat, India. Given the goat's limited production potential and the value of its milk, genetic modification through the implementation of Artificial Insemination (AI) programs based on semen preservation is crucial. This is only possible with the long-term storage of high-quality bucks' sperm with adequate extenders for the success of AI programs. In mammals, seminal plasma primarily protects sperm from oxidative damage. Many researchers are now interested in medicinal herbs because of their high antioxidative qualities (Krishnaiah *et al*., 2011) [1], more effective bioactive chemicals, and lower toxicity when compared to manufactured drugs (Ardeshirnia *et al*., 2017) [2]. Portulaca oleracae (Portulacaceae), popularly known as Purslane, is an annual edible green-grass plant that humans consume raw or cooked, and is used in traditional medicine in many countries (Uddin *et al*., 2014) [3]. The World Health Organization (WHO) refers to this plant as 'Global Panacea', as it is one of the most widely utilized medical plants. Purslane contains substances such as flavonoids, terpenoids, phenolic acids, alkaloids, saponins, omega-3 fatty acids, carotene, vitamins, glutathione, and melatonin (Erkan, 2012) [4]. The purslane leaves water extract had the highest total flavonoid and ascorbic acid concentration. Purslane's major components (phenols and flavonoids) may be responsible for its antioxidant properties (Yang *et al*., 2009) [5]. Looking into the numerous qualities of purslane leaves and active ingredient flavonoids, a study was conducted to investigate the effect of Purslane (*Portulaca Oleracea*) leaves aqueous extract in tris egg yolk citrate extender on the sperm motility of cryopreserved Surti buck semen.

2. Materials and Methods
2.1 Selection and management of bucks: A total of four Surti bucks, over one year old and kept under the All India Coordinated Research Project (AICRP) on Goat at Kamdhenu

University, Navsari, Livestock Research Station, were chosen. They appeared to be in good health. The chosen bucks were raised in consistent food and management environments. The chosen bucks were kept in a shared covered pen with the South Gujarati climate and photoperiod as it naturally exists. The animals were fed high-quality feed ad libitum coupled with 500gm of concentrate each animal per day, and they were permitted to graze from 2:30 PM to 4:30 PM. They received frequent vaccinations against common diseases like Foot and Mouth Disease (FMD) and Peste des Petits Ruminants (PPR) and were dewormed four times a year using various types of dewormers. The chosen bucks were kept apart from the female and kept in a shared covered pen. Using a female (doe) dummy, the bucks were trained to donate semen in an artificial vagina. Following a roughly one-month training phase, semen was routinely collected twice a week from each buck using an artificial vagina for a maximum of eight weeks. In all, sixteen ejaculates of semen were collected from each buck.

2.2 Preparation of Purslane (*Portulaca oleracea*) leaves aqueous extract: The Purslane (*Portulaca Oleracea*) plant was collected from surrounding area of Navsari. To remove dust and grime, the leaves were gently washed in clean water. They were allowed to air dry for four days at room temperature, and then a mixer grinder was used to ground them into a fine powder. A glass container sealed with aluminum foil had 100g of powdered dried leaves, which were extracted with water. The container was left at room temperature for 24 hours, stirring often, until the soluble material had dissolved. Using the liquid partition method, an aqueous extract of Purslane (*Portulaca Oleracea*) leaves was produced. Before sample preparation for ensuing analyses, the aqueous extract was placed in a vial and refrigerated at -20 °C.

2.3 Semen collection, experimental group and cryopreservation: All of the chosen bucks' semen was collected early in the morning, between 6.30 and 7.30 AM, using an eight-inch artificial vagina (AV) to maintain an internal temperature between 40 °C and 42 °C and enough pressure. Every component of the artificial vagina is sterile, and each buck has its own artificial vagina. Additionally, a buck apron is worn during the collection process to stop any further contamination and preserve the integrity of the semen. All four bucks' ejaculates were combined to boost the volume of semen and remove variability among the bucks. Semen samples were only processed further if their initial motility was at least 70%. 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 progressive sperm motility (%) was evaluated at just after dilution (Initial), Pre-freeze and Post thaw stage (24 hours after cryopreservation) using standard methods. The motility degradation rate (MDR) (%) was calculated as per the formula gave by Campos *et al*., 2004 [6].

