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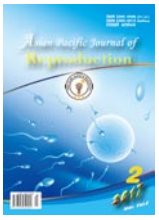

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Preservability of rabbit semen after chilled storage in tris based extender enriched with different concentrations of Propolis ethanolic extract (PEE)

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ABSTRACT

Objective: To maintain semen quality of male rabbits during chilled storage by enrichment the tris based diluent with different concentrations of propolis ethanolic extracts. **Methods:** Total phenolic and total flavonoid contents, as well as antioxidant activity was determined in propolis ethanolic extract (PEE). The extract was analysed by HPLC for separation and identification of target metabolites. Semen was collected from 10 rabbit bucks, pooled, then divided into five aliquots (each of 500 μL) and diluted each in 5 mL Tris-citric acid-glucose-egg yolk extender (TCGY). The 1st aliquot served as control while PEE was added at concentration of 0.8, 1.2, 1.6 and 2.0 mg/5 mL tris extender in the aliquot 2, 3, 4 and 5 respectively. Diluted semen samples were subjected to cooling at 4 ℃ for 72 h. Sperm motility, sperm viability, sperm abnormality, sperm membrane integrity and acrosome integrity were evaluated in chilled semen allover the chilling period. **Results:** The resluts revealed presence of a considerable amount of total phenolic compounds (98.67 mg GAE/g extract) and total flavonoids (70.16 mg CE/g extract) which were parallel to an antioxidant activity assessed as ABTS, DPPH and FRAP (198.65, 180.18 and 306.17 mM TE/g extract respectively). The dominant phenolic acid was chlorogenic acids (3.959 mg/g extract). Other compounds were found in less amounts rosmarinic acid (3.959 mg/g extract), myrcetin (1.946 mg/g extract), kaempferol (1.089 mg/g extract) and apeginin-7-glucoside (1.113 mg/g extract). Obtained results clearly demonstrated that the addition of 1.2-1.6 mg PEE in the chilled extended rabbit semen proved to be beneficial for maintaining semen characteristics compared to control and the addition of 0.8 and 2 mg PEE. **Conclusions:** The enrichment of rabbit semen tris-basic extender with 1.2-1.6 mg PEE/5 mL tris-extender (as the best and safe concentrations) maintain the sperm characteristics in good condition all over 72 h of chilling.

1. Introduction

Developing and improving methods for semen preservation would provide adequate fertility rates that maintain the high production rates for rabbit industry to be economically beneficial. Several studies had been conducted to improve the preservation protocols and the extender composition[1-10]. Unfortunately, rabbit spermatozoa had a restricted capacity to withstand the chilling[5,6] or frozen storage[11] is limited. This is partially due to the membrane lipid peroxidation caused by an excess level of reactive oxygen species (ROS), which in turn would affect lipids, proteins, nucleic acids and sugars within the sperm[12,13].

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In folk medicine, fruits, vegetables and their seeds extracts exerted an ancient life secure for their high contents of remedies compounds[14,15]. Some of these extracts were used in animal semen preservation[16–18]. Their cryopreserving properties are a hidden meaning in their strong antioxidant capacity, that enabled spermatozoa to overcome the outside/inside damage during cryopreservation process[19,20].

Bees have been extensively employed for their products since ancient due to their miracle pharmacological activity[21]. Propolis or "bee glue" is one of the bee products that possess various biological activities such as antibacterial[22,23], antifungal and antiviral[24,25], anti-protozoal[26], anti-inflammatory[27], antitumor[28], immunostimulating[29] and antioxidant properties[30–32]. Propolis contains some minerals (Mg, Ca, I, K, Na, Cu, Zn, Mn and Fe), some vitamins (B1, B2, B6, C and E) and a number of fatty acids. In addition, it contains some enzymes as succinic dehydrogenase, glucose-6-phosphatase, adenosine triphosphatase and acid phosphatase[33]. Propolis, also contains more than 300 biochemical constituents, including mostly a mixture of polyphenols, flavonoids, terpenoids, steroids, sugars, amino acids and others[34].The antioxidant activity of propolis is mainly attributed to its flavonoid content, that is capable of scavenging free radicals and thereby it protects cell membrane against lipid peroxidation[35]. Propolis also induces the activation of antioxidant enzymes such as superoxide dismutase[36] and catalase[37] against free radicals.

Thence, the present study aimed to maintain semen quality of male rabbits during chilled storage by enrichment the tris based diluent with different concentrations of propolis ethanolic extracts to be used in chilling of the extended semen.

2. Material and Methods

2.1. Collection of samples and chemicals

2.1.1. Propolis samples

Propolis samples were obtained from colonies of honeybees located in Dakahlia Governorate, Egypt, in the summer of 2015. The sample was kept in the dark at -20 ℃ up to its processing.

