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Comparative study on effect of ethanolic extract of *Ocimum gratissimum* leaves as a supplement to extender on chilled dog semen

Manish Kumar, Sumit Prakash Yadav, Pooja and Krishan Yadav

Abstract
The aim of study was to examine the efficiency of Egg yolk-tris-fructose extender (EYTF) and Egg yolk-tris-fructose extender (EYTF) +100µg/ml ethanolic extract of Ram Tulsi (*Ocimum gratissimum*) in preserving semen of dogs (n=6) at different time slots (0, 24, 48, and 72 hours) at refrigeration temperature (4 °C). Total 24 semen ejaculates were collected by digital manipulation at weekly intervals. The fresh semen was examined for macroscopic (volume, colour, consistency and pH) and microscopic parameters (mass motility, individual motility, sperm concentration, sperm abnormalities, viability and HOST) immediately after collection. Extended samples were evaluated for individual sperm motility, viability, ABNORMALITIES, and sperm function test (HOST). The average individual sperm motility, live sperm percentage and HOST were reduced significantly (p<0.05) in Egg yolk-tris-fructose extender (EYTF) and Egg yolk-tris-fructose extender (EYTF) +100 µg/ml ethanolic extract of Ram Tulsi (*Ocimum gratissimum*) from 0 to 72 hours. When individual sperm motility, abnormal spermatozoa and HOST percentage was compared between Egg yolk-tris-fructose extender (EYTF) and Egg yolk-tris-fructose extender (EYTF) +100 µg/ml ethanolic extract of Ram Tulsi (*Ocimum gratissimum*) at 0 hour and 24 hour there was non-significant difference but at 48- and 72-hours significant difference was observed. The percentage of live spermatozoa differed significantly at 24, 48, and 72 hours (p<0.05) between extenders. The percentage of abnormal spermatozoa significantly increased from 0 to 72 hours of preservation both in Egg yolk-tris-fructose extender (EYTF) and Egg yolk-tris-fructose extender (EYTF) +100 µg/ml ethanolic extract of Ram Tulsi (*Ocimum gratissimum*). In conclusion, the Egg yolk-tris-fructose extender (EYTF) +100 µg/ml ethanolic extract of Ram Tulsi (*Ocimum gratissimum*) was found better than Egg yolk-tris-fructose extender (EYTF) the extender in terms of preserving the motility, viability, and membrane integrity of refrigerated canine semen for up to 72 hours, suggesting it a simple, economical, and efficient extender for canine semen refrigeration.

Keywords: Dog semen, HOST, Egg-yolk-tris-fructose extender, Egg-yolk-tris-fructose extender with ethanolic extract of Ram Tulsi

Introduction
Due to less expensive preparation, shipping, and frequently simpler regulations than with frozen semen, chilled, extended semen is growing in popularity in canine breeding (Ponglowhapan *et al.*, 2004) [20]. Compared to frozen-thawed canine semen, chilled canine semen has a higher fertility rate when placed in the vagina (Linde-Forsberg, 1991) [11]. *Ocimum gratissimum* is a herbaceous plant that belongs to the family Lamiaceae. *Ocimum* leaves are rich in 3.5% of essential oils (Trevisan *et al.*, 2006) [28]. *Ocimum gratissimum* leaves oil contains bioactive components made up of eugenol, α-bisabolene, β-selinene, 1, 8-cineole, and thymol (Silva *et al.*, 2004) [25]. These phytochemicals have antioxidant properties (Chiu *et al.*, 2013; Mahapatra and Roy, 2014) [5, 12]. Hence, by adding *Ocimum gratissimum* leaves extract to the extender can improve quality of chilled canine sperm by reducing sperm lipid peroxidation during storage (Vui *et al.*, 2019) [29]. Our study aimed to compare the effects of egg yolk tris fructose with 100 µg/ml ethanol extract of *Ocimum gratissimum* leaves as a supplement to extender on chilled canine semen at 0, 24, 48, and 72 hours of preservation under refrigeration temperature (4 °C).

