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Cryopreservative Impact of Thyme (*Thymus vulgaris*) Extract on Buffalo Sperm Quality

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Abstract

THE objective of the current investigation was exploring the influence of extract of Thyme on sperm freezability in buffaloes. Preparation of Thyme ethanolic extract, Evaluation of its antioxidant activity by Diphenyl-1- plcrylhydrazyl (DPPH), High Performance Liquid Chromatography (HPLC) analysis to assess the concentrations of its phenolics and flavonoids. Semen from five mature buffalo bulls was obtained. The main extender was Tris-citric acid- fructose egg yolk (TCFY). Tris diluent with zero Thyme extract was considered as a control. The other extenders are Tris containing ethanolic Thyme extract at concentrations 4, 8, 16 mg/dl as experimental extenders. The semen specimens were supplementary and diluted semen was exposed to the freezing process. Extended semen was estimated for the sperm parameters. The total phenolic content of Ethanolic extract of Thyme (20422.51 µg/g of the extract, mostly Rosmarenic acid, Ferulic acid). The total flavonoids content of Thyme extract (3369.94µg/g of extract, mostly Coffeic acid). The DPPH of Thyme extract (96.5%) with 1000µg/ml and decreased with decreasing concentration. HPLC chromatograms of Thyme extract with peaks of Rosmarenic acid, Ferulic acid and Ferulic acid. Sperm measures were promoted post cooling and thawing in the lower concentration of Thyme (TT_1) if compared to the control and/or the other treatments (TT_2 and TT_3). Enhancement of conception rate was obvious in TT_1 with elevated antioxidants and decreased Malondialdehyde(MDA). It could be fulfilled that, Thymus extract improved post-thawing buffalo sperm quality, the antioxidant status and conception rate (55 %) when used in a low concentration (4 mg /dl).

Keywords: Tris, Thyme, Buffalo, Semen, Freezing.

Introduction

Infertility represents the major reproductive harms in the animal life and roughly thirty percent of this hazard is attributed to male effects [1]. Sperm freezing plays an important role in assisted reproductive knowledge in humans, and bovines. However, no matter what type of extender and storing conditions are applied, the management and preservation of sperm harmfully affect its value. As well, oxidative effects, which often occur during the freezing process, significantly reduce sperm function and reduce the sperm's ability to fertilize. This oxidative damage causes nucleic acid, lipid and protein compounds to be deteriorated. Recently, plant extracts were explored as a cost-effective natural base of enrichment to maintain and ameliorate spermatozoa survival throughout the preservation process [2].

According to Ros-Santaella and Eliana Pintus [2], the factors that influence the preservation of semen include the plant substances used, the extraction technique, the dose, the likely toxic effects, and the antibacterial properties. Frozen-thawed sperm is found as a reproductive technique in the artificial reproduction of farm animals. This method depends on the principle of partial permanent harm to the sperm, which may lead to a decrease in sperm motion, flexibility of the plasma membrane, vitality, and eventually bull fertilization potential [3,4]. So, the primary purpose of the current investigation was to augment the impact of endogenous antioxidants on sperm value. Endogenous antioxidants are not sufficient to scavenge free radicals, therefore, there is a strong need for more harmless and essential natural antioxidants to avoid the toxic effects of synthetic ones. Natural antioxidants have an enhancing effect on sperm value [4-7]. The Thyme plant is a flowering aromatic plant having medicinal effects, it is a part of

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the La-miaceae family [8-10] and has anti-bacterial and anti-mycotic properties [11]. It exists in the genus Thymus, which is a member of the phytosanitary plant family (Thymusaceae) predominating in a variety of plants, including the mint. This plant has an antioxidant property due to its phenolic and flavonoids composition. It has some ingredients like caffeic acid, labiatic acid, thymol and flavonoids[12] .In addition, the diluent supplemented with intermediate quantities of the plant extract Thyme improves dose-dependently the value of the ram sperm post-freezing and post-thaw [11] .The present study objective was to assess the impact of Thyme extract as herbal antioxidant on freeze-thawed buffalo sperm value.

Material and Methods

Preparation of Thymus vulgaris extract

Collected Thyme plants were dried out at ambient temperature for ten days. Then, 100 g of the dried plants were changed into powder soaked in half a liter of ethanol (60%) for a day and the mixture was exposed to filtration. The extraction of *Thymus vulgaris* was done using Soxhlet equipment. Evaporation of ethanol from the extract using a rotatory evaporator at 50 °C, then refrigeration of the extract at 4 °C for waiting its use [11].

