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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 mg/5 mL tris extender in the aliquot 2, 3, 4 and 5 respectively. Diluted semen samples were subjected to cooling at 4°C 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 results 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 apigenin-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 on preservation protocols and extender composition have been carried out [1–10]. Unfortunately the ability of rabbit sperm to

survive *in vitro* after chilled [5–6] or frozen storage [11] is limited. This is in part due to lipid peroxidation caused by a supra-physiological level of reactive oxygen species (ROS), which affects sperm lipids, proteins, nucleic acids and sugars [12,13].

Natural extracts and infusions from fruits and vegetables and their seeds maintain life due to their high contents of remedies compounds essential for health [14,15]. Some of these extracts were used in semen extenders for preserving animal's sperms [16–18]. The cryopreserving properties are mainly attributed to their strong antioxidant capacity, thus protecting spermatozoa from damage during cryopreservation process [19,20].

Natural products from bees have been extensively employed since ancient time because of its broad pharmacological activity

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[21]. Propolis or “bee glue” is one of the bee products that exhibits numerous 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 such as Mg, Ca, I, K, Na, Cu, Zn, Mn and Fe as well as some vitamins like 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 °C up to its processing.

2.1.2. Phenolic acids standards

Gallic, protocatechuic, gentisic, chlorogenic, vanillic, caffeic, syringic, p-coumaric, ellagic, ferulic, sinapic, rosmarinic, and cinnamic acids and 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-ethyl-benzothiazoline-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 2000, Germany) under reduced pressure at 40 °C. The extract was reconstituted in 10 mL dimethylsulfoxide (DMSO) and stored at -80 °C 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]. Briefly, the extract (100 µL) was transferred into a test tube and the volume adjusted to 3.5 mL with distilled water and oxidized with the addition of 250 µL of Folin-Ciocalteu reagent. After 5 min, the mixture was neutralized with 1.25 mL of 20% aqueous sodium carbonate (Na₂CO₃) solution. After 40 min, the absorbance was measured at 725 nm against the solvent blank. The total phenolic content was determined by means of a calibration curve prepared with gallic acid, and expressed as milligrams of gallic acid equivalent (mg GAE)/g of extract. Additional dilution was done if the absorbance value measured was over the linear range of the standard curve.

2.3.2. Determination of total flavonoid content

The total flavonoid content was determined according to Zilic *et al.* [39] using aluminum chloride (AlCl₃) colorimetric assay. Briefly, 300 µL of 5% sodium nitrite (NaNO₂) was mixed with 100 µL of extract. After 6 min, 300 µL of a 10% AlCl₃ solution was added and the volume was adjusted to 2.5 mL using distilled water. After 7 min, 1.5 mL of 1 mol/L NaOH was added, and the mixture was centrifuged at 5 000 g for 10 min. Absorbance of the supernatant was measured at 510 nm against the solvent blank. The total flavonoid content was determined by means of a calibration curve prepared with catechine, and expressed as milligrams of catechin equivalent (mg CE) per g of extract. Additional dilution was done if the absorbance value measured was over the linear range of the standard curve.

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 final concentration was 200 µmol/L for DPPH and the final reaction volume was 3.0 mL. The absorbance was measured at 517 nm against a blank of pure methanol after 60 min of incubation in a dark condition. The standard curve was prepared using Trolox. Results were expressed as milligrams of Trolox equivalent (mg TE) per g of

extract. Additional dilution was needed if the DPPH value measured was over the linear range of the standard curve.

2.4.2. Determination of ABTS radical scavenging activity

The stock solutions of ABTS reagent was prepared according to Hwang and Do Thi [40] by reacting equal quantities of a 7 mmol/L aqueous solution of ABTS with 2.45 mmol/L potassium persulfate for 16 h at room temperature (25°C) in the dark. The working solution was then prepared by diluting 1 mL ABTS solution with 60 mL of ethanol: water (50:50, v/v) to obtain an absorbance of 1.0 ± 0.02 units at 734 nm using the spectrophotometer. Extracts (50 μ L) were allowed to react with 4.95 mL of the ABTS solution for 1 h in a dark condition. Then the absorbance was taken at 734 nm using the spectrophotometer. The standard curve was prepared using Trolox. Results were expressed as milligrams of Trolox equivalent (mg TE) per g of extract. Additional dilution was needed if the ABTS value measured was over the linear range of the standard curve.

