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Effect of Purslane (*Portulaca Oleracea*) Leaves Extract in Tris Egg Yolk Citrate Extender on Oxidative Stress Parameters of Cryopreserved Surti Buck Semen

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Abstract

This research aimed to investigate the antioxidant properties of Purslane (Portulaca Oleracea) leaf extract in the Tris egg yolk citrate extender on the cryopreserved Surti buck semen. A total of 64 semen samples were collected from four Surti bucks, with 16 samples from each buck, twice a week using the artificial vagina method. The semen samples were diluted with the Tris egg yolk citrate extender and the Purslane (Portulaca Oleracea) leaves aqueous extract was added at different concentrations, namely 0% (T1), 1% (T2), 2% (T3), and 3% (T4), to maintain the final concentration of 100x106 sperm/ml with a pH of 6.5-6.8. The levels of lipid peroxidation and reduced glutathione were evaluated at three stages: just after dilution (initial), pre-freeze, and post-thaw (24 hours after cryopreservation) stages. Results showed that Lipid peroxidation (MDA) decreased with an increase in Purslane (Portulaca Oleracea) leaves aqueous extract concentration at initial, pre-freeze, and post-thaw stages, with the lowest level observed in the 3% Purslane (Portulaca Oleracea) leaves aqueous extract group. Additionally, Glutathione (GSH) values increased with an increase in Purslane (Portulaca Oleracea) leaves aqueous extract concentration at the initial, pre-freeze, and post-thaw stages, with the highest GSH activity observed in the 3% Purslane (Portulaca Oleracea) leaves aqueous extract in tris egg yolk citrate extender group. These results indicated that Purslane (Portulaca Oleracea) leaves aqueous extract is an effective antioxidant that enhances the sperm antioxidant defense in cryopreserved Surti buck semen.

Keywords: Cryopreserved Semen, Purslane (*Portulaca Oleracea*) Leaves Aqueous Extract, Antioxidant, Surti Buck Semen.

Introduction

The Surti goat is an important milk-producing breed among the different goat breeds in Gujarat, India. Goat 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). Nowadays medicinal herbs have attracted the attention of many researchers owing to their highly antioxidative properties (Krishnaiah *et al.*,2011), having more effective bioactive compounds, and being less toxic compared to synthetic medications (Ardeshirnia *et al.*,2017). *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). As one of the most commonly used medicinal herbs, this plant is named 'Global Panacea' by the World Health Organization (WHO) (Anthony, 2001). Purslane contains compounds such as flavonoids, terpenoids, phenolic acids, alkaloids, saponins, omega-3 fatty acids, carotene, vitamins, glutathione, and melatonin (Erkan, *et al*., 2012). The water extract of the purslane leaf contained the highest number 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). Looking into the various properties of purslane leaves and active compound flavonoids, the study has been undertaken to investigate the effect of Purslane (*Portulaca Oleracea*) leaves aqueous extract in tris egg yolk citrate extender on oxidative stress parameters of cryopreserved Surti buck semen.

Materials and Methods

Selection And Management of Bucks

A total of four apparently healthy Surti bucks above one year of age maintained under the All India Coordinated Research Project (AICRP) on Goat at Livestock Research Station, Kamdhenu University, Navsari were selected. The selected bucks were managed under uniform management and feeding conditions. The selected bucks were housed in a common covered pen and under naturally existing photoperiod and air temperature of South Gujarat. The animals were allowed to graze between 2:30 PM to 4:30 PM and fed with good quality fodder ad libitum along with 500gm of concentrate per animal per day. They were dewormed four times a year using different types of dewormer and regularly vaccinated against common diseases *viz. Peste des Petits Ruminants* (PPR) and Foot and Mouth Disease (FMD). The selected bucks were housed in a common covered pen and separated from females. The bucks were trained to donate their semen in an artificial vagina by using a female (doe) as a dummy. After completion of the training period of about one month, semen was collected regularly by using an artificial vagina twice a week from each buck for up to 8 weeks, and a total of 64 semen ejaculates (16 ejaculates from each buck) were collected.

Preparation of Purslane (Portulaca Oleracea) Leaves Aqueous Extract

The Purslane (*Portulaca Oleracea*) plant was collected from the 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 a mixer grinder. 100g of dried leaves powdered was extracted with water in a glass container covered with aluminum foil and allowed to stand at room temperature for a period of 24 hours with frequent agitation until the soluble matter had dissolved. Hot water extraction was carried out at 80 °C for 30 min to optimize the extraction medium. The mixture was cooled down and strained and finally, the aqueous extract was clarified by filtration. The filtrate was exhaustively extracted by a liquid partition with hexane using a separating funnel and concentrated plant leaves aqueous extract was stored in a vial and was kept in a refrigerator at -20°C before sample preparation for subsequent analyses.