Assessment of antioxidant activity using DPPH radical scavenging method

Free radical scavenging activity of Thymus vulgaris extract was calculated by 1, 1- diphenyl-2-picryl hydrazyl (DPPH) according to Huang [13]. In brief, 0.1 mM solution of DPPH in ethanol was set. This solution (1 ml) was supplementary to 3 ml of the extract in ethanol at different concentrations (3.9, 7.8, 15.62, 31.25, 62.5, 125, 250, 500, 1000 µg/ml). Here, only those extracts are utilized which are Solubilize in ethanol and their various concentrations were all set by dilution method. The mixture was stirred vigorously and allowed to stand at room temp for 30 min. then, absorbance was calculated at 517 nm by using spectrophotometer (UV-VIS milton roy). Reference standard compound used was ascorbic acid and experiment was completed in triplicates. The IC 50 value of the sample, which is the concentration of sample required to inhibit 50% of the DPPH free radical, was designed using Log dose inhibition curve. Lower absorbance of the reaction mixture expressed higher free radical activity. The percent DPPH scavenging effect was computed by using following equation:

DPPH scavenging effect (%) or

Percent inhibition = A0 - A 1 / A0 × 100.

Where A0 was the absorbance of control reaction and A1 was the absorbance in presence of test or standard sample.

HPLC conditions

HPLC analysis was done using an Agilent 1260 series. The separation was performed using Zorbax Eclipse Plus C8 column (4.6 mm x 250 mm i.d., 5 µm). The mobile phase consisted of water (A) and 0.05% trifluoroacetic acid in acetonitrile (B) at a flow rate 0.9 ml/min. The mobile phase was planned successively in a linear gradient as follows: 0 min (82% A); 0–1 min (82% A); 1-11 min (75% A); 11-18 min (60% A); 18-22 min (82% A) ; 22-24 min (82% A). The multi-wavelength detector was kept at 280 nm. The injection volume was 5 µl for each of the sample solutions. The column temperature was kept at 40 °C [14] with little modifications.

Buffalo bulls

Five buffalo bulls (aged 3.5 - 5 years) kept at the Buffalo Semen Freezing Center will be be used in semen collection. The animals were reserved on normal feeding and management conditions. They have normal sanitary status (body weight, 600-800 kg), free from common and genital illnesses. Summer feeding, selected bulls were hold cool and relaxed through water sprinkling for minimum 3-4 times daily, sheltered from storm, kept in comfortable environment having minimum moisture, fed through cold times and have a plenty of fresh drinking water. The animals feeding: in summer, 6 kg dry matter plus 2kg roughage and 3.5 kg dried clover per animal daily, in winter, 6 kg dry matter plus 2 kg roughage and 28 kg clover per animal daily. Temperature humidity indicator: 72-78.

Semen Collection and principal Evaluation

Semen samples were harvested using prepared artificial vagina weekly for eighteen weeks. Semen samples were mostly assessed for sperm forward motility and sperm concentration. Semen samples with (70%) or more spermatozoal motility and standard morphological sperm percent were collective to get enough semen to eliminate the bull individual variation. Keeping semen for 10 min at 37°C in the water bath before extension.

Sperm freezing procedures

The fundamental extender was Tris-citric acid-fructose (TCF) which was composed on the line of Foote [15] .The TCFYG diluent was formulated by dissolving 3.028 g Tris, 1.678 g citric acid and 2.000 g fructose in 100 mL bi-distilled water and then 20% egg yolk was included and 7% glycerol with a penicillin-streptomycin mixture as antibiotic at 0.01 ml/ml of the extender as documented by Ijaz et al. [16] .

Tris diluent with zero Thyme extract was designed as a control. The other extenders are Tris containing ethanol extract of Thyme at concentrations 4, 8, 16 mg/dl as experimental diluents. The semen specimens were supplementary and neat sperm concentration 60 × 10^6/mL was done. Diluted semen was gradually cooled

(about for two hours) to 5°C and subjected to two hours equilibration. Semen was packed into 0.25 ml polyvinyl French straws that were kept in a horizontal position on a special rack and exposed to liquid nitrogen vapor 4 cm part from its surface for ten minutes and next immediately dipped in liquid nitrogen [17].

Evaluation of Semen Quality Criteria

The assessment will be implemented for cooled and frozen spermatozoa. The frozen straws of semen were exposed to thawing at 37°C for one minute. The features estimated are motility, alive percentage, sperm abnormalities, acrosome and sperm membrane integrities.