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 Individual Sperm Motility (%): The initial mean individual sperm motility (%) was differed non-significantly between T_1 (80.00 ± 1.12), T_2 (80.31 ± 1.07), T_3 (81.56 ± 1.18) and T_4 (82.19 ± 1.20) group (Table 1). Pre-freeze mean individual sperm motility (%) was significantly (p<0.01) higher in T_4 (58.44 ± 1.18) group as compared to T_1 (39.69 ± 1.80), T_2 (47.94 ± 1.28) and T_3 (53.44 ± 1.48) group. Similarly, post-thaw mean individual sperm motility (%) was also significantly (p<0.01) higher in T_4 (43.44 ± 1.97) group when compared with T_1 (23.13 ± 1.11), T2 (31.88 ± 1.28) and T_3 (38.75 ± 1.41). The individual sperm motility (%) was significantly differed among all the groups during pre-freeze and post thaw stage.

The corresponding overall mean individual sperm motility (%) irrespective of time interval was significantly higher (p<0.01) in T_4 (61.35 ± 2.48) group as compared to T_1 (47.60 ± 3.57) group. While overall mean individual sperm motility (%) was non- significantly differed among T_2, T_3 and T_4 groups.

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

Groups	Individual Sperm Motility (%) (n=16)			Overall (n= 48)	F value	P value
	Initial	Pre-freeze	Post-thaw			
T_1	$80.00\pm1.12_x$	$39.69\pm1.80^{d}_y$	$23.13\pm1.11^{d}z$	47.60 ± 3.57^{b}	450.63**	0.00
T_2	$80.31\pm1.07_x$	$47.94\pm1.28^{c}_y$	$31.88\pm1.28^{c}z$	53.38 ± 3.02^{ab}	414.20**	0.00
T_3	$81.56\pm1.18_x$	$53.44\pm1.48^{b}_y$	$38.75\pm1.41^{b}z$	57.92 ± 2.70^{a}	255.27**	0.00
T_4	$82.19\pm1.20_x$	$58.44\pm1.18^{a}_y$	$43.44\pm1.97^{a}z$	61.35 ± 2.48^{a}	169.64**	0.00
Overall (n=64)	$81.02\pm0.57_x$	$49.88\pm1.13_y$	$34.3\pm1.20_z$	--	557.66**	0.00
F value	0.81	30.59**	35.69**	4.01**	--	--
P value	0.49	0.00	0.00	0.00	--	--

[a-d] Means with different superscript within a column (between the groups) differs significantly at p<0.05.

[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 individual sperm motility (%) in T_1, T_2, T_3, T_4 groups were significantly (p<0.01) higher at initial phase (80.00 ± 1.12, 80.31 ± 1.07, 81.56 ± 1.18 and 82.19 ± 1.20) as compared to pre-freeze (39.69 ± 1.8, 47.94 ± 1.28, 53.44 ± 1.48 and 58.44 ± 1.18) and post thaw (23.13 ± 1.11, 31.88 ± 1.28, 38.75 ± 1.41 and 43.44 ± 1.97) stage. Furthermore, mean individual sperm motility (%) among initial, pre-freeze and post-thaw stage were differed significantly(p<0.01) in all the groups.

The corresponding overall mean individual motility irrespective of treatment groups were reduced with increasing preservation time at initial stage (81.02 ± 0.57) followed by pre-freeze (49.88 ± 1.13) and post-thaw (34.3 ± 1.20) stages.

Highest mean individual sperm motility (%) was found at initial, pre-freeze and post-thaw stage in T_4 (82.19 ± 1.20, 58.44 ± 1.18 and 43.44 ± 1.97) group followed by T_3 (81.56 ± 1.18, 53.44 ± 1.48 and 38.75 ± 1.41) group and T_2 (80.31 ± 1.07, 47.94 ± 1.28 and 31.88 ± 1.28) group. While lowest mean individual sperm motility (%) was found in T_1 group at initial (80.00 ± 1.12), pre-freeze (39.69 ± 1.8) and post-thaw (23.13 ± 1.11) stages.

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 individual sperm motility in Surti buck semen was observed in all stages of preservation (initial, pre freezing, post-thaw) in Purslane (*Portulaca Oleracea*) leaves aqueous extract supplemented group as compare to control group, moreover the individual sperm motility was found higher with the increase the concentration of Purslane (*Portulaca Oleracea*) leaves aqueous extract in tris based extender was in agreement with, Awan *et al*. (2018) [7], who also found significantly higher (p<0.05) sperm motility in post-cooling and post-thaw semen in *Nigella sativa* extract added group (0%, 1%, 2%, 3%, 4% and 5%) as compared to control group in buffalo semen. Similarly, Ahmed *et al.* (2019) [8] reported higher post thaw progressive motility in different concentration of quercetin (T_1-50 mM, T_2-100 mM, T_3-150 mM, T_4-200 mM) supplemented group as compared to control group in buffalo bull semen.

