Biochemical changes of chilled rabbit semen diluted in tris based extender enriched with different concentrations of *Moringa olifera* leaves extract

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A B S T R A C T

The present study aimed to assess the effects of *moringa olifera* leaves extract (MOLE) on lipid peroxidation and antioxidant enzymes activities of diluted rabbit semen during chilled storage. Total phenolic and total flavonoid contents, as well as antioxidants activities were determined in MOLE. The extract was analysed by high performance liquid chromatography (HPLC). Semen was collected from 10 rabbit bucks, pooled, then divided into five aliquots and diluted each in 5 ml Tris-citric acid-glucose-egg yolk extender (TCGY). The 1st aliquot served as control, while MOLE was added with different concentrations (1.6, 2.0, 2.4 and 2.8 mg/5 ml TCGY extender) for the aliquots 2, 3, 4 and 5, respectively. Diluted semen samples were refrigerated at 5°C for 72 hours. Lipid peroxidation (MDA), H_2O_2 concentration, SOD activity, CAT activity and GSH concentration were evaluated in chilled semen during the chilling period. Total phenolic contents of MOLE were 19.78 mg GAE /g extract, while total flavonoid contents were 11.94 mg CE / g extract. The MOLE sample exhibited various antiradical activities measured towards DPPH (34.37 mM TE/g) and ABTS (53.47 mM TE/g) and the reducing power was determined by FRAP method (64.59 mM TE/g). This was interpreted by HPLC analysis against 24 standard metabolites. The most effective compound in the MOLE was rutin, rosmarinic acid and pyrogallol. Other compounds were found in fewer amounts as *p*-hydroxybenzoic acid, hisperdin, caffeic acid, apeginin-7-glucoside, myrcetin and naringeen. Obtained results clearly demonstrated that the addition of 2.0 – 2.8 mg MOLE in the chilled extended rabbit semen proved to be beneficial for minimizing MDA and H_2O_2 concentration, in addition they increased SOD, CAT activities and GSH concentration compared to the control.

Key words: Male rabbits, Semen extender, Moringa extract, Antioxidants, HPLC.

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1. INTRODUCTION

The demand for using stored semen in artificial insemination (AI) programs of livestock animals is increasing (Paulenz et al., 2010; Xu et al., 2009). Therefore, Developing and improving methods for semen preservation would provide adequate fertility rates that maintain the high production rates for rabbit industry. Several studies on preservation protocols and extender composition have been carried out (Di lorio et al., 2014; Iaffaldano et al., 2010; Johinke et al., 2014; Rosato et al., 2012; Rosato and Iaffaldano, 2011). Unfortunately the ability of rabbit sperm to survive *in vitro* after chilled (Iaffaldano et al., 2010; Rosato and Iaffaldano, 2011) or frozen storage (Moce and Vicente, 2009) 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 (Bansal and Bilaspuri, 2010; Kim et al., 2011). The majority of ROS are continuously neutralized by antioxidants contained in rabbit semen itself (Mourvaki et al., 2010), while the endogenous antioxidants are insufficient to counteract the lipid peroxidation that occurs during sperm storage (Castellini et al., 2000). Several attempts have been made with dietary supra-nutritional antioxidants supplementation aimed to enhance rabbit sperm quality (Gliozzi et al., 2009; Yousef et al., 2003) or its survival during refrigeration (Castellini et al., 2000). However, reports evaluating the efficacy of the addition of natural antioxidants in the rabbit seminal extenders are lacking.

Natural extracts and infusions from fruits and vegetables were used in semen extenders for

preserving animal's sperms (El-Nattat et al., 2016; El-Sheshtawy et al., 2016). Plants and their products are potential sources of phytochemicals that have been found to counteract free radicals due to their antioxidant activity (Khalafalla et al., 2010). *Moringa oleifera Lam (Family: Moringaceae),* commonly known as drumstick tree or horseradish tree is a highly valued plant in tropical and subtropical countries where it is mostly cultivated. The leaves are highly nutritious, being a good source of proteins, β- carotene, vitamins (such as A, B, C and E, riboflavin, nicotinic acid, follic acid, pyridoxine), amino acids, minerals and various antioxidant compounds (polyphenols, flavonoids, proanthocyanidins, flavonols) (Anwar et al., 2007; Khalafalla et al., 2010). These polyphenols, flavonoids, proanthocyanidins, flavonols, vitamin C, vitamin E, β-carotene, zinc and selenium have been documented to possess strong antioxidant potential (Aqil et al., 2006).