Materials and Methods

Six healthy privately owned large breed dogs of German Shepherd (04), Labrador (01) and Golden retriever (01) breeds; 2-6 yrs of age, with good libido were used to collect ejaculate by digital manipulation. Total 24 semen ejaculates were collected at weekly intervals. The fresh sperm-rich fraction was examined for macroscopic examination included volume, colour, consistency and pH, while the microscopic examination included mass motility, individual motility, sperm concentration, sperm abnormalities, live count and hypo-osmotic swelling test (HOST). Following the preliminary evaluations, the sperm-rich fraction of the sperm sample was divided into two equal aliquots; each aliquot was diluted 1:4 in both extender groups; Group I (Egg yolk-tris-fructose extender (EYTF).) and Group II - Egg yolk-tris-fructose extender (EYTF) +100 µg/ml ethanolic extract of Ram Tulsi (*Ocimum gratissimum*) leaves at room temperature. The extended semen samples were kept in a beaker with water at 37 °C before being chilled to 4 °C in a refrigerator. Diluted samples were examined for individual motility, sperm abnormalities, live & dead count, and HOST at 0, 24, 48, and 72 hours.

Table 1: Composition of extenders

S. No.	Content	Quantity
1.	Tris (hydroxyl methyl) amino methane	3.025gram
2.	Citric acid	1.7gram
3.	D-Fructose	1.25gram
4.	Benzyl penicillin	100mg
5.	Dihydro streptomycin sulphate	100mg
6.	Freshly collected egg yolk	20% (v/v)
7.	Distilled water	100ml

Mass motility was observed by drop of fresh semen on a pre-warmed glass side under a microscope (10x). Individual sperm motility and sperm concentration were evaluated as per the standard procedures described Payan-Carreira *et al.* (2011) [19]. On nigrosin/eosin stained slides, the number of live spermatozoa (%) and abnormal sperm (%) were counted using an oil immersion objective microscope (100x). The spermatozoa (%) with intact plasma membrane were determined using the HOST (Jeyendran *et al.*, 1984) [7].

Statistical analysis

Data obtained were subjected to analysis by completely randomized design (CRD) by one-way analysis of variance technique (Snedecor and Cochran, 1989) [26] using the statistical package SPSS software 20 version. The mean of different experimental groups were tested for statistical significance by Duncan`s Multiple Range Test (Duncan, 1995) [6].

Results and Discussion

The volume, pH, sperm concentration, mass motility, individual sperm motility, live sperm, abnormal sperm, and hypo-osmotic swelling test results for fresh sperm samples were 1.81±0.06 ml, 6.23±0.02, 372.92±12.19 million per millilitre, 4.21±0.13, 92.92±0.27%, 93.33±0.50%, 7.44±0.08% and 89.92±0.27%, respectively. The colour and consistency of semen samples were observed as creamy to milky and thin to thick, respectively.

Khye *et al.* (2021) [10] and Patti *et al.* (2021) [18] reported a higher volume of sperm-rich fraction than the present work. These variations in semen volume may be caused by differences in dog size, age, body weight, and breeds and frequency of semen collection. Patti *et al.* (2021) [18] found the colour of sperm-rich fraction varied from white to milky white which is similar to the present finding.

The consistency of sperm rich fraction in the present study was slightly Thin/thick to the reported by Barve (2014) [2], while Srinivas *et al.* (2022) [27] found that the sperm-rich fraction of canine semen was thin milky in consistency in their study.

Shalini and Antoine (2018) [23] found similar pH value of semen. Khye *et al.* (2021) [10] and Martnez-Barbitta and Rivera (2022) [13] reported lower pH than the present study. Sperm concentration results of the present study were in agreement with Shalini and Antoine (2018) [23]. However, Martnez-Barbitta and Rivera (2022) [13] reported higher sperm concentration than the present study. These differences may be due to the number of spermatozoa per ejaculate varies with age, testicular weight, sexual activity, and dog size.

The mass motility observed during the present study was higher than reported by Shalini and Antoine (2018) [23]; and Srinivas *et al.* (2022) [27], while lower mass motility compared to present study was reported by Dostal *et al.* (2001) [4]. The individual sperm motility in the present study was in accordance with Kawakami *et al.* (2005) [9]. The sperm motility reported by Silva *et al.* (2009) [24] was higher, while Khye *et al.* (2021) [10] and Srinivas *et al.* (2022) [27] reported lower sperm motility than the present study findings. Findings of live spermatozoa count are in agreement with Michael *et al.* (2009) [14]. Puja *et al.* (2018) [21] reported higher, while Srinivas *et al.* (2022) [27] and Martnez-Barbitta and Rivera (2022) [13] reported lower live sperm percentages than the present findings. The average HOST of fresh semen observed in the present study was similarly as recorded by Sanchez-Calabuig *et al.* (2017). Slightly higher values were reported by Patti *et al.* (2021) [18]. Dobranic *et al.* (2005) [3], they reported an average hypo-osmotic positive sperm as 87.94±1.57% in fresh dog semen which are lower and hence not in accordance with findings for HOST as observed in the present study.