Motility

Progressive forward motility will be measured in each semen sample (5 µL) diluted with 2.9% Sodium Citrate dehydrate solution. The drop was located on a slide with cover slip, then observed with the warm stage microscope (400X). As a minimum two hundred spermatozoa from different microscopic fields were counted. Sperm motility percent was calculated [18].

Sperm viability and morphological abnormality

A smear from diluted semen was prepared on a glass slide and was stained by eosin nigrosin stain and examined using the warm stage microscope (400X). Abnormal sperm percent was counted from examined two hundred sperms in five microscopic fields [19].

Sperm membrane integrity [Hypo-osmotic swelling test (HOST)]

The hypo-osmotic solution (125 mosmol/1) will be composed of 6.25 gm of sodium citrate dihydrate and 11.25 gm of fructose per liter of pure distilled water. A mixture of 10µl semen and 1 ml of solution was incubated for sixty minutes at 37°C. Post incubation, 5 µL of this solution was examined using the warm stage microscope (400X). Sperm percent positive to HOST (with swollen or curled tail) was counted from examined two hundred sperms in different microscopical fields [20].

Percent of normal acrosome

Semen samples were stained by Trypan blue/Giemsa staining with minor modifications [21] .Trypan blue was practical for staining at a concentration of 0.27%, one drop (5 µl) of diluted semen and one drop (5 µl) of Trypan blue gently mixed on a slide and for every semen droplet 2 smears were examined. Slides are air-dried in upright position and then put into 10% buffered formol saline (9 gm Nacl, 6.5 gm Na_2H PO_4, 4 gm NAH_2PO_4 for fixing at 37^0C for 30 min. Slides were located into jars having the Giemsa solution and left for 12 hours. The Giemsa staining solution was recently organized by adding 14.3% (v/v) of Giemsa stock solution (Sigma GS-500) to distilled water. Slides were gently washed again in distilled water, air-dried in upright position and coverslipped. Intact acrosomes are purple, the frontal part of the sperm head having no acrosome are light purple.

Viability index

The post- thawing viability indices were computed as recorded by Milovanov [22]to be equivalent to half of the post-thaw sperm motility plus the sum of sperm forward motilities post first, second, and third hours of thawing.

Measuring of oxidant/antioxidant parameters

Semen will be collected then centrifuged at 2773 ×g for 5 min at 4°C using a cooling centrifuge (Sigma 3-18KS, Germany). The seminal plasma was separated and stored at -80°C. The level of total antioxidant capacity (TAC) in the seminal plasma was determined according to the method of Koracevic et al. [23] ,lipid peroxidation compounds as malondialdehyde (MDA) according to the method of Satoh [24] using test kits that were manufactured by Biodiagnostic Co., Egypt. All measurements were done using Double Beam UV/Visible Spectrophotometer, Model T80, UK.

In vivo conception rate (CR)

The number of female buffaloes (360) will be inseminated artificially with the frozen post- thawed TCFY as a control and the other experimental extenders. Conception rate was detected through rectal palpation after two months from the artificial insemination. The inseminated animals were administered through the cooperation with Beni-Suef Governorate. The insemination of animals were implemented using the insemination gun and semen was pushed inside the uterus. The CR was estimated following the equation:

$$CR = \frac{\text{no.of pregnant buffaloes}}{\text{total no.of inseminated buffaloes}} \times 100$$

Statistical analysis

Statistical analysis data were computed using the SPSS [25] computerized program v. 14.0 to calculate the analysis of variance (ANOVA) for the different criteria between control and experimental groups. Major difference among the mean values was designed by means of Duncan test at $P<0.05$.

Results

Table (1) reveals the total phenolic content of Ethanolic extract of Thyme (20422.51 µg/g extract, Rosmarenic acid, Ferulic acid).

Table (2) exhibits the total flavonoids content of Thyme extract (3369.94µg/g extract, Coffeic acid).

Fig (1)A: reveals the DPPH of Thyme Extract (96.5%) with 1000µg/ml and decreased with decreasing concentration. Fig (1) B:Standard.

Fig.2: shows HPLC chromatograms of Thyme extract with peaks of Rosmarenic acid, Ferulic acid, Coffeic acid.

Table (3) revealed that, Thyme enrichment to Tris extender ameliorated semen parameters as indicated by greater post- cooled sperm motility and alive sperms ,higher percent of normal sperm membrane (HOST) and acrosome , lower sperm abnormalities and sperm premature capacitation in TT1 if compared to the control and/or the other treatments (TT2 and TT3).