2.4.3. Ferric reducing activity power (FRAP) assay

The FRAP assay was done according to Hwang and Do Thi [40]. The stock solutions included 300 mmol/L acetate buffer [3.1 g sodium acetate ($C_2H_3NaO_2 \cdot 3H_2O$) and 16 mL glacial acetic acid ($C_2H_4O_2$), pH 3.6], 10 mmol/L TPTZ solution in 40 mmol/L HCl, and 20 mmol/L ferric chloride ($FeCl_3 \cdot 6H_2O$) solution. The fresh working solution was prepared by mixing 25 mL acetate buffer, 2.5 mL TPTZ solution, and 2.5 mL $FeCl_3 \cdot 6H_2O$ solution and then warmed at 37 °C before using. 50 μ L of PEE were allowed to react with 3.95 mL of the FRAP solution for 30 min in the dark condition. Readings of the colored product [ferrous tripyridyltriazine complex] were then taken at 593 nm. The standard curve was prepared using Trolox. Results were expressed as milligrams of Trolox equivalent (mg TE) per g of extract. Additional dilution was needed if the FRAP value measured was over the linear range of the standard curve.

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

Samples were injected automatically into an HP 1100 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 \times 250 mm) with a spherical particle size of 5 μ m, which was kept at 25 °C. 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 °C. The injection volume was 20 μ L [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 weeks and 2.3–2.9 kg initial weight. 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 °C. Semen was collected twice weekly. Each ejaculate was assessed for initial semen quality; only those that were white, > 200 μ L in volume, $\geq 300 \times 10^6$ cells/mL in concentration and with $\geq 70\%$ motile spermatozoa were included in the study.

2.7. Experimental design

2.7.1. Pilot experiment for selection of useful extract concentrations

(1) Immediately after semen collection, selected ejaculates were pooled to avoid individual differences and to obtain sufficient volume for each treatment. (2) The pooled sample was divided into 11 aliquots (each of 500 μ L). (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]. 3% 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 \pm 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 \pm 1.67	73.33 ^B \pm 1.67	60.00 ^B \pm 2.89	33.33 ^E \pm 1.67
0.4	87.50 ^B \pm 1.45	67.50 ^C \pm 1.45	47.50 ^{CD} \pm 1.45	32.50 ^E \pm 1.45
0.8*	87.50 ^B \pm 1.45	72.50 ^B \pm 1.45	57.50 ^B \pm 1.45	42.50 ^C \pm 1.45
1.2*	92.50 ^A \pm 1.45	82.50 ^A \pm 1.45	67.50 ^A \pm 1.45	52.50 ^B \pm 1.45
1.6*	92.50 ^A \pm 1.45	82.50 ^A \pm 1.45	72.50 ^A \pm 1.45	57.50 ^A \pm 1.45
2.0*	92.50 ^A \pm 1.45	82.50 ^A \pm 1.45	67.50 ^A \pm 1.45	52.50 ^B \pm 1.45
2.4	87.50 ^B \pm 1.45	67.50 ^C \pm 1.45	52.50 ^C \pm 1.45	37.50 ^D \pm 1.45
2.8	87.50 ^B \pm 1.45	62.50 ^D \pm 1.45	47.50 ^{CD} \pm 1.45	32.50 ^E \pm 1.45
3.2	82.50 ^C \pm 1.45	57.50 ^E \pm 1.45	42.50 \pm 1.45	22.50 ^F \pm 1.45
3.6	82.50 ^C \pm 1.45	57.50 ^E \pm 1.45	37.50 ^F \pm 1.45	20.00 ^{FG} \pm 0.00
4.0	80.00 ^C \pm 0.00	52.50 ^F \pm 1.45	32.50 ^F \pm 1.45	17.50 ^G \pm 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 μ L) 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 °C 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 will be placed in pre-warmed slide (37°C) and covered with cover slip. Sperm motility subjectively will be assessed by using phase contrast hot stage microscope set at magnification of 400 × and equipped with a heating plate (37°C).