In accordance to present study Ismail *et al.* (2020) [9] reported significantly (p<0.05) higher motility 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) [10] 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 progressive sperm motility as compared to control group.

While, Zhang *et al*. (2022) [11] reported significantly (p<0.05) higher sperm progressive motility in proline supplemented group as compared to control group in basic extender on post thaw period of Loshan buck semen. Altyeb *et al*. (2022) [12] reported higher post thawed motility in higher concentration of cysteine and L-carnitine supplemented group as compared to other and control groups in Zaraibibuck semen, similarly, Azimi *et al*. (2020) [13] reported purslane (*Portulaca Oleracea*) leaves extract added group showed significantly (p<0.05) higher post thaw individual sperm motility in Markhoze goat semen as compared to control group. Moreover, Torkamanpari *et al*. (2023) [14] observed sperm motility at lower concentration of purslane hydroalcoholic extract (50 mg/l) supplemented in extender group was significantly (p<0.05) higher than control group of human spermatozoa after vitrification.

In present study we found significantly (p<0.01) higher post thaw motility in T_4 (3% purslane (*Portulaca Oleracea*) leaves aqueous extract supplemented) group followed by T_3 (2% purslane (*Portulaca Oleracea*)) as compare to control group. In accordance to these findings Awan *et al*. (2018) [7] reported significantly (p<0.05) higher post thawed sperm motility in 3% Nigella sativa extract added group than control group. Moreover, Ahmed *et al*. (2019) [8]; Ismail *et al.* (2020) [9]; Hassan *et al*. (2021) [10]; Zhang *et al*. (2022) [11] and Altyeb *et al*. (2022) [12] reported higher concentration of different antioxidant additives (quercetin; mint, thyme, or curcumin; *Turraeafis cheri;* proline and cysteine) supplemented in tris-based extender showed significantly (p<0.05) higher post thaw motility as compared to others and control group of buffalo bull; Baladi buck; Laoshan bucks; Zaraibi buck semen, respectively. Moreover, Khalil *et al*. (2023) [15] also reported significantly (p<0.05) higher post thaw motility 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.

Contrary to present findings Inanc *et al.* (2019) [16] reported higher post thaw motility in bull semen at lower concentration of catechin (5µg/ml) supplemented group as compared to control, catechin10µg/mL, catechin25µg/ml and Catechin 50µg/mL supplemented in extender groups. Similarly, Azimi *et al*. (2020) [17] also found significantly (p<0.05) higher post thaw sperm motility in Markhozebuck semen at lower concentration of purslane (*Portulaca oleracea*) leaves extract added group (PAE50µg/ml) as compared to control, PAE25µg/ml, and PAE100µg/ml groups.

Purslane (*Portulaca oleracea*) is an essential source of natural antioxidants and can be used effectively to scavenge free radicals and/or inhibit ROS. Hence, Purslane in a native form or nano-formulation is considered a natural antioxidant in Tris-extender of goat cryopreserved semen for improving sperm freezing ability and protecting spermatozoa from cryodamage by increasing the semen antioxidant capacity (Khalil *et al.* 2023) [15]. The 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) [18]. This discrepancy in sperm motility might have been due to thymoquinone (active compound of *Nigella sativa*) that exhibits antioxidant properties at low concentrations that protect spermatozoa from ROS versus pro-oxidant properties at high concentrations that enhances the production of ROS and ultimately results in reduced sperm motility (Burits and Bucar, 2000) [19].

3.2 Motility degeneration rate (%)

The pre-freeze mean motility degeneration rate (%) was significantly lower (p<0.01) in T4 (28.69 ± 1.73) group as compared to T_3 (34.43 ± 1.66), T_2 (40.27 ± 1.53) and T_1 (50.41 ± 2.08) group (Table 2). Moreover, pre-freeze mean motility degeneration rate (%) was significantly (p<0.01) differed among all the groups. Post-thaw mean motility degeneration rate (%) was significantly lower (p<0.01) in T_4 (47.11 ± 2.28) group as compared to T_1 (70.91 ± 1.58) and T_2 (60.37 ± 1.42) groups, whereas it was non-significantly differed between T_3 and T_4 group.

Table 2: Effect of different concentrations of Purslane (*Portulaca oleracea*) leaves aqueous extract on Motility Degeneration Rate (MDR) percent of Surti buck semen at various stages of cryopreservation (Mean±SE).