Table (4) revealed that, Thyme enrichment to Tris extender inTT_1 exhibited better post- thawing sperm forward motility, higher spermatozoal membrane (HOST) and acrosomal integrities and viability index and lowered sperm abnormalities and sperm premature capacitation if compared to the control and/or the other treatments (TT_2 and TT3) that showed deterioration of post- thaw semen characteristics.

Table (5) exhibited significant decrease in post-thawing sperm motility with the advance of time in all the used Thyme concentrations. The sperm motility was higher in TT_1 in each of the post-thawing times relative to the control and other used Thyme concentrations.

Table (6) showed significant elevation of the total antioxidant (TAC) accompanied by the lower decrease of malondialdehyde (MDA) in TT_1 relative to the control and other used Thyme concentrations.

Table (7) showed the superior conception ratio (CR) in TT_1(55.0%) and the lowest in TT_2 and TT_3 (2.5% and 2.2%, respectively).

Discussion

Spermatozoa freezability can be influenced by a variety of factors, including: Osmotic stress Ice crystal formation Cryoprotectant toxicity Individual variation [26, 27] .Among several cases, oxidative hazard has been known to influence the fertility potential and functioning of frozen/thawed spermatozoa [28 , 29, 30] .Oxidative stress erupts in the presence of a difference between the rate at which the oxygen free radicals are generated and the cell's antioxidant capacity [31] .Too much difference can damage the sperm cells [32] .Low levels of reactive molecules are necessary for sperm capacitation, the process by which spermatozoa acquire their reproductive potential [33] .By the effect of oxidative stress, spermatozoa suffer extensive damage such as peroxidation of membrane lipids, DNA fragmentation, [34-36] , low mitochondrial membrane activity [37, 38] , inactivation of enzymes associated with motility[39] and Loss of sperm motility due to damage of sperm cell membrane [35, 36] .

Antioxidant enzymes such as SOD, CAT, and GSH represent a wide range of antioxidants. However, their antioxidant capacity decreases over time as the freezing process progresses. Consequently, it is suggested to add antioxidant supplements to the sperm diluents [40] .

There is a worldwide interest searching for the synergistic property of the various ingredients of natural supplements if compared to the single functional fractions [41]. Spermatozoa freezing causes damage to the sperm cells , consequential in a decrease in the semen value [42] .However, it is important to preserve the excellent genetic characteristics of the local breeds of bovine animals. Semen cryopreservation is associated with cryoinjury due to the excessive production of Oxygen Free radicals [43]. Therefore, the natural additive added to the extender improves the antioxidant outcome and improves the fertilization capacity of the frozen spermatozoa [44] .Premature capacitation of the sperm used for artificial insemination (AI) is a cause of the male subfertility, and may be a good indicator for the detection of low fertile bovine bulls [45] .

Thyme enrichment to freezing extender TT1 (Low concentration, 4 ml/dL) improves post-thaw post-sperm motility, live sperm percent, sperm abnormalities and viability index, which are markers of sperm resistance, with a significant reduction of percent premature capacitation in TT1. Thyme compounds are recognized to be active antioxidants and metal chelators, and free radicals are eliminated by the phenolic compounds [11].These results could be documented by the eminent increase in TAC and the lower decrease in MDA in TT1, and are in line with the results demonstrated by ismail et al. [46] .Higher concentrations of thyme extract degrade post-sperm quality.

Our results are in line with those of vahedi et al. [11] on ram semen, who found that addition of 2 and 4 ml/dL Thyme extract at extender stage increased entire forward motility percentage and increased the percentage of viability compared to control and high extract groups, and plasma membrane fluidity in 4 and 8 ml/dL extract groups was superior to that of the control group and 12 and 16 ml/ LDL extract groups. They also noted that 16 ml/dL extract notably affected all assessed traits.

El-Gindy [47] found that feeding bucks a diet enriched with Thyme improved their sperm quality and storage life due to its antioxidant properties. Ismail et al. [46]found that adding 50 µg or 100 µg of Thyme nano into a Tris extender improved cryopreservation goat semen quality as indicated by an ameliorated progression of sperm motility, viability and plasma membrane integrity compared to the control extender after equilibration (5 ◦C for 2 h)

and thawing (37 ◦C for 30 s) and decreased the apoptosis, malondialdehyde level, and chromatin dispersion of sperm cells, while increased the total antioxidant capacity and catalase activity in the frozen/thawed extender with no effect on sperm abnormality and acrosome integrity .