Hence, the present study was designed to assess the effects of MOLE, supplementation to a basic rabbit semen extender, on lipid peroxidation, H_2O_2 concentration, some antioxidant enzymes activities and reduced glutathione concentration in chilled rabbit semen over 72 hours.

2. MATERIAL AND METHODS

2.1. Collection of samples and chemicals:

2.1.1. Moringa leaves samples:

Moringa oleifera leaves were air-dried, kept under shade for 7 days, milled into powder and passed through a mesh sieve-40 and packed in polyethylene bags to be stored at until use.

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, diethyl ether, ethyl acetate, tetrahydrofuran and methanol (analytical grade) were purchased from Tedia Company, Inc., Fairfield, 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:

MOLE was prepared by mixing 30 grams of raw material with 300 mL methanol (1:10 w/v) and stirred at room temperature. The solution was kept at 4 °C for 24 h. The extract obtained was filtered, then dried using rotary evaporator under reduced pressure at 40 °C and the final yield of extract was recorded (Roopalatha and Nair, 2013). The extract was reconstituted in 10 mL 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 contents were determined according to the Folin-Ciocalteu procedure (Zilic et al., 2012). 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 contents were determined according to Zilic et al., (2012) using aluminum chloride ($AlCl_3$) colorimetric assay. Total flavonoid contents were 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 extract was determined using the stable DPPH according to Hwang and Do Thi (2014). 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 g 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 (2014). The standard curve was prepared using

Trolox (Y= 2.965 + 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 g of extract.

2.4.3. Ferric reducing activity power (FRAP) assay:

The FRAP assay was done according to Hwang and Thi (2014). The standard curve was prepared using Trolox (Y= 0.041+ 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 g of extract.

2.5. Separation and identification of phenolic acids by HPLC:

Samples were injected automatically into an HP 1100 series HPLC system (Hewlett-Packard, GmbH, Germany) equipped with a diode array detector (DAD). 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 °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 µL (Gayosso-García Sancho et al., 2011).

2.6. Animals management and semen collection:

2.6.1. Ten 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.

2.6.2. 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 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:

- Immediately after semen collection, selected ejaculates were pooled to avoid individual differences and to obtain sufficient volume for each treatment.
- The pooled sample was divided into 11 aliquots (each of 500 µL).
- 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, Roca et al. 2000). 3% egg yolk was added to the basic extender as a modification (TCGY).
- The other 10 aliquots were diluted 1:10 in the TCGY basic extender enriched with ten concentrations of MOLE as shown in table 1.

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:

1. The first aliquot was diluted 1:10 in TCGY basic extender and served as control.
2. The other four aliquots were diluted 1:10 in the TCGY extender supplemented with the selected 4 concentrations of the MOLE that were obtained from the pilot experiment (Table 1).

2.8. Assessment of oxidative/antioxidants status:

The diluted semen samples were refrigerated in an incubator at 4 °C for 72 h. Oxidative/antioxidant status will be measured in diluted semen during chilling periods (2, 24, 48 and 72 hours post chilling) using commercially kits obtained from Bio Diagnostic Research office (Dokki, Giza, Egypt).

2.8.1. Determination of Lipid peroxidation (nmol/ml):

Thiobarbituric acid-reactive substances (TBARS) were determined according to the method of Ohkawa et al. (1979).

2.8.2. Determination of Hydrogen peroxide (mM/L):

The H_2O_2 level was determined according to the method of Aebi (1984).

2.8.3. Determination of Superoxide Dismutase (SOD) activity (U/ml):

The activity of SOD was determined according to Nishikimi et al. (1972).

2.8.4. Determination of Catalase activity (U/L):

In The activity of catalase was determined according to Aebi (1984).

2.8.5. Determination of Reduced glutathione (mg/dl):

Reduced glutathione concentration was assayed.