2.1.2. Phenolic acids standards

Gallic, protocatechuic, gentisic, chlorogenic, vanillic, caffeic, syringic, p-coumaric, ferulic, sinapic, rosmarinic and cinnamic acid, catachine, scoplatine, rutin, naringeen, hisperdin, myrcetin, quercetin, apegnin and kaempferol were purchased from Sigma-Aldrich, Inc. (Louis, USA).

2.1.3. Radical precursor and folin

DPPH (2,2-Diphenyl-1-picryl-hydrazyl), ABTS (2,2-azino-bis/3-ethil-benothiazoline-6-sulfonic acid), TPTZ (2, 4, 6- tripyridyl-s-triazine) and Folin-Ciocalteu reagent were purchased from Sigma-Aldrich, Inc. (Louis, USA).

2.1.4. Solvents and other chemicals

Acetonitrile (HPLC grade) was purchased from Aldrich Chemical (GmbH & Co KG, Steinheim, Germany). Petroleum ether, diethyl ether, ethyl acetate, tetrahydrofuran and methanol (analytical grade) were purchased from Tedia Company, Inc., Fairfield, OH 45014, USA. Other chemicals used in this study *i.e.* sodium hydroxide, potassium persulphate, dinitrosalicylic acid, aluminum chloride, sodium nitrite, sodium carbonate, hydrochloric acid, sulphuric acid and acetic acid were of analytical grade.

2.2. Preparation of extract

Ethanolic extract of propolis was prepared as described by Cvek *et al.*[38] with some modification by cutting 50 g of crude propolis sample into small pieces, grounded, extracted with 500 mL of 80% ethanol (1:10 w/v) and stirred continuously by shaking incubator (150 r/min) in the absence of light at room temperature for a week. After extraction the sample was filtered, evaporated to remove the residual solvent using a rotary evaporator (Heidolph VV 2 000, Germany) under reduced pressure at 40 ℃. The extract was reconstituted in 10 mL dimethylsulfoxide (DMSO) and stored at -80 ℃ till further use.

2.3. Determination of major phytochemicals in prepared extract

2.3.1. Determination of total phenolic content

The total phenolic content was determined according to the Folin-Ciocalteu procedure[39]. Total phenolics content was expressed as mg/g gallic acid equivalent using a derived equation from the calibration curve: Y= 0.034x + 0.111, R^2 = 0.999, where x is the absorbance and Y is the gallic acid equivalent (mg/g).

2.3.2. Determination of total flavonoid content

The total flavonoids content was determined according to Zilic *et al.*[39] using aluminum chloride ($AlCl_3$) colorimetric assay. Total flavonoids content was calculated as catechin equivalent (mg/g) using the following equation from the calibration curve: Y= 0.012x + 0.008, R^2 = 0.998, where x is the absorbance and Y is the catechin equivalent (mg/g).

2.4. Determination of antioxidant activity of prepared extracts

2.4.1. Determination of DPPH radical scavenging activity

Free radical scavenging capacity of extracts were determined using the stable DPPH according to Hwang and Do Thi[40]. The standard curve was prepared using Trolox (Y= 2.441x + 2.113, R^2 = 0.999, where x is the inhibition % and Y is the trolox equivalent mg/g).

Results were expressed as mg of Trolox equivalent per gram of extract.

2.4.2. Determination of ABTS radical scavenging activity

ABTS radical scavenging capacity of extract was determined according to Hwang and Do Thi[40]. The standard curve was prepared using Trolox (Y= 2.965x + 0.693, R^2 = 0.999, where x is the inhibition % and Y is the trolox equivalent mg/g). Results were expressed as mg of Trolox equivalent per gram of extract.

2.4.3. Ferric reducing activity power (FRAP) assay

The FRAP assay was done according to Hwang and Do Thi[40]. The standard curve was prepared using Trolox (Y= 0.041x + 0.006, R^2 = 0.999, where x is the inhibition % and Y is the trolox equivalent mg/g). Results were expressed as mg of Trolox equivalent per gram of extract.

2.5. Separation and identification of phenolic acids by High-performance liquid chromatography (HPLC)

Samples were injected automatically into an HP 1 100 series HPLC system (Hewlett-Packard, GmbH, Germany) equipped with a diode array detector (DAD). Absorption spectra for the main peaks were recorded at 280 and 320 nm. The HPLC system was equipped with a Xterra RP18 reverse phase column (4.6 mm × 250 mm) with a spherical particle size of 5 μm, which was kept at 25 ℃. The mobile phase was composed of 1% formic acid (A) and acetonitrile (B), and the elution gradient was 2 to 100% (B) in 40 min at a flow rate of 0.5 mL/min and 25 ℃. The injection volume was 20 μL[41].