Thyme consists of a wide range of compounds, including thymol, flavonoids, caffeic acid, labiatic acid, carvacrol [11].

HPLC is measured to be the most effective method for isolating the polar composites in Thyme that are well dissolved in water. In the present study, the total phenolic content of the ethanolic thymus extract is 20422.51 µg/g extract (Rosmarenic Acid, Ferulic Acid) and it contains 3369.94 µg/g of total flavonoids (Coffeic Acid).

The use of Rosmarenic Acid in the Semen Extender improves the quality and fertilization capacity of the pig sperm after thawing because it provides a safety net for the boar's spermatozoa against oxidative damage in cryopreservation due to their efficient antioxidant properties, as evidenced by the decrease in MDA concentration [48] .

Ferulic acid, an effective fraction in various natural herbs, was explored to be highly potent in scavenging oxygen anions and improving the cellular cAMP, and cGMP [49] .The use of Ferulic acid improves the quality of the boar sperm when cryopreserved and has been established to be a high-efficiency antioxidant, protecting the sperm from freeze damage [50] .Ferulic acid may enhance the deleterious effect (as marked by assaying the MDA) and nitrosative (as indicated by assaying the nitrate-nitrite) reactions during the preservation of rooster semen by refrigeration [51] .

Adding of 100 µM of caffairic acid reduces the total of malondialdehyde and improves the quality of the water buffalo's sperm after thawing [52] .

In this study, the free radicals scavenging activity of the extract of Thyme was 96.5% with 1000 µg/ml (DPPH assay) and decreased as the concentration decreased. The improved fertility potential of the extract can be accredited to its antioxidant activity, as flavonoids, which are known to reduce the presence of reactive oxygen species in the body (ROS). Phenolic compounds, which are the dominant antioxidants widely distributed in plants, are able to directly scavenge free radicals, so that their antioxidant activity surpasses that of synthetic antioxidants, as well as vitamins C and E [11, 53] .

Referring to the fact that oxygen free radicals alter sperm vitality during freezing processes [54] .It was also documented that flavonoids, as antioxidants, could lower ROS levels [55] .Phenolic compounds are dominant antioxidants extensively distributed in plants, being capable of scavenging free radicals directly so that their antioxidative power is higher than that of synthetic antioxidants and of vitamins C and E [56, 57] .This property of phenolic compounds can be linked to their ability to donate hydrogen ions (H+) [58] .

Lipid results in decreased motility and deteriorated membrane fluidity throughout damage of the lipid matrix composition on sperm cell membranes.

Thymus extract showed strong anti-radiation scavenging activity in this study. There is a strong association between total phenolic and antioxidant activity, which confirms that the total phenolic is responsible for the free radical scavenging activity [59] .

In this study, it can be fulfilled that Thymus vulgaris extract as natural antioxidant improves post-thaw buffalo sperm quality and antioxidant status, as well as conception rate(55%), when used with low concentrations(4mg/dl)due to its potent antiradiation scavenging activity and high total phenolics.

Ethical Approval

The experimental plan was approved by the Medical Research Ethics Committee of the National Research Centre, Dokki, Egypt and its registration number is 19/104 and its date is 10/10/2019.

Authorship

The author had performed all the items of the experimental design, the collection of semen, the diluting concentrations, the freezing process, semen evaluation and the preparing of the manuscript.

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Conflict of interest

The authors announce that, there isn’t any conflict of interest.

TABLE 1. HPLC analysis of Polyphenolic compounds concentration in Thyme extract

Compound	Concentration of compounds (µg / g Thyme extract)
Gallic acid	267.91
Chlorogenic acid	225.00
Methyl gallate	116.43
Syringic acid	448.23
Pyrocatechol	0.00
Ellagic acid	72.70
Coumaric acid	28.52
Vanillin	158.22
Ferulic acid	3020.71
Rosmarenic acid	16081.34
Cinnamic acid	3.45
Total	20422.51

TABLE 2. HPLC analysis of Flavonoid compounds concentration in Thyme extract

Compound	Concentration of compounds (µg / g Thyme extract)
Catechin	309.32
Coffeic acid	1355.84
Rutin	710.20
Naringenin	121.82
Daidzein	198.24
Querectin	190.54
Kaempferol	255.21
Hesperetin	228.77
Total	3369.94

TABLE 3. Impact of Tris diluent augmented with Thyme extract on the post-cooled sperm characteristics of diluted buffalo bull semen (Mean±SE).