Table 1: Extended semen parameters (Mean ± SE) during preservation (4 °C) Group I (Egg yolk-tris-fructose extender (EYTF).) and Group II - Egg yolk-tris-fructose extender (EYTF) +100 µg/ml(C100) ethanolic extract of Ram Tulsi (*Ocimum gratissimum*) leaves

Parameter	Extender	0 hour	24 hours	48 hours	72 hours
Individual sperm motility	EYTF	88.12^{aD}±0.72	86.87^{aC}±0.66	84.58^{aB}±0.73	79.17^{aA}±0.65
	EYTF+ C100	89.37^{bD}±0.81	88.12^{bC}±0.66	85.83^{bB}±0.72	80.62^{bA}±0.62
Live Sperm	EYTF	89.88^{aD}±0.23	84.5^{aC} ±0.25	80.92^{aB}±0.19	76.71^{aA}±0.25
	EYTF+ C100	90.42^{dD}±0.19	87.79^{dC}±0.24	84.62^{dB}±0.27	82.12^{dA}±0.34
Abnormal Sperm	EYTF	7.21^{aA} ±0.19	8.71^{aB} ±0.16	10.50^{aC}±0.15	11.92^{aD}±0.16
	EYTF+ C100	7.71^{bA} ±0.15	8.92^{bB} ±0.15	11.21^{bC}±0.15	11.50^{bD}0.10
Hypo-Osmotic Swelling Test	EYTF	89.87^{aD}±0.50	78.79^{aC}±0.64	75.58^{aB}±0.53	73.08^{aA}±0.48
	EYTF+ C100	89.37^{dD}±0.53	87.00^{dC}±0.49	85.08^{dB}±0.49	83.87^{dA}±0.43

Mean values having different superscripts in a row (a, b, c, d) and in a column (A, B) differ significantly ($p \leq 0.05$)

Nguyen *et al.* (2020) [15] reported slightly higher individual sperm motility during their study on the antioxidant effect of different levels (Group I (Egg yolk-tris-fructose extender (EYTF).) and Group II - Egg yolk-tris-fructose extender (EYTF) +100 µg/ml ethanolic extract of Ram Tulsi (*Ocimum gratissimum*) leaves) of essential oils from *Ocimum gratissimum* leaves as a supplement to extender on chilled canine semen. However, present findings were slightly lower than the finding of Joseph *et al*. (2019) [8] investigated the effects of methanol and oil extracts of *Ocimum gratissimum* on testicular morphology and epididymal sperm reserve in Wistar rats. Their results revealed no significant difference in livability, the differences in sperm motility between the references and the present study could be due to the difference in the higher initial motility in fresh semen before dilution and difference in contents of the dilutors and environmental variations.

The variation in abnormality at 100 µg/mL ethanolic extract of Ram Tulsi (*Ocimum gratissimum*) leaves group than another group in present study was due to its high mass activity, which increased friction between the spermatozoa and their surrounding media as mentioned by Otite (2012) [16] in his study.

The present study findings were slightly different from observation of Nguyen *et al.* (2020) [15] investigation on the antioxidant effect of different levels ((Group I (Egg yolk-tris-fructose extender (EYTF).) and Group II - Egg yolk-tris-fructose extender (EYTF) +100 µg/ml ethanolic extract of Ram Tulsi (*Ocimum gratissimum*) leaves) of essential oils from *Ocimum gratissimum* leaves in extender on chilled canine spermatozoa throughout the duration of 12 days storage. The differences in sperm motility between the references and the present study could be due to the difference in the higher initial motility in fresh semen before dilution and difference in contents of the dilutors and environmental variations.