2.6. Animals management and semen collection

Twenty sexually mature and fertile New Zealand White (NZW) male rabbits were obtained from the same herd in a commercial farm, for the purpose of this study. Rabbits aged 26-30 wk and weighing 2.3-2.9 kg. Bucks were individually housed in metal wire mesh cages provided with separate facilities for feeding and water supply. All bucks were fed a commercial diet and provided with food and water *ad libitum*.

Rabbit Bucks were trained to mount teaser female and then ejaculated in artificial vagina (IMV, France) adapted at 40-42 ℃. Semen was collected twice weekly. Each ejaculate was assessed for initial semen quality; only those that were white, >200 μL in volume, ≥300 × 10^6 cells/mL in concentration and with ≥70% motile spermatozoa were included in the study.

2.7. Experimental design

2.7.1. Pilot experiment for selection of useful extract concentrations

(1) After semen collection, selected ejaculates were immediately pooled to avoid individual differences and to obtain sufficient volume for each treatment. (2) The pooled samples were divided into 11 aliquots (each of 500 μL). (3) The first aliquot was diluted 1:10 in Tris-citrate-glucose (TCG) basic extender (250 mM Tris-hydroxymethylaminomethane, 88 mM citric acid, 47 mM glucose[1]. Three percent egg yolk was added to the basic extender as a modification (TCGY). (4) The other 10 aliquots were diluted 1:10 in the TCGY basic extender enriched with ten concentrations of PEE as shown in Table 1.

Table 1
Sperm motility percentages (Mean±SE) of rabbit semen after chilled storage in TCG extenders enriched with different concentrations (mg/5ml extender) of propolis ethanolic extract (PEE).

Concentration (mg/5 mL)	Chilling duration			
	2 h	24 h	48 h	72 h
Control (0.0)	88.33^{B}±1.67	73.33^{B}±1.67	60.00^{B}±2.89	33.33^{E}±1.67
0.4	87.50^{B}±1.45	67.50^{C}±1.45	47.50^{CD}±1.45	32.50^{E}±1.45
0.8*	87.50^{B}±1.45	72.50^{B}±1.45	57.50^{B}±1.45	42.50^{C}±1.45
1.2*	92.50^{A}±1.45	82.50^{A}±1.45	67.50^{A}±1.45	52.50^{B}±1.45
1.6*	92.50^{A}±1.45	82.50^{A}±1.45	72.50^{A}±1.45	57.50^{A}±1.45
2.0*	92.50^{A}±1.45	82.50^{A}±1.45	67.50^{A}±1.45	52.50^{B}±1.45
2.4	87.50^{B}±1.45	67.50^{C}±1.45	52.50^{C}±1.45	37.50^{D}±1.45
2.8	87.50^{B}±1.45	62.50^{D}±1.45	47.50^{CD}±1.45	32.50^{E}±1.45
3.2	82.50^{C}±1.45	57.50^{E}±1.45	42.50±1.45	22.50^{F}±1.45
3.6	82.50^{C}±1.45	57.50^{E}±1.45	37.50^{E}±1.45	20.00^{FG}±0.00
4.0	80.00^{C}±0.00	52.50^{F}±1.45	32.50^{F}±1.45	17.50^{G}±1.45
F	9.28	54.10	64.16	95.10
P	0.000 1	0.000 1	0.000 1	0.000 1

Different superscripts (A, B…. etc) within the same column indicate significant difference using Waller Duncan K-ratio (P<0.05). * Selected concentrations will be used in the experimental design.

2.7.2. Experimental design to select the optimal extract enriched extender

Immediately after semen collection, selected ejaculates were pooled so as to allow sufficient volume for each treatment. The pooled sample was splitted in five subsamples (each of 500 μL) to prepare one of the five treatments as follows: The first aliquot was diluted 1:10 in TCGY basic extender and served as control. The other four aliquots were diluted 1:10 in the TCGY extender supplemented with the selected 4 concentrations of the PEE that were obtained from the pilot experiment (Table 1).

2.8. Semen evaluation

The diluted semen samples were refrigerated in an incubator at 4 ℃ for 72 h. Forward motility, sperm viability, sperm membrane integrity and acrosome integrity % were assessed after 2, 24, 48 and 72 h post-chilling.

2.8.1. Sperm motility

A drop of semen was placed on a pre-warmed slide (37 ℃) and covered with a cover slip. Sperm motility subjectively was assessed

using a phase contrast hot stage microscope set at a magnification of 400 × and equipped with a heating plate (37 ℃).

2.8.2. Sperm morphology and viability

Stained smear was prepared as soon after ejaculation using an eosin nigrosine staining mixture at 1:4 dilution rate[42]. The principle of these techniques is dye exclusion as red eosin stains dead sperm head while nigrosine provides a blue-black background.