Diluent	motility	alive	abnormality	HOST	acrosome	capacitation
Control(TCFYG)	68.33±1.66^{c}	73.66±1.33^{b}	19.66±0.33^{b}	55.00±2.88^{b}	92.00±2.00^{b}	35.00±2.88^{a}
TT_1(4 mg/dL)	48.33±1.66^{b}	71.00±1.00^{ab}	16.00±1.00^{a}	58.76±1.23^{b}	89.66±0.33^{b}	38.66±1.33^{a}
TT_2(8 mg/dL)	16.66±3.33^{a}	68.33±4.40^{ab}	20.66±0.66^{b}	52.00±2.00^{b}	76.00±3.78^{a}	42.66±2.66^{a}
TT_3(16 mg/dL)	13.33±3.33^{a}	60.00±5.77^{a}	21.33±1.85^{b}	39.20±5.09^{a}	72.33±5.04^{a}	51.50±1.50^{b}
p-value	0.000	0.007	0.000	0.000	0.019	0.013

Means bearing different superscripts within column differ at 5% levels of probability. Control Tris-citrate-fructose-egg yolk-glycerol (TCFYG); TT1(TrisT1); TR2(TrisT2); TR3(TrisT3)

TABLE 4. Impact of Tris diluent augmented with Thyme extract on the post-thawed sperm characteristics of diluted buffalo bull semen (Mean±SE).

Diluent	Motility	Alive	Abnormalities	HOST	Acrosome	Viability index	capacitation
Control (Tris extender)	35.00±5.00^{b}	78.33±1.66^{d}	24.33±0.66^{b}	50.66±0.66^{b}	89.66±2.60^{b}	69.10±0.86^{b}	59.00±3.78^{a}
TT_1 (4 mg /dL)	41.66±1.66^{b}	62.00±2.00^{c}	16.33±1.33^{a}	51.66±1.66^{b}	87.33±1.20^{b}	98.61±0.83^{c}	50.00±5.77^{a}
TT_2 (8 mg /dL)	6.66±1.66^{a}	41.66±1.66^{b}	22.66±1.45^{b}	39.00±2.08^{ab}	73.33±3.33^{a}	3.11±0.11^{a}	55.66±6.98^{a}
TT_3 (16 mg /dL)	5.66±0.66^{a}	23.33±3.33^{a}	24.00±1.00^{b}	28.33±8.33^{a}	67.33±5.04^{a}	2.91±0.08^{a}	51.66±4.40^{a}
p-value	0.000	0.000	0.004	0.017	0.004	0.000	(NS)0.653

Means different superscripts (a, b, c) within column differ at P<0.05.Control Tris-citrate-fructose-egg yolk-glycerol (TCFYG) ; TT1(TrisT1); TT2(TrisT2); TT3(TrisT3).NS: Non-significant.

TABLE 5. Effect of Tris extender enriched with Thyme extract on post-thaw total motility % of frozen-thawed buffalo bull spermatozoa.

Hours	Control	TT_1 (4mg /dL)	TT_2 (8 mg /dL)	TT_3 (16 mg /dL)	p-value
0	35.00±5.00^b	41.66±1.66^b	6.66±1.66^a	5.66±0.66^a	0.001
1	25.88±0.48^b	32.33±1.45^c	0.00±0.00^a	0.00±0.00^a	0.001
2	17.77±0.39^b	25.33±0.33^c	0.00±0.00^a	0.00±0.00^a	0.001
3	8.27±0.14^b	19.00±0.57^c	0.00±0.00^a	0.00±0.00^a	0.001

Means different superscripts (a, b, c) within rows differ at P<0.05.Control Tris-citrate-fructose-egg yolk-glycerol (TCFYG) ; TT1(TrisT1); TT2(TrisT2); TT3(TrisT3).

TABLE 6. Effect of Tris diluent augmented with Thyme extract on Antioxidant concentration-TAC (mM) and MDA concentration (µM).

Diluent	TAC	MDA
Control (Tris extender)	0.04±.01^b	6.38±0.06^b
TT_1 (4 mg /dL)	0.35±.01^c	4.92±0.01^a
TT_2 (8 mg /dL)	0.01±.001^a	9.77±0.04^d
TT_3 (16 mg /dL)	0.01±.001^a	8.52±0.03^c
p-value	0.000	0.000

Means different superscripts (a, b, c) within column differ at P<0.05.Control Tris-citrate-fructose-egg yolk-glycerol (TCFYG) ; TT1(TrisT1); TT2(TrisT2); TT3(TrisT3).

TABLE 7. Effect of Tris extender enriched with Thyme extract on pregnancy rate in buffalo.