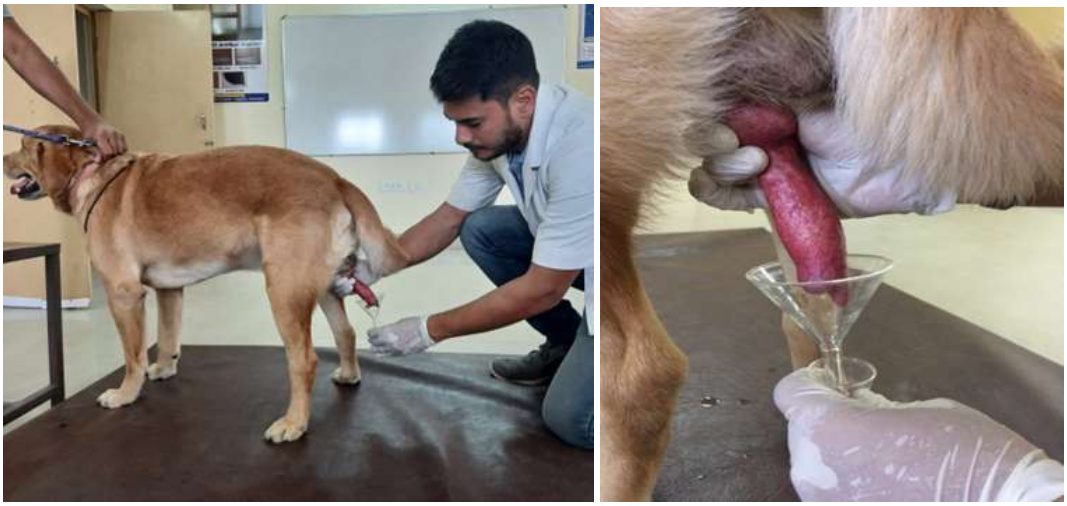
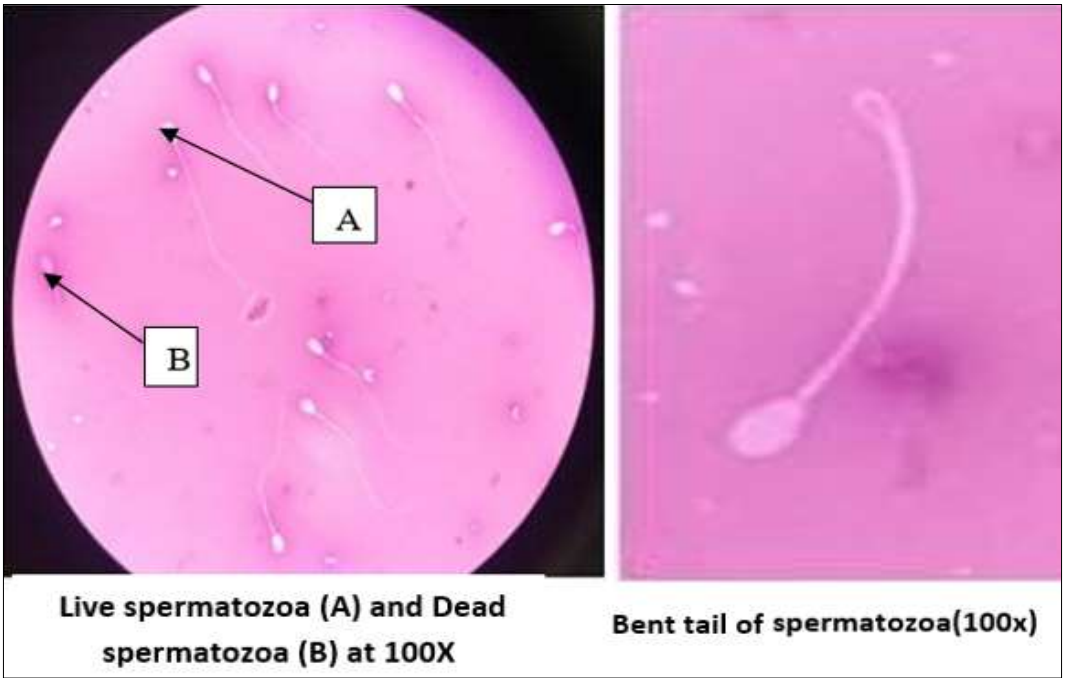
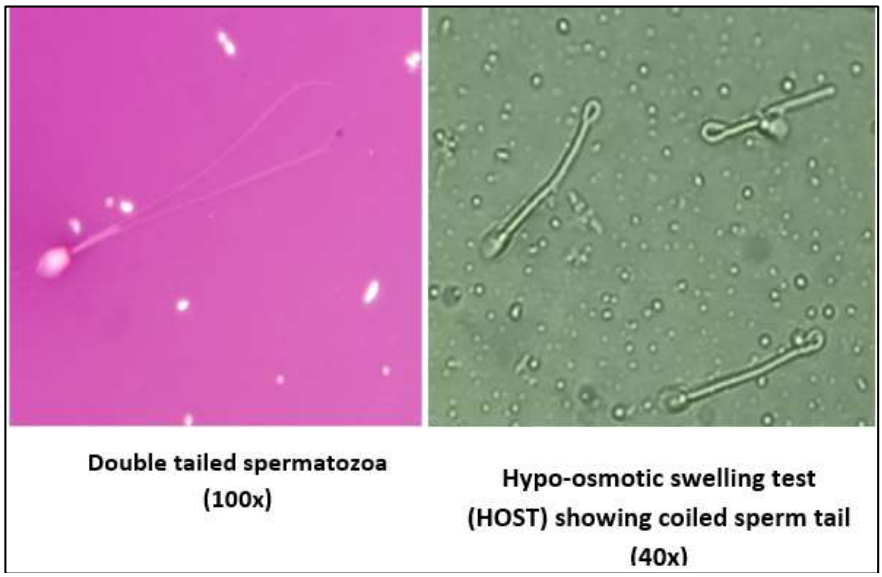