2.8.3. Sperm membrane integrity: Hypo-osmotic swelling test (HOST)

HOST is a relatively simple test developed for rabbit spermatozoa to evaluate the functional integrity of the spermatozoa membrane[43] against a hypo-osmotic solution of 60 mOsmol/L. The swollen spermatozoa characterized by a coiled tail which is an indication on the sperm intact plasma membrane.

2.8.4. Acrosome integrity

In the present study, Giemsa was used to stain the acrosome dark purple. Staining technique[44] was as follow: (1) Fresh ejaculate was diluted at 1:5 in warm normal saline. (2) Diluted semen was smeared and air-dried. (3) Smear was fixed in 10% neutral formal saline for 15 min. (4) Fixed smear was washed in running water for 20 min. (5) Fixed smear was immersed in Giemsa working solution overnight. (6) Stained smear was rinsed in two changes of distilled water and air-dried. One hundred spermatozoa per sample were examined with an oil immersion at 1 000 × magnification for acrosome integrity in each stained smear.

2.9. Statistical analysis

Statistical analysis was analyzed using the SAS computerized program v. 9.2[45] to calculate the analysis of variance (ANOVA) for the different parameters between control and additives replications. Significant difference between means was calculated using Duncan multiple range test at $P<0.05$.

3. Results

3.1. Total phenolic content, total flavonoids content, antioxidant activities and HPLC analysis of Propolis ethanolic extract

Data revealed the presence of a considerable amount of total phenolic compounds [(98.67±1.33) mg GAE/g extract] and total flavonoids [(70.16±1.27) mg CE/g extract] which are parallel to an antioxidant activity assessed as ABTS, DPPH and FRAP [(198.65±1.44), 180.18±1.72) and (306.17±3.83) mmol/L TE/g extract respectively]. This was interpreted by HPLC analysis against 24 standard metabolites (Table 2, Figure 1). The most effective compound in the prolopolis ethanolic extract was the chlorogenic acids represented in 36.906 mg/g extract. Other compounds were found in less amounts rosmarinic acid (3.959 mg/g extract), myrcetin (1.946 mg/g extract), kaempferol (1.089 mg/g extract) and apeginin-7-glucoside (1.113 mg/g extract). There were further compounds lower than 1 mg/g extract as protochatchuic acid (0.165 mg/g extract), ferulic acid (0.131 mg/g extract), quercetin (0.271 mg/g extract), apeginin (0.544 mg/g extract) (Figure 2).

Table 2
HPLC analysis of polyphenolic compounds in propolis ethanolic extract (PEE).

Compound	Retention Time (min)	Concentration (μg/g extract)
Pyrogallol	4.90	0.00
Gallic acid	5.90	0.00
Protochatechuic acid	10.03	164.96
P-hydroxybenzoic acid	15.22	0.00
Catachine	18.37	0.00
Chlorogenic acid	20.28	36 906.00
Caffeic acid	21.08	0.00
Syringic acid	22.52	0.00
Vanillic acid	24.82	0.00
Scoplatine	31.07	0.00
Ferulic acid	32.17	131.15
Sinapic acid	33.56	0.00
Rutin	36.18	0.00
P-coumaric acid	36.95	0.00
Naringeen	38.07	0.00
Hisperdin	38.60	0.00
Apeginin-7-glucoside	38.96	1 112.67
Myrcetin	40.24	1 945.59
Rosmarinic acid	40.95	3 959.41
Cinnamic acid	41.52	0.00
Quercetin	43.01	271.48
Apegnin	43.72	544.07
Kaempferol	46.22	1 089.00
Chyrsin	52.24	0.00

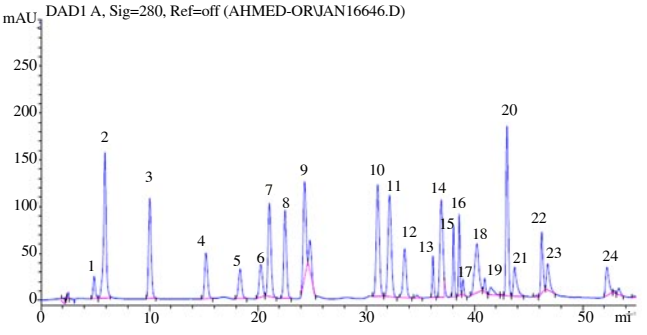

Figure 1. HPLC Chromatograms of standard metabolites showing signal from diode array detector at wavelength 280

Peak 1, Pyrogallol; 2, Gallic acid; 3, Protochatechuic acid; 4, P-hydroxybenzoic acid; 5, Catachine; 6, Chlorogenic acid; 7, Caffeic acid; 8, Syringic acid; 9, Vanillic acid; 10, Scoplatine; 11, ferulic acid; 12, Sinapic acid; 13, Rutin; 14, p-coumaric acid; 15, Naringeen; 16, Hisperdin; 17, Apeginin-7-glucoside; 18, Myrcetin; 19, Rosmarinic acid; 20, Cinnamic acid; 21, Quercetin; 22, Apegnin; 23, Kaempferol ; 24, Chyrsin.