Semen Collection, Experimental Group and Cryopreservation

Semen was collected from all the selected bucks in the early morning between 6.30 AM to 7.30 AM with the help of an Eight-inch Artificial Vagina (AV) maintaining the inner temperature of 40°C to 42°C and sufficient pressure. To maintain the quality of semen, all the parts of the artificial vagina were properly sterilized and for each buck separate Artificial vagina was used during collection buck apron was applied to prevent further contamination. To

increase the semen volume as well as to 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 a tris egg yolk citrate extender to achieve a 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 T1), 1% (T2), 2% (T3) and 3% (T4) (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 a micropipette having a 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 for about 4 hours. After equilibration, the floating rack holding the straws was placed in a manual vapour freezing unit (Minitube, Germany) for 10 minutes in such a way that the straws remained 5 cm above the liquid nitrogen in the vaporous phase. After completion of freezing the straws were directly and quickly plunged into a liquid nitrogen container. Lipid Peroxidation and Reduced Glutathione were evaluated at just after dilution (Initial), Pre-freeze, and Post thaw stage (24 hours after cryopreservation). Lipid peroxidation of spermatozoa was measured by determining the concentration of Malondialdehyde (MDA) production based on Thiobarbituric acid reaction (TBA) as an indicator for lipid peroxidation according to the method Banday *et al*. (2017). Reduced glutathione (GSH) was estimated as per the method described by Sedlak and Lindsay (1968).

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 the above parameters was made by analysis of variance (ANOVA) and the mean difference between the groups was tested by using Duncan's new Multiple Range Test (DNMRT) at 5 and 1 percent levels of significance.

Results and Discussion

Lipid Peroxidation (MDA)

The mean initial MDA levels (nmol/ml) were non-significantly differed between the groups (Table 1). Pre-freeze mean MDA level (nmol/ml) was significantly lower ($p<0.01$) in T4 (5.47 ± 0.11) group as compared to T1 (6.55 ± 0.08), T2 (6.21 ± 0.09) and T3 (5.88 ± 0.15) groups, moreover, it was significantly ($p<0.01$) differed among all the groups. Similarly, Post-thaw mean MDA level (nmol/ml) was significantly lower ($p<0.01$) in T4 (6.50 ± 0.10) group as compared to T1 (8.14 ± 0.09), T2 (7.77 ± 0.07) and T3 (7.21 ± 0.07) groups, moreover, it was significantly ($p<0.01$) differed among all the groups.

Table 1: Antioxidant Effect of different concentrations of Purslane (*Portulaca Oleracea*) leaves aqueous extract on Lipid Peroxidation (MDA nmol/ml) level of Surti buck semen at various stages of cryopreservation (Mean±SE).

Groups	Lipid Peroxidation (MDA) (nmol/ml) (n=16)			Overall (n= 48)	F value	P value
	Initial	Pre-freeze	Post-thaw			
T1	$4.91\pm0.07^{a}_{z}$	$6.55\pm0.08^{a}_{y}$	$8.14\pm0.09^{a}_{x}$	6.53 ± 0.20^{a}	414.35**	0.00
T2	$4.82\pm0.08^{a}_{z}$	$6.21\pm0.09^{b}_{y}$	$7.77\pm.07^{b}_{x}$	6.27 ± 0.18^{ab}	326.86**	0.00
T3	$4.71\pm0.11^{a}_{z}$	$5.88\pm0.15^{c}_{y}$	$7.21\pm0.07^{c}_{x}$	5.93 ± 0.16^{bc}	123.46**	0.00
T4	$4.68\pm0.13^{a}_{z}$	$5.47\pm0.11^{d}_{y}$	$6.50\pm0.10^{d}_{x}$	5.55 ± 0.13^{c}	66.09**	0.00
Overall (n=64)	$4.78\pm0.05_{z}$	$6.03\pm0.07_{y}$	$7.40\pm0.09_{x}$	--	331.96**	0.00
F value	1.14	18.14**	75.95**	6.37**	--	--
P value	0.33	0.00	0.00	0.00	--	--

a-d Means with different superscripts within a column (between the groups) differ significantly at p<0.01.
*x-z Means with different subscripts between a column (Between various stages) differs significantly at p<0.01. **p<0.01*
***T1** – control, **T2** – 1% Purslane (Portulaca Oleracea) leaves aqueous extract, **T3** - 2% Purslane (Portulaca Oleracea) leaves aqueous extract, **T4** - 3% Purslane (Portulaca Oleracea) leaves aqueous extract.*