Treatment	No of inseminated females	No of conceived females	In vivo fertility rate (CR, %)
Control (Tris extender)	100	50	50%
TT_1 (4 mg /dL)	90	50	55.0%
TT_2 (8 mg /dL)	80	2	2.5%
TT_3 (16 mg /dL)	90	2	2.2%

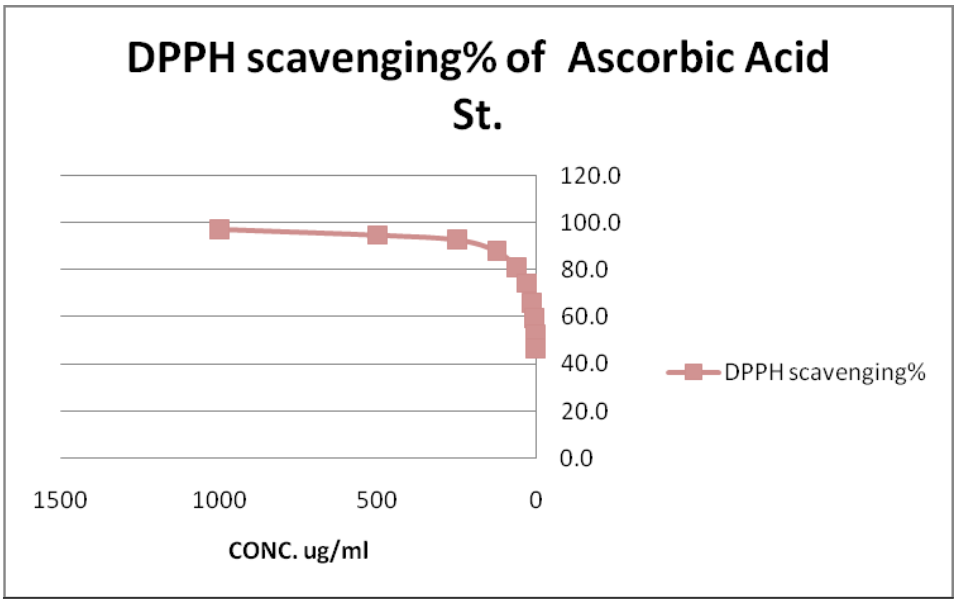

Fig.1.A . Standard

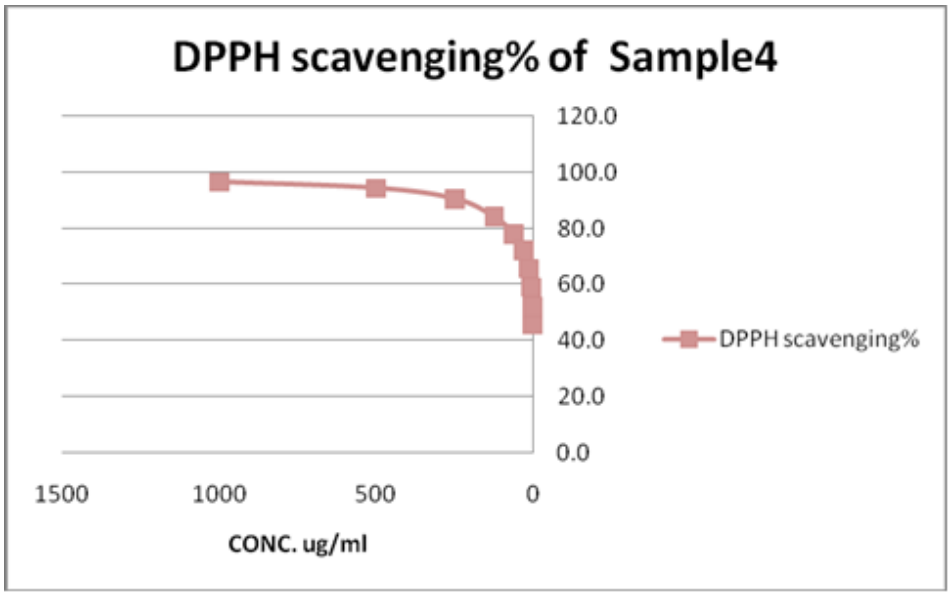

Fig.1.B. Sample

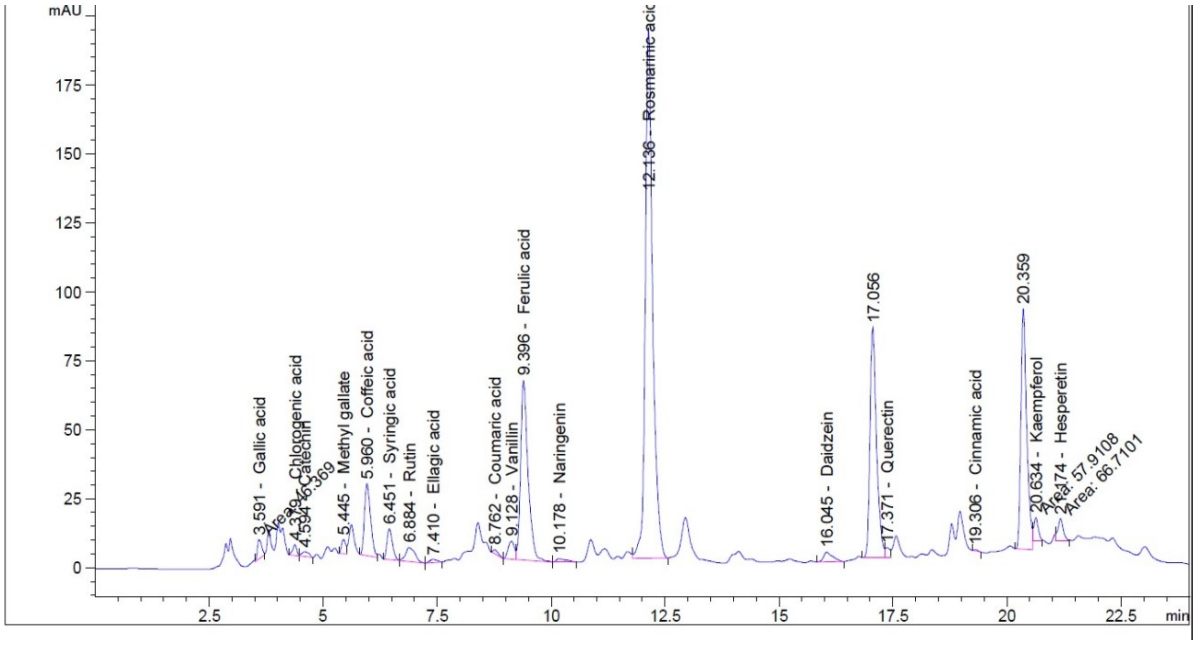

Fig. 2. HPLC Chromatograms of standard metabolites showing signal from diode array detector at wavelength 280 nm. Peak: Rosmarenic acid, Ferulic acid, Coffeic acid.

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تأثير المستخلص الايثانولى للثيم على كفاءة الحفظ بالتجميد للحيوانات المنوية فى طلائق الجاموس

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الملخص

تهدف تلك الدراسة الى التوصل الى تأثير المستخلص الايثانولى للثيم على كفاءة الحفظ بالتجميد للحيوانات المنوية والخصوبة فى طلائق الجاموس. تم تحضير المستخلص الايثانولى للثيم وتقييم نشاط مضادات