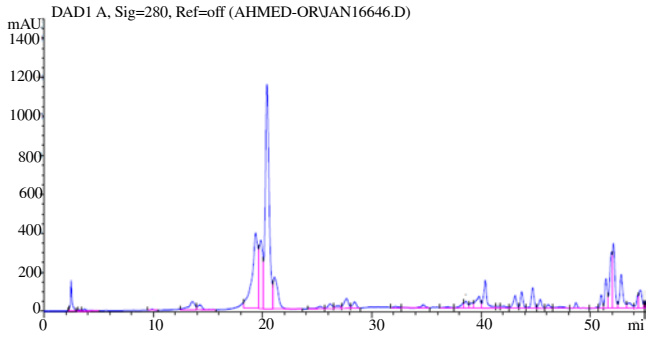

Figure 2. HPLC Chromatograms of propolis extract showing signal from diode array detector at wavelength 280.

Peak 1, Protochatchuic acid; 2, Chlorogenic acid; 3, Ferulic acid; 4, Apeginin-7-glucoside; 5, Myrcetin; 6, Rosmarinic acid; 7, Quercetin; 8, Apegnin; 9, Kaempferol

3.2. Sperm motility percentage

Data output in Table 3 showed that the chilling time had a regression effect which was significantly (P<0.000 1) represented by the gradual lowering of the overall mean of motile sperm from 94.58% after 2 h to 49.17% after 72 h. This coincided the sperm motility% within every treatment (within column) (control (0), 0.8, 1.2, 1.6 and 2.0 mg PEE/5 mL tris extender) from 2 to 72 h.

Concerning the enrichments of 5 mL tris extender with different concentrations of PEE within rows (Table 3), the concentrations of 1.2, 1.6 and 2.0 mg PEE/5 mL tris extender were significantly (P<0.000 1) the best PEE enrichments that maintained higher motility% from 2 to 72 h compared to the control (0 mg PEE) and the concentration of 0.8 mg PEE/5 mL tris extender. This was approved by the analogous overall means with their respective concentrations.

3.3. Sperm livability percentage

Data output in Table 4 showed that the chilling time had a regression effect which was significantly (P<0.000 1) represented by the gradual lowering of the overall mean of live sperm percentage from 93.00 % after 2 hours to 89.67 % after 72 h. This coincided the sperm livability % within treatment (within columns) (control (0), 1.6 and 2.0 mg PEE/5 mL tris extender) from 2 to 72 h.

Concerning the enrichments of 5 mL tris extender with different concentrations of PEE (within rows) (Table 4), there is no significant difference between different concentrations of PEE compared to the control. This was approved by the analogous overall means with their respective concentrations.

3.4. Sperm abnormality percentage

Data output in Table 5 showed that the overall mean of abnormal sperm percentage was significantly (P<0.000 1) increased from 14.25 % after 2 h to 17.67 % after 72 h. This coincided the sperm abnormality % within treatment (within columns) (control (0), 0.8, 1.6 and 2.0 mg PEE/5 mL tris extender) from 2 to 72 h.

Concerning the enrichments of 5 mL tris extender with different concentrations of PEE (within rows) (Table 5), there is a significant (P<0.034 7) difference between the concentrations 1.2 and 1.6 of PEE in tris extender compared to the control after 72 h. The overall mean of treatment with the concentrations 1.2 and 1.6 PEE in tris extender were significantly (P<0.001 0) different with the concentrations of 0.8 and 2.0 of PEE.

Table 3

Sperm motility percentages (Mean±SE) of rabbit semen after chilled storage in TCG extender enriched with different concentrations (mg/5 ml extender) of propolis ethanolic extract (PEE).

Chilling Duration (h)	Control (TCGY)	Concentrations of tris-extender enriched with PEE (mg/5 mL)				F-cal	P<	Overallmean*	P<
		0.8	1.2	1.6	2.0				
2	91.67^{Aa}±1.67	93.33^{Aa}±1.67	95.00^{Aa}±0.00	95.00^{Aa}±0.00	95.00^{Aa}±0.00	2.00	0.170 5	94.58^{k}	0.0001
24	76.67^{Cb}±1.67	81.67^{BCb}±1.67	88.33^{Aa}±1.67	86.67^{ABb}±1.67	85.00^{ABb}±2.89	5.50	0.013 2	85.42^{l}	
48	61.67^{Cc}±1.67	63.33^{BCc}±1.67	71.67^{ABb}±3.33	73.67^{Ac}±3.33	70.00^{ABCc}±2.89	3.73	0.041 5	69.58^{m}	
72	35.00^{Cd}±2.00	41.33^{BCd}±1.67	51.67^{ABc}±3.33	53.33^{Ad}±3.33	50.00^{ABd}±2.89	5.17	0.016 1	49.17^{n}	
f-cal	70.08	183.33	59.85	52.85	61.33	Interaction: time*concentration= 0.637 8			
P<	0.000 1	0.000 1	0.000 1	0.000 1	0.000 1				
Overallmean*		70.00^{L}	76.67^{K}	77.08^{K}	75.00^{K}				
P<				0.000 5					

Overall mean concern the 2 way analysis without the control.