The corresponding overall mean MDA level (nmol/ml) irrespective of time interval was significantly lower ($p<0.01$)

in the T4 (5.55 ± 0.13) group as compared to T1 (6.53 ± 0.20) and T2 (6.27 ± 0.18) groups, whereas it was non-significantly differed between T1 vs. T2; T2 vs. T3 and T3 vs. T4 groups. Moreover, in T1, T2, T3, T4 groups mean MDA level (nmol/ml) was significantly lower ($p<0.01$) at initial (4.91 ± 0.07, 4.82 ± 0.08, 4.71 ± 0.11 and 4.68 ± 0.13) stage as compared to pre-freeze (6.55 ± 0.08, 6.21 ± 0.09, 5.88 ± 0.15 and 5.47 ± 0.11) and post-thaw (8.14 ± 0.09, 7.77 ± 0.07, 7.21 ± 0.07 and 6.5 ± 0.10) stage. Furthermore, the mean MDA level (nmol/ml) among initial, pre-freeze, and post-thaw stages differed significantly ($p<0.01$) in all the groups. The corresponding overall mean MDA level (nmol/ml) irrespective of treatment groups was increased with increasing preservation time at the initial (4.78 ± 0.05), pre-freeze (6.03 ± 0.07), and post-thaw (7.40 ± 0.09) stage. The overall mean MDA level (nmol/ml) irrespective of different treatment groups significantly ($p<0.01$) differed between various stages of cryopreservation.

The lowest mean MDA level (nmol/ml) was found at initial, pre-freeze and post-thaw stage in T4 group (4.68 ± 0.13, 5.47 ± 0.11 and 6.5 ± 0.10) followed by T3 (4.71 ± 0.11, 5.88 ± 0.15 and 7.21 ± 0.07) and T2 (4.82 ± 0.08, 6.21 ± 0.09 and 7.77 ± 0.07) group. While highest mean MDA level was found in the T1 group at the initial (4.91 ± 0.07), pre-freeze (6.55 ± 0.08), and post-thaw (8.14 ± 0.09) stages. In the present study the lowest MDA level (nmol/ml) 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 compared to the control group. Similarly, Mehdipour *et al.* (2016); Seifi-Jamadi *et al.* (2017); Azimi *et al*. (2020); Altyeb *et al*. (2022); El-Seadawy *et al*. (2022) and Khalil *et al*. (2023) reported different antioxidant additives (*Camellia sinensis*; quercetin; *Portulaca Oleracea;* cysteine and L-carnitine and moringa leaf extract) supplemented in tris based extender showed significantly ($p<0.05$) lower MDA level as compared to control group of Ghezel rams; Markhoze goat; Zaraibi buck; Awassi ram and Damascus buck semen, respectively.

In the present study, significantly ($p<0.01$) lower pre-freeze and post-thaw MDA levels in Surti buck semen were found in the T4 group as compared to the T2, T3, and control groups. It's shown that MDA levels depend on the concentration of purslane (*Portulaca Oleracea*) leaves aqueous extract added in the extender. Similarly, Altyeb *et al*. (2022) also reported lower MDA levels in higher concentrations of cysteine and L-carnitine supplemented group as compared to other control groups in Zaraibi buck semen. Khalil *et al*. (2023) also reported significantly ($p<0.05$) lower MDA levels in Damascus bucks' semen in a higher concentration of ethanolic purslane (*Portulaca oleracea*) leaf extract (100µg) supplemented group as compared to other and control group.

Contrasting to the present findings, Mehdipour *et al.* (2016) reported MDA level was significantly lower ($p<0.05$) in the 10 mg/l group (1.4 ± 0.2) followed by the 5 mg/l group (2.4 ± 0.2) as compared to control (3.1 ± 0.2) group of *Camellia sinensis* extract added in tris-based extender in Ghezel rams semen. Likewise, Seifi-Jamadi *et al.* (2017) observed significantly ($p<0.001$) lower freeze-thaw MDA (nmol/ml) level in quercetin 10 µM (5.81 ± 0.44) group as compared to 20 µM (6.78 ±0.17) and control (6.96±0.22) group in Mahabadi goat semen. Azimi *et al*. (2020) also reported lower levels of MDA in lower concentrations of purslane aqueous extract (PAE50µg/ml) added in the basic extender group as compared to PAE25µg/ml, PAE100µg/ml, and control groups in Markhoze goat semen. Moreover, El-Seadawy *et al*. (2022) observed MDA level was significantly ($p<0.05$) lower in lower concentration of moringa leaf methanolic extract (MLME 0.48 mg/ml) supplemented group as compared to other (MLME 0.56 mg/ml and MLME 0.64 mg/ml) and control group in Awassi ram semen.