الأكسدة DPPH تحليل الكرومتوجرافى السائل HPLC لمعرفة تركيزات الفينولات والفلافونيدات فى المستخلص. تم تجميع السائل المنوى من خمسة طلائق من الجاموس ناضجة الأنابيب المحتوية على التريس فقط تعتبر ككنترول والأخرى المحتوية على تركيزات الثيم 4و8و16 ملجم/100مل تعتبر كمعاملات.تمت اضافة السائل المنوى لنصل الى تركيز 60 مليون حيوان منوى/مل.تم تعبئة السائل المنوى المخفف فى قصيبات 0.25 مل وتم عمل خطوات التجميد والتقييم للسائل المنوى المخفف. أظهرت النتائج أن التركيز الكلى للفينولات 20422.51 ميكروجرام /مل وبصفة أساسية .Rosmarenic acid, Ferulic acid وكان التركيز الكلى للفلافونويدس 3369.94 وبصفة أساسية Coffeic acid تحسنت خصائص الحيوانات المنوية بعد التبريد والتجميد مع التركيز الأول 4ملجم/100مل وكذلك تحسنت نسبة الحمل وتركيز مضادات الأكسدة .

الخلاصة: تحسن خصائص السائل المنوى باضافة المستخلص الايثانولى للثيم الى مخففاته ويظهر هذا التحسن بعد التبريد والتجميد مع التركيز الأول 4ملجم/100مل.

الكلمات الدالة: تريس وثيم و طلائق الجاموس و السائل المنوى.