Different superscripts (A, B, C, D, E) within the same row indicate significant difference using Duncan's multiple range test (P<0.05).

Different superscripts (a, b, c, d, e) within the same column indicate significant difference using Duncan's multiple range test (P<0.05).

Different superscripts (K, L, M, N) of overall means within rows indicate significant difference using Duncan's multiple range test (P<0.05).

Different superscripts (k, l, m, n) of overall means within columns indicate significant difference using Duncan's multiple range test (P<0.05).

3.5. Sperm membrane integrity percentage

Data output in Table 6 showed that the overall mean of sperm membrane integrity (HOST) percentages was significantly (P<0.000 1) decreased from 74.08 % after 2 h to 70.75 % after 72 h. This coincided the Sperm membrane integrity % within treatment (within columns) (control (0) and 0.8 mg PEE/5 ml tris extender) from 2 to 72 h.

Concerning the enrichments of 5 mL tris extender with different concentrations of PEE (within rows) (Table 6), there is a significant (P<0.000 1 - P<0.002 7) difference between the concentrations 0.8, 1.2, 1.6 and 2.0 of PEE in tris extender compared to the control after 2, 24, 48 and 72 h. The overall mean of treatment with the concentrations 1.2 and 1.6 PEE in tris ex;tender were significantly (P<0.003 5) different with the concentrations of 0.8 and 2.0 of PEE.

3.6. Sperm acrosome integrity percentage

Data output in Table 7 showed that the overall mean of sperm acrosome integrity percentages was significantly (P<0.000 1) decreased from 95.33 % after 2 h to 93.00 % after 72 h. This coincided the Sperm acrosome integrity % within treatment (within columns) (control (0) and 0.8 mg PEE/5 mL tris extender) from 2 to 72 h.

Concerning the enrichments of 5 mL tris extender with different concentrations of PEE (within rows) (Table 7), there is a significant (P<0.000 1 - P<0.015 9) difference between the concentrations 0.8, 1.2, 1.6 and 2.0 of PEE in tris extender compared to the control after 2, 48 and 72 h. The overall mean of treatment was not significantly different between all the concentrations of PEE.

4. Discussion

A good semen quality is a main target requested from the male reproduction. This is in need for a physiological boundary of ROS to accomplish its role. Whereas, high levels of ROS is sticky related with the hindrance of sperm fertilizing capability[46]. The feed

Table 4

Sperm livability percentages (Mean±SE) of rabbit semen after chilled storage in TCG extender enriched with different concentrations (mg/5 ml extender) of propolis ethanolic extract (PEE).

Chilling duration (h)	Control (TCGY)	Concentrations of tris-extender enriched with PEE (mg/5 mL)				F-cal	P<	Overall mean*	P<
		0.8	1.2	1.6	2.0				
2	92.33^{Aa}±0.33	92.67^{Aa}±0.33	93.00^{Aa}±0.58	93.33^{Aa}±0.33	93.00^{Aa}±0.58	0.72	0.596 2	93.00^{k}	0.000 1
24	90.67^{Aa}±0.67	90.67^{Aab}±0.67	91.67^{Aa}±0.88	92.00^{Aab}±0.00	91.33^{Aab}±0.67	0.84	0.529 3	91.42^{l}	
48	90.00^{Aab}±0.00	90.00^{Ab}±1.15	91.00^{Aa}±1.53	91.33^{Aab}±0.67	90.00^{Abc}±0.00	0.53	0.727 7	90.58^{lm}	
72	88.00^{Ab}±1.15	89.33^{Ab}±0.67	90.00^{Aa}±1.15	90.00^{Ab}±1.15	89.33^{Ac}±0.67	0.68	0.620 3	89.67^{m}	
f-cal	6.80	3.56	1.33	4.08	8.58	Interaction: time*concentration = 0.999 5			
P<	0.013 6	0.067 2	0.332 2	0.049 7	0.007 0				
Overall mean*		90.67^{K}	91.42^{K}	91.67^{K}	90.92^{K}				
P<				0.290 0					

Overall mean concern the 2 way analysis without the control.
Different superscripts (A, B, C, D, E) within the same row indicate significant difference using Duncan's multiple range test (P<0.05).
Different superscripts (a, b, c, d, e) within the same column indicate significant difference using Duncan's multiple range test (P<0.05).
Different superscripts (K, L, M, N) of overall means within rows indicate significant difference using Duncan's multiple range test (P<0.05).
Different superscripts (k, l, m, n) of overall means within columns indicate significant difference using Duncan's multiple range test (P<0.05).