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]. Two hundred spermatozoa per sample were examined for morphology and viability in stained smear at 1 000 × magnification (oil immersion). 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 to evaluate the functional integrity of the spermatozoa membrane [43] developed this assay for rabbit spermatozoa. Where a volume of 10 µL of semen was gently mixed with 2mL 60 mOsm/L sucrose solution and incubated for 30 min in a water bath at 37 °C. After incubation, 20 µL of the solutions containing semen were placed on a microscope slide and covered with a cover glass. A total of 200 spermatozoa were counted in at least five different fields and examined for swallowed coiled tail at 1 000 magnification. The swollen spermatozoa characterized by coiling of the tail are considered to have an 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 at 1 000x 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 mg GAE/g extract) and total flavonoids (70.16 mg CE/g extract) which are parallel to an antioxidant activity assessed as ABTS, DPPH and FRAP (198.65, 180.18 and 306.17 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 propolis 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
Protochatchuic 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

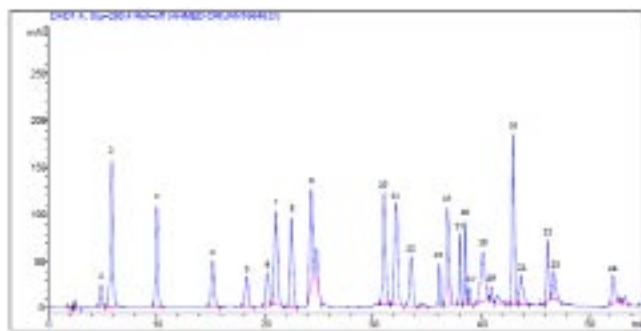


Figure 1. HPLC Chromatograms of standard metabolites showing signal from diode array detector at wavelength 280

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.

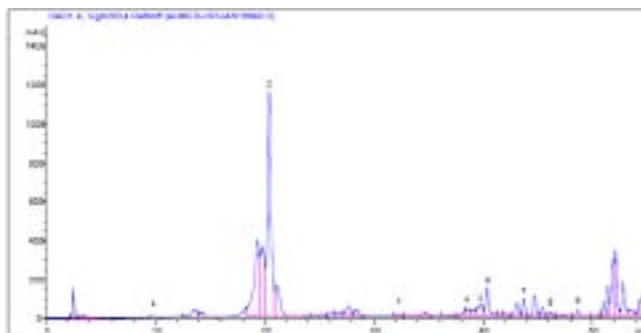


Figure 2. HPLC Chromatograms of propolis extract showing signal from diode array detector at wavelength 280

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

Table 3

Sperm motility percentages (Mean \pm 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} \pm 1.67	93.33 ^{AA} \pm 1.67	95.00 ^{AA} \pm 0.00	95.00 ^{AA} \pm 0.00	95.00 ^{AA} \pm 0.00	2.00	0.170 5	94.58 ^k	0.0001
24	76.67 ^{CB} \pm 1.67	81.67 ^{BCB} \pm 1.67	88.33 ^{AA} \pm 1.67	86.67 ^{ABB} \pm 1.67	85.00 ^{ABB} \pm 2.89	5.50	0.013 2	85.42 ^l	
48	61.67 ^{CC} \pm 1.67	63.33 ^{BCC} \pm 1.67	71.67 ^{ABB} \pm 3.33	73.67 ^{AC} \pm 3.33	70.00 ^{ABCC} \pm 2.89	3.73	0.041 5	69.58 ^m	
72	35.00 ^{Cd} \pm 2.00	41.33 ^{BCd} \pm 1.67	51.67 ^{ABC} \pm 3.33	53.33 ^{Ad} \pm 3.33	50.00 ^{ABd} \pm 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 ($P<0.05$).