Fig 1: Collection of sperm rich fraction





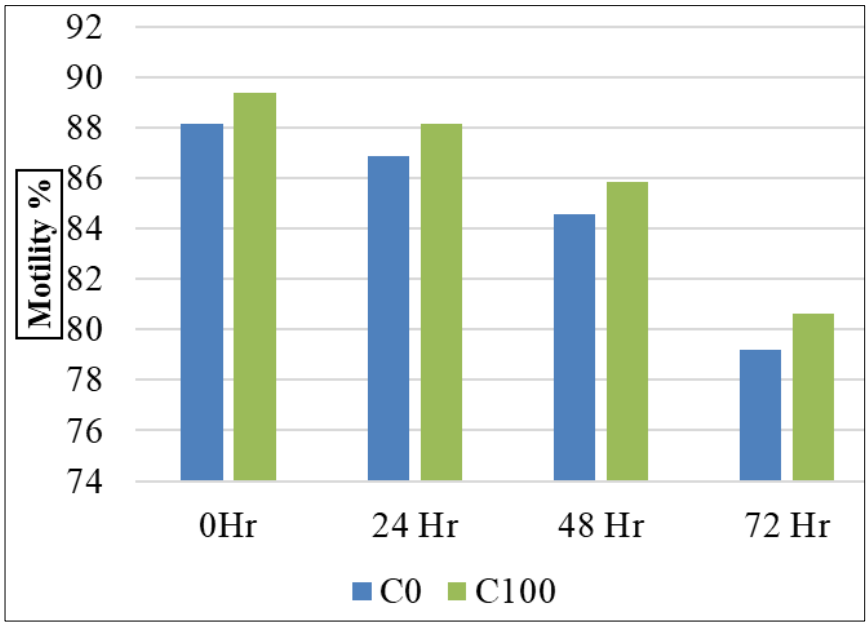


Fig 2: Individual sperm motility of refrigerated aliquots (n=24)

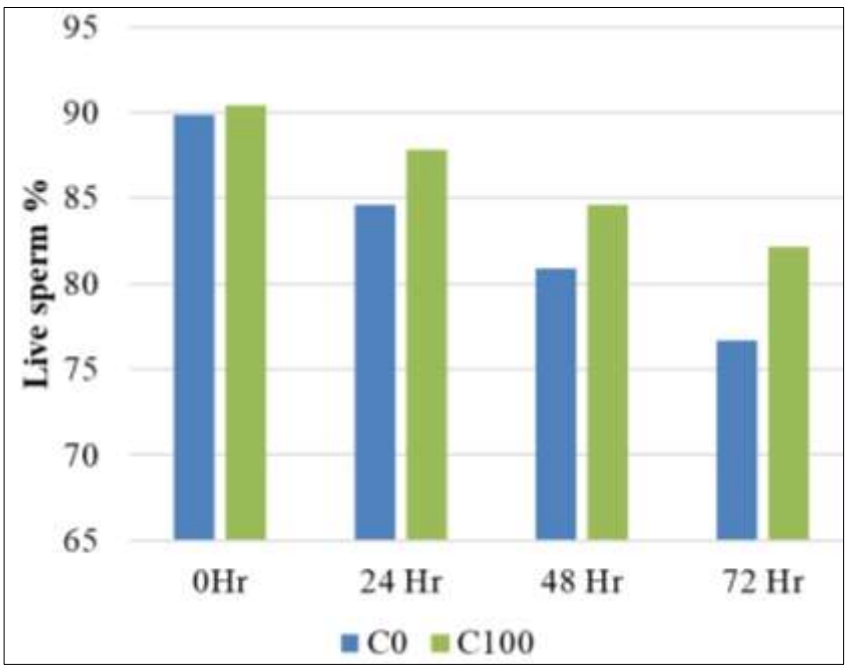


Fig 3: Percentage of live spermatozoa of refrigerated aliquots (n=24)