Table 5

Sperm abnormality percentages (Mean±SE) of rabbit semen after chilled storage in TCG extender enriched with different concentrations (mg/5 ml extender) of propolis ethanolic extract (PEE).

Chilling duration (h)	Control (TCGY)	Concentrations of tris-extender enriched with PEE (mg/5 mL)				F-cal	P<	Overall mean*	P<
		0.8	1.2	1.6	2.0				
2	14.00^{Ac}±0.58	14.67^{Ab}±0.67	14.00^{Aa}±0.58	13.67^{Ab}±0.88	14.67^{Ac}±0.33	0.50	0.736 8	14.25^{m}	0.000 1
24	17.00^{Ab}±1.00	17.00^{Aab}±0.58	15.00^{Aa}±0.58	15.00^{Aab}±0.58	16.33^{Ab}±0.33	2.42	0.117 1	15.83^{l}	
48	18.00^{Aab}±0.58	18.00^{Aa}±1.15	15.67^{Aa}±1.33	15.33^{Aab}±0.33	16.67^{Ab}±0.33	2.15	0.148 6	16.42^{l}	
72	20.33^{Aa}±0.88	19.00^{ABa}±0.58	16.67^{Ba}±1.33	16.67^{Ba}±0.33	18.33^{ABa}±0.33	3.98	0.034 7	17.67^{k}	
f-cal	11.27	5.64	1.19	4.56	20.33	Interaction: time*concentration = 0.977 0			
P<	0.003 0	0.022 6	0.372 3	0.038 4	0.000 4				
Overall mean*		17.17^{K}	15.33^{L}	15.17^{L}	16.50^{K}				
P<				0.001 0					

Overall mean concern the 2 way analysis without the control.
Different superscripts (A, B, C, D, E) within the same row indicate significant difference using Duncan's multiple range test (P<0.05).
Different superscripts (a, b, c, d, e) within the same column indicate significant difference using Duncan's multiple range test (P<0.05).
Different superscripts (K, L, M, N) of overall means within rows indicate significant difference using Duncan's multiple range test (P<0.05).
Different superscripts (k, l, m, n) of overall means within columns indicate significant difference using Duncan's multiple range test (P<0.05).

on natural products as prescribed in folk medicine improved the motility and fertilizing capability of sperm, through facing up to the ROS deteriorating effects, as they enclose in their folds polyphenolic compounds, minerals, vitamins, enzymes and other antioxidants that play a role in scavenging free radicals and up-regulate certain metal chelation reactions[47]. The chilling is one of the detrimental factors that induce the production of ROS in extended semen of rabbits[5,6]. Propolis was used in this study as a natural additive to semen extender owing to its high contents of polyphenolic compounds, vitamins, minerals and other antioxidants in addition to its antinflammatory, antibacterial, immunoregulatory and strong cytoprotective effect against some toxic stimuli[48-52].

The present results showed that the addition of 0.8-2.0 mg PEE/5 mL Tris extender had significantly maintained the sperm quality (motility, viability, membrane and acrosome integrity and lowered the sperm abnormality to its minimal attendance) in a good condition during chilling till 72 h compared to the control treatment. The safe and best effective concentrations of PEE in Tris based extender were trapped between 1.2-1.6 mg PEE/5 mL Tris extender. This was attributed mainly to the high content of chlorogenic acid (36.9 mg/g extract) which has a potent reducing power against the lipid peroxidation of sperm membrane chilled for 72 h. This coincided with the addition of chlorogenic acid to Beltsville extender for boar semen stored for 72 h at 15 ℃[53]. Whereas, the presence of some other phenolic compounds as rosmarinic acid (3.959 mg/g extract), myricetin (1.946 mg/g extract), Kaempferol (1.089 mg/g extract) and apeginin-7-glucoside (1.113 mg/g extract) that have scavenging antioxidant activity against free radicals interpret the benefit beyond the enrichment of rabbit semen tris-based extender to overcome the lipid peroxidation process induced via chilling period[54-59]. This elongates the period of chilling in rabbit extended semen above 72 h in agreement with Di Lorio *et al.*[9] and Johinke *et al.*[10]. On the

Table 6

Sperm membrane integrity percentages (HOST) (Mean±SE) of rabbit semen after chilled storage in TCG extender enriched with different concentrations (mg/5 ml extender) of propolis ethanolic extract (PEE).