3.2. 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 6), there is a significant ($P<0.034$) 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 ($P<0.001$) different with the concentrations of 0.8 and 2.0 of PEE.

3.5. Sperm membrane integrity percentage

Data output in Table 6 showed that the overall mean of sperm membrane integrity (HOST) percentages was significantly ($P<0.000$) 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 ($P<0.000$ – $P<0.002$) 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 extender were significantly ($P<0.003$) 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 ($P<0.000$) decreased from 95.33 % after 2 h to 93 % 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 6), there is a significant ($P<0.000$ – $P<0.015$) difference between the concentrations 0.8, 1.2, 1.6 and 2.0 of PEE in tris extender compared to the control after

Table 4

Sperm livability percentages (Mean \pm 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} \pm 0.33	92.67 ^{AA} \pm 0.33	93.00 ^{AA} \pm 0.58	93.33 ^{AA} \pm 0.33	93.00 ^{AA} \pm 0.58	0.72	0.596 2	93.00 ^k	0.000 1
24	90.67 ^{AA} \pm 0.67	90.67 ^{AB} \pm 0.67	91.67 ^{AA} \pm 0.88	92.00 ^{AB} \pm 0.00	91.33 ^{AB} \pm 0.67	0.84	0.529 3	91.42 ^l	
48	90.00 ^{AB} \pm 0.00	90.00 ^{AB} \pm 1.15	91.00 ^{AA} \pm 1.53	91.33 ^{AB} \pm 0.67	90.00 ^{ABC} \pm 0.00	0.53	0.727 7	90.58 ^{lm}	
72	88.00 ^{AB} \pm 1.15	89.33 ^{AB} \pm 0.67	90.00 ^{AA} \pm 1.15	90.00 ^{AB} \pm 1.15	89.33 ^{AC} \pm 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 \pm 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} \pm 0.58	14.67 ^{AB} \pm 0.67	14.00 ^{AA} \pm 0.58	13.67 ^{AB} \pm 0.88	14.67 ^{AC} \pm 0.33	0.50	0.736 8	14.25 ^m	0.000 1
24	17.00 ^{AB} \pm 1.00	17.00 ^{AB} \pm 0.58	15.00 ^{AA} \pm 0.58	15.00 ^{AB} \pm 0.58	16.33 ^{AB} \pm 0.33	2.42	0.117 1	15.83 ^l	
48	18.00 ^{AB} \pm 0.58	18.00 ^{AA} \pm 1.15	15.67 ^{AA} \pm 1.33	15.33 ^{AB} \pm 0.33	16.67 ^{AB} \pm 0.33	2.15	0.148 6	16.42 ^l	
72	20.33 ^{AA} \pm 0.88	19.00 ^{ABA} \pm 0.58	16.67 ^{BA} \pm 1.33	16.67 ^{BA} \pm 0.33	18.33 ^{ABA} \pm 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 ($P<0.05$).

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 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 antiinflammatory, 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°C [53]. Whereas, the presence of some

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 Control (TCGY)		Concentrations of tris-extender enriched with PEE (mg/5 mL)				F-cal	P<	Overall mean*	P<
Duration (h)	(TCGY)	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 Control (TCGY)		Concentrations of tris-extender enriched with PEE (mg/5 mL)				F-cal	P<	Overall mean*	P<
duration (h)	(TCGY)	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 ($P<0.05$).

other phenolic compounds as rosmarinic acid (3.959 mg/g extract), myricetin (1.946 mg/g extract), Kaempferol (1.089 mg/g extract) and apigenin-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 Johnke *et al.* [10]. On the 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

We declare that we have no conflict of interest.

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