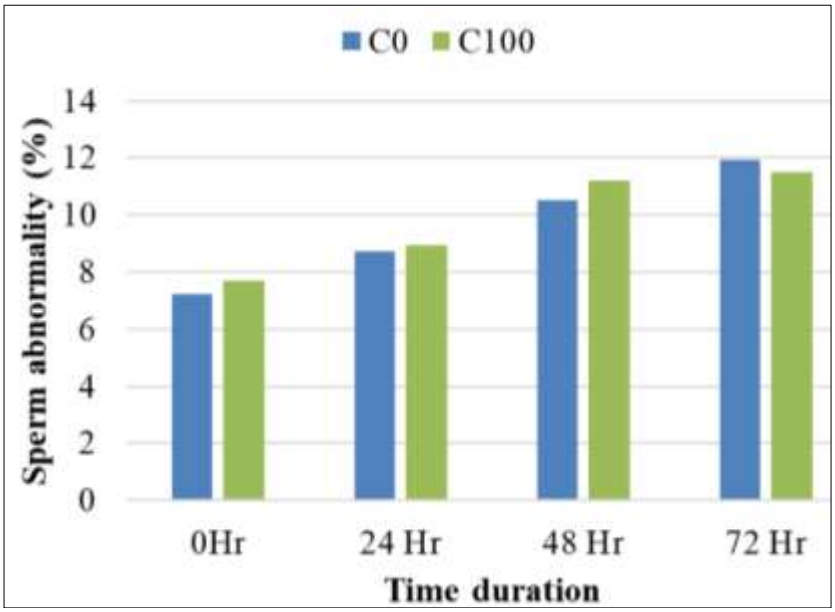


Fig 4: Morphological sperm abnormalities of refrigerated aliquots (n=24)

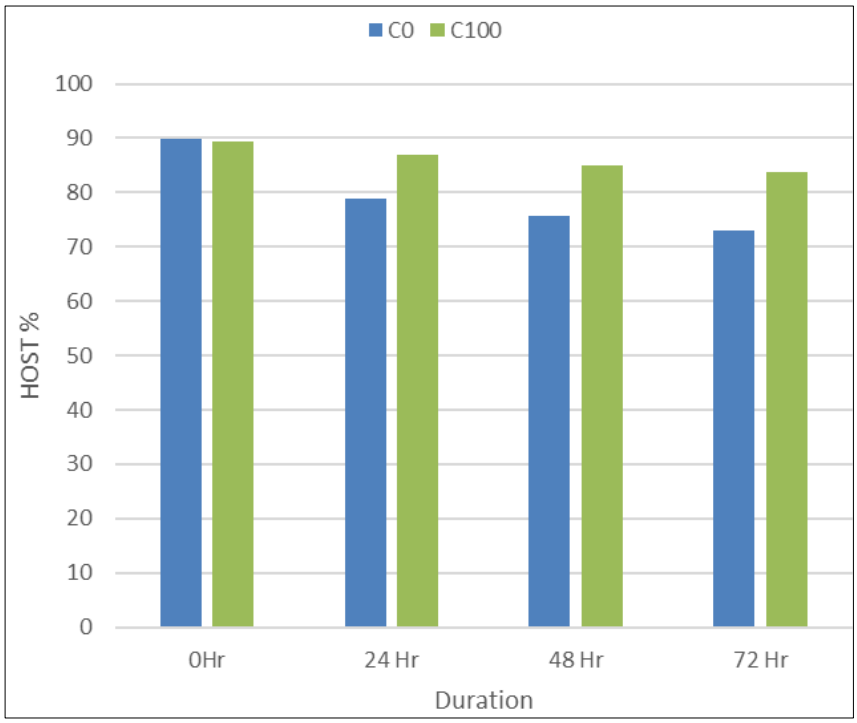


Fig 5: HOST percentage of refrigerated aliquots (n=24)

Conclusion

EYTF+100 µg/ml ethanolic extract of Ram Tulsi (*Ocimum gratissimum*) leaves) of essential oils is comparatively better than the EYTF group extender in terms of preserving the motility, viability, and membrane integrity of refrigerated canine semen for up to 72 hours, suggesting it a simple, economical, and efficient extender for canine semen refrigeration.

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