Chilling Duration (h)	Control (TCGY)	Concentrations of tris-extender enriched with PEE (mg/5 mL)				F-cal	*P*<	Overall mean*	*P*<
		0.8	1.2	1.6	2.0				
2	67.67^{Ba}±1.45	72.67^{Aa}±0.33	75.00^{Aa}±1.00	75.33^{Aa}±1.45	73.33^{Aa}±0.33	8.70	0.002 7	74.08^{k}	0.000 1
24	64.00^{Ca}±1.00	70.67^{Bab}±0.67	73.33^{Aab}±0.67	73.00^{Aab}±0.58	72.33^{ABab}±0.33	32.02	0.000 1	72.33^{l}	
48	61.00^{Bab}±2.08	71.33^{Aab}±0.67	72.33^{Aab}±1.20	71.67^{Ab}±0.88	71.67^{Aab}±0.88	14.94	0.000 3	71.75^{lm}	
72	55.00^{Bb}±2.89	69.33^{Ab}±0.67	71.67^{Ab}±0.88	71.33^{Ab}±0.67	70.67^{Ab}±0.67	24.13	0.000 1	70.75^{m}	
f-cal	7.27	5.33	2.29	3.60	3.49	Interaction: time*concentration = 0.927 2			
P<	0.011 3	0.026 0	0.154 8	0.065 6	0.070 0				
Overall mean*		71.00^{L}	73.08^{K}	72.83^{K}	72.00^{KL}				
P<				0.003 5					

Overall mean concern the 2 way analysis without the control.
Different superscripts (A, B, C, D, E) within the same row indicate significant difference using Duncan's multiple range test (*P*<0.05).
Different superscripts (a, b, c, d, e) within the same column indicate significant difference using Duncan's multiple range test (*P*<0.05).
Different superscripts (K, L, M, N) of overall means within rows indicate significant difference using Duncan's multiple range test (*P*<0.05).
Different superscripts (k, l, m, n) of overall means within columns indicate significant difference using Duncan's multiple range test (*P*<0.05).

Table 7

Sperm acrosome integrity percentages (Mean±SE) of rabbit semen after chilled storage in TCG extender enriched with different concentrations (mg/5 ml extender) of propolis ethanolic extract (PEE).

Chilling duration (h)	Control (TCGY)	Concentrations of tris-extender enriched with PEE (mg/5 mL)				F-cal	*P*<	Overall mean*	*P*<
		0.8	1.2	1.6	2.0				
2	92.33^{Ba}±0.33	95.00^{Aa}±0.58	95.67^{Aa}±0.88	96.00^{Aa}±0.58	94.67^{Aa}±0.33	6.23	0.008 8	95.33^{k}	0.000 1
24	93.00^{Aa}±0.58	94.00^{Aa}±0.58	94.33^{Aa}±0.88	94.33^{Aab}±0.67	94.00^{Aab}±0.58	0.67	0.624 4	94.17^{l}	
48	90.67^{Bb}±0.67	93.67^{Aab}±0.33	93.33^{Aa}±0.8	94.67^{Aab}±0.67	93.00^{Aab}±0.58	5.18	0.015 9	93.67^{lm}	
72	88.00^{Bc}±0.00	92.33^{Ab}±0.33	93.33^{Aa}±0.67	93.67^{Ab}±0.67	92.67^{Ab}±0.67	18.27	0.000 1	93.00^{m}	
f-cal	22.33	5.46	1.76	2.31	2.76	Interaction: time*concentration = 0.971 4			
P<	0.000 3	0.0245	0.2323	0.1528	0.1117				
Overall mean*		93.75^{K}	94.17^{K}	94.67^{K}	93.58^{K}				
P<				0.097 9					

Overall mean concern the 2 way analysis without the control.
Different superscripts (A, B, C, D, E) within the same row indicate significant difference using Duncan's multiple range test (*P*<0.05).
Different superscripts (a, b, c, d, e) within the same column indicate significant difference using Duncan's multiple range test (*P*<0.05).
Different superscripts (K, L, M, N) of overall means within rows indicate significant difference using Duncan's multiple range test (*P*<0.05).
Different superscripts (k, l, m, n) of overall means within columns indicate significant difference using Duncan's multiple range test (*P*<0.05).

contrary, El-Nattat *et al.*[60] had used an antioxidant (L-carnitin) in rabbit semen tris-extender that doesn't exceed the 48 h chilling, while the incorporation of L-carnitine in the Galap (commercial extender, IMV, France) extended the chilling period to 72 h. In conclusion, the enrichment of rabbit semen tris-basic extender with (1.2-1.6) mg PEE/5 mL tris-extender (as the best and safe concentrations) maintain the sperm characteristics in good condition all over 72 h of chilling.

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Conflict of interest statement

The authors declare that they have no conflict of interest.

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