Groups	MDR (%) (n=16)		Overall (n= 32)	F value	P value
	Pre-freeze	Post-thaw			
T_1	$50.41\pm2.08^{a}{}_{y}$	$70.91\pm1.58^{a}{}_{x}$	60.66 ± 2.24^{a}	1.12**	0.00
T_2	$40.27\pm1.53^{b}{}_{y}$	$60.37\pm1.42^{b}{}_{x}$	50.32 ± 2.08^{b}	0.07**	0.00
T_3	$34.43\pm1.66^{c}{}_{y}$	$52.27\pm2.02^{c}{}_{x}$	43.35 ± 2.05^{c}	0.05**	0.00
T_4	$28.69\pm1.73^{d}{}_{y}$	$47.11\pm2.28^{c}{}_{x}$	37.9 ± 2.17^{c}	1.75**	0.00
Overall (n=64)	$38.45\pm1.33_{y}$	$57.67\pm1.45_{x}$	--	0.49**	0.00
F value	27.72**	31.17**	21.07**	--	--
P value	0.00	0.00	0.00	--	--

[a-d] 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 motility degeneration rate (%) irrespective of time interval was significantly lower ($p<0.01$) in T4 (37.9 ± 2.17) as compared to T_1 (60.66 ± 2.24) and T_2 (50.32 ± 2.08) group, whereas it was non-significantly differed between T_3 and T_4 group.

Moreover, mean motility degeneration rate (%) in T_1, T_2, T_3 and T_4 groups were significantly lower ($p<0.01$) at pre-freeze (50.41 ± 2.08, 40.27 ± 1.53, 34.43 ± 1.66 and 28.69 ± 1.73) stage as compared to post-thaw (70.91 ± 1.58, 60.37 ± 1.42, 52.27 ± 2.02 and 47.11 ± 2.28) stage. Furthermore, mean motility degeneration rate (%) among pre-freeze and post-thaw stage was differed significantly ($p<0.01$) in all the groups.

The corresponding overall mean motility degeneration rate (%) irrespective of treatment groups were increased with increasing preservation time at pre-freeze (38.45 ± 1.33) and post-thaw (57.67 ± 1.45) stage. The overall mean motility degeneration rate (%) irrespective of different treatment groups were significantly ($p<0.01$) differed among various stages of cryopreservation.

Lowest mean motility degeneration rate (%) was found at pre-freeze and post-thaw stage in T_4 (28.69 ± 1.73 and 47.11 ± 2.28) group followed by T_3 (34.43 ± 1.66 and 52.27 ± 2.02) and T_2 (40.27 ± 1.53 and 60.37 ± 1.42) group. While highest mean motility degeneration rate (%) was found in T_1 group at pre-freeze (50.41 ± 2.08) and post-thaw (70.91 ± 1.58) stage.

In the present study the lower MDR was observed at pre-freeze and post thaw stage in Purslane (*Portulaca oleracea*) leaves aqueous extract added in tris-based extender group as compare to control group. Moreover, significantly ($p<0.01$) lower MDR was observed at pre-freeze and post thaw stage in T_4 3% purslane (*Portulaca Oleracea*) leaves aqueous extract added in tris-based extender group as compared to control group. Likewise, Aguiar *et al*. (2013) [20] reported significantly ($p<0.05$) higher MDR at 2 hours after cooling than that of 48 hours after cooling during dry season in non-defined breed of bucks. Lima *et al*. (2013) [21] reported lower MDR in coconut water supplemented egg yolk extender (CW-EY) group as compared to other extenders and control group. Atara *et al.* (2019) [22] reported average MDR percent showing an increasing trend in adult bucks with increase the preservation time as 30 (6.65 ± 0.24), 60 (13.67 ± 0.42) and 120 (28.77 ± 0.79) minutes when maintained at 37 °C.

Similarly, Patel, (2019) [23] reported significantly ($p<0.01$) higher MDR in control (45.61±2.56) group as compared to groups supplemented with 1% (37.35±2.63), 2% (32.96±2.63), 3% (30.44±2.71) and 4% (24.15±2.74) honey in tris egg yolk citrate extender in Surti buck semen. Whereas, Baldaniya *et al.* (2020) [24] reported lower MDR percentage irrespective of preservation time in a group supplemented with 5% coconut water (24.03±3.74%) followed by 10% (34.92±3.79%), 15% (47.03±3.93%), 0% (51.59±4.41%) and 20% (53.16±3.86%) group to Surti buck semen in tris egg yolk citrate extenders.

4. Conclusions

Addition of 3% Purslane (*Portulaca Oleracea*) leaves aqueous extract in tris egg yolk citrate extender maintained sperm motility above 55% at pre freeze and 40% at post thaw stage signify its favorable effect on cryopreservation of Surti buck semen.

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