Azimi *et al.* (2020) reported a higher concentration of purslane aqueous extract PAE100µg/ml (3.18±0.23) showed a non-significantly lower MDA level than the control (2.91 ± 0.21) 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 the physiological actions of sperm (Mata-Campuzano *et al.* 2015). Moreover, Inanc *et al*. (2021) reported a low concentration of thymoquinone (TQ) TQ25 (4.56±0.67) added in tris-based extender showed a non-significantly lower MDA level than the control (4.17±0.17) group in Sonmez ram semen.

Previous research has attributed the antioxidant effect of purslane to three phenolic alkaloids (oleracein A, B, and E), which lead to free radical scavenging and prevent lipid peroxidation in rats (Sanja *et al*. 2009). Many *in vitro* and *in vivo* studies documented the antioxidant and protective effects of different extracts of purslane against oxidative stress the main compounds of purslane (phenols and flavonoids) might be responsible for its antioxidant effects (Yang *et al*. 2009).

Reduced Glutathione (GSH)

The mean initial GSH levels (nmol/ml) were non-significantly differed between the groups (Table 2). Pre-freeze mean GSH level (nmol/ml) was significantly ($p<0.01$) higher in T4 (5.72 ± 0.10) group as compared to T1 (4.29 ± 0.10), T2 (4.68 ± 0.11) and T3 (5.13 ± 0.10) groups, moreover, it was significantly ($p<0.01$) differed among all the groups. Similarly, post-thaw mean MDA level (nmol/ml) was significantly higher ($p<0.01$) in the T4 (4.55 ± 0.12) group as compared to T3 (4.10 ± 0.10), T2 (3.80 ± 0.10) and T1 (3.47 ± 0.10) groups. Post-thaw mean GSH (nmol/ml) level was significantly ($p<0.01$) differed among all the groups. The corresponding overall mean GSH level (nmol/ml) irrespective of time interval was significantly ($p<0.01$) higher in T4 (5.44 ± 0.11) group as compared to T1 (4.50 ± 0.15) T2 (4.76 ± 0.13) and T3 (5.02 ± 0.12) groups, whereas it was non-significantly differed between T1 vs. T2 and T2 vs. T3 groups. Moreover, in T1, T2, T3, T4 groups mean GSH level (nmol/ml) was significantly higher ($p<0.01$) at initial (5.74 ± 0.10, 5.80 ± 0.12, 5.82 ± 0.08 and 6.03 ± 0.08) stage as compared to pre-freeze (4.29 ± 0.10, 4.68 ± 0.11, 5.13 ± 0.10 and 5.72 ± 0.10) and post-thaw stage (3.47 ± 0.10, 3.80 ± 0.10, 4.10 ± 0.10 and 4.55 ± 0.12) stage. Furthermore, the mean GSH level (nmol/ml) among initial, pre-freeze and post-thaw stage was differed significantly ($p<0.01$) in all the groups.

Table 2. Antioxidant Effect of different concentrations of Purslane (*Portulaca Oleracea*) leaves aqueous extract on Reduced Glutathione (GSH nmol/ml) level of Surti buck semen at various stages of cryopreservation (Mean±SE).

Groups	Reduced Glutathione (GSH) (nmol/ml) (n=16)			Overall (n= 48)	F value	P value
	Initial	Pre-freeze	Post-thaw			
T1	$5.74\pm0.10^{a}_{x}$	$4.29\pm0.10^{d}_{y}$	$3.47\pm0.10^{d}_{z}$	4.50 ± 0.15^{c}	136.04**	0.00
T2	$5.80\pm0.12^{a}_{x}$	$4.68\pm0.11^{c}_{y}$	$3.80\pm0.10^{c}_{z}$	4.76 ± 0.13^{bc}	82.52**	0.00
T3	$5.82\pm0.08^{a}_{x}$	$5.13\pm0.10^{b}_{y}$	$4.10\pm0.10^{b}_{z}$	5.02 ± 0.12^{b}	83.21**	0.00
T4	$6.03\pm0.08^{a}_{x}$	$5.72\pm0.10^{a}_{y}$	$4.55\pm0.12^{a}_{z}$	5.44 ± 0.11^{a}	60.54**	0.00
Overall (n=64)	$5.85\pm0.05_{x}$	$4.96\pm0.08_{y}$	$3.98\pm0.07_{z}$	--	179.55**	0.00
F value	1.77	36.38**	19.34**	9.79**	--	--
P value	0.16	0.00	0.00	0.00	--	--

a-d Means with different superscripts within a column (between the groups) differ significantly at p<0.01.
*x-z Means with different subscripts between a column (between various stages) differ significantly at p<0.01. **p<0.01*
***T1** – control, **T2** – 1% Purslane (Portulaca Oleracea) leaves aqueous extract, **T3** - 2% Purslane (Portulaca Oleracea) leaves aqueous extract, **T4** - 3% Purslane (Portulaca Oleracea) leaves aqueous extract.*

The corresponding overall mean GSH level (nmol/ml) irrespective of treatment groups were decreased with increasing preservation time at the initial (5.85 ± 0.05), pre-freeze (4.96 ± 0.08), and post-thaw (3.98 ± 0.07) stage. The overall mean GSH level(nmol/ml) irrespective of different treatment groups significantly ($p<0.01$) differed among various stages of cryopreservation. The highest mean GSH level (nmol/ml) was found at an initial, pre-freeze and post-thaw stage in T4 (6.03 ± 0.08, 5.72 ± 0.10 and 4.55 ± 0.12) group followed by T3 (5.82 ± 0.08, 5.13 ± 0.10 and 4.10 ± 0.10) and T2 (5.80 ± 0.12, 4.68 ± 0.11 and 3.80 ± 0.10) group.

In the present study the highest GSH level (nmol/ml) 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 compared to the control group. Similarly, Perumal *et al.* (2013) reported significantly ($p<0.05$) higher mean GSH (nmol/ml) levels in the taurine-treated group as compared to the control group in the semen of Mithun bull. Likewise, Zhang *et al.* (2022) also reported significantly ($p<0.05$) higher GSH levels in the proline-supplemented group as compared to the control group in Loshan buck semen.

In the present study, the significantly ($p<0.01$) higher pre-freeze and post-thaw GSH levels in Surti buck semen were found in the T4 (3% purslane (*Portulaca Oleracea*) leaves aqueous extract) group as compared to T2 (1%), T3 (2%) and control group. It's shown that GSH levels depend on the concentration of purslane (*Portulaca Oleracea*) leaves aqueous extract added in the extender. Similarly, Perumal *et al.* (2013) reported the highest mean of GSH (nmol/ml) in the taurine-treated group as compared to the control group and differed significantly ($p<0.05$) between the group during liquid storage (5^{0}C). Zhang *et al*. (2022) also reported significantly ($p<0.05$) higher GSH levels in 2 mM proline supplemented in the basic extender group as compared to other (0.5, 1, and 4 mM) and

control groups in Laoshan buck semen.

Contrary to present findings, Sariozkan *et al.* (2009) recorded significantly lower ($p<0.01$) GSH levels (nmol/ml) in Taurine 2mM (0.4 ± 0.1) and non-significantly lower in cysteine 2mM (1.1 ± 0.1) added in Bioxcell® extender groups as compared to control (1.4 ± 0.3) group in frozen-thawed bull semen. Inanc *et al.* (2021) also reported GSH level (nmol/ml) was non-significantly higher ($p>0.05$) in 100 µg/mL thymoquinone (TQ) added in basic extender group (8.92 ± 0.33) followed by 50 µg/mL (8.87 ± 0.27) as compared to control group (8.38 ± 0.09) in Sonmez ram semen.

Mammalian sperm is prone to the effects of increased intracellular ROS and lipid peroxidation products due to low levels of antioxidants in the cytoplasm and high levels of saturated fatty acids in the plasma membrane. The presence of these products can disrupt the normal function of sperm. Though small amounts of ROS are required for sperm physiological activity, high amounts of ROS are toxic to sperm. Purslane extract can thus be effective in converting anion superoxide to hydrogen peroxide and thereby reducing intracellular ROS products, therefore the observation of increased enzymatic capacity of antioxidants, which leads to the removal of ROS within sperm cells. Sperm mitochondria are the main site of ROS production due to their oxidative phosphorylation activity Thuwanut *et al*. (2011). Research evidence shows that phenols and flavonoids, the main components of purslane extract, are mainly responsible for its antioxidant activity Sanja *et al*. (2019).

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Contribution by Authors

Equal contribution. All authors declared that ‘written informed’ consent was obtained from the approved parties for the publication of this article and accompanying images.

Conflict of Interests

There is no conflict of interest.

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