2.9. Statistical analysis:

Statistical analysis was analyzed using the SAS computerized program v. 9.2 (SAS, 2008) 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 Moringa Olifera Leaves Extract:

Total phenolic contents of MOLE were 19.78 mg GAE /g extract, while total flavonoid contents were 11.94 mg CE / g extract. The MOLE sample exhibited various antiradical activity measured towards ABTS (53.47 mM TE/g) and DPPH (34.37 mM TE/g) while the reducing power was determined by FRAP method (64.59 mM TE/g /g) (Table 2). These results were interpreted by HPLC analysis against 24 standard metabolites (Table 3). The most effective compounds in the MOLE were Rutin (8.278 mg / g extract), Rosmarinic acid (1.364 mg / g extract) and Pyrogallol (1.104 mg / g extract). Other compounds were found in fewer amounts as *P*-hydroxybenzoic acid (0.671 mg / g extract), Hisperdin (0.545 mg / g extract), Caffeic acid (0.218 mg / g extract), Apeginin-7-glucoside (0.214 mg / g extract), Myrcetin (0.190 mg / g extract) and Naringeen (0.187 mg / g extract). There were further compounds found in trace amounts as Vanillic acid (0.039 mg / g extract), Kaempferol (0.037 mg / g extract), Chlorogenic acid (0.036 mg / g extract) and Cinnamic acid (0.033 mg / g extract).

3.2. Biochemical analysis of tris extender diluted semen enriched with MOLE:

3.2.1. MDA concentration (nmol/ml):

Data output in table (4) showed no significant difference in the overall mean of MDA concentration from 2 hours to 72 hours. This coincided within treatment (within columns) (1.6, 2.0, 2.4 and 2.8 mg MOLE / 5 ml tris extender) from 2 to 72 hours while the control (0 mg MOLE) showed a significant ($P<0.0125$) increase in MDA concentration from 2.69 nmol/ml after 2 hours to 4.16 nmol/ml after 72 hours.

Concerning the enrichments of 5 ml tris extender with different concentrations of MOLE within rows (Table 4), the concentrations of 1.6, 2.0, 2.4 and 2.8 mg MOLE / 5 ml tris extender showed significant ($P<0.0099$- $P<0.0007$) decrease in MDA concentration after 48-72 hours compared to the control (0 mg MOLE). The concentration of 2.0 mg MOLE /5 ml tris extender is the best MOLE enrichments that maintained lower MDA concentration from 2 to 72 hours. This was analogous to the overall mean with its respective concentration.

3.2.2. H_2O_2 concentration (mmol/l):

Data output in table (5) showed that the overall mean of H_2O_2 concentration was significantly ($P<0.0002$) increased from 0.060 mmol/l after 2 hours to 0.097 mmol/l after 72 hours. Results within treatment (within columns) (control, 2.0 and 2.4 mg MOLE / 5 ml tris extender), significantly ($P<0.0026$- $P<0.0030$) and irregularly increased from 2 to 72 hours while the concentration of 1.6 and 2.8 mg showed no significant differences.

Concerning the enrichments of 5 ml tris extender with different concentrations of MOLE within rows (Table 5), the concentrations of 2.0 mg MOLE / 5 ml tris extender were significantly ($P<0.0026$- $P<0.0081$) the best MOLE enrichments that maintained lower H_2O_2 concentration compared to the control (0 mg MOLE) from 2 to 48 hours. After 72 hours, the significant ($P<0.0024$) concentrations were 2.4 and 2.8 mg/5 ml compared to the control. The overall mean of the concentration (2.0 mg/5 ml) was significantly ($P<0.0186$) different from the other concentrations.

3.2.3. SOD activity (U/ml):

Data output in table (6) showed that there is no significant difference concerning the overall mean of SOD activity from 2 hours to 72 hours. This coincided within treatment (within columns) (1.6, 2.0, 2.4 and 2.8 mg MOLE / 5 ml tris extender) from 2 to 72 hours. Only, the control (0 mg MOLE) showed a significant ($P<0.0027$) increase in SOD activity from 87.03 U/ml after 2 hours to 113.94 U/ml after 72 hours.

Concerning the enrichments of 5 ml tris extender with the different concentrations of MOLE (within rows) (Table 6), the concentrations of 2.8 mg was the best MOLE enrichments that maintained highest SOD activity after 48 hours compared to the concentrations of 1.6 and 2.4 mg MOLE ($P<0.0018$). The overall means of the concentration 2.8 mg MOLE was the highest significant ($P<0.0001$) result compared to the other treatments concerning the SOD activity.

3.2.4. CAT activity (U/L):

Data output in table (7) showed that the trend of overall mean for the CAT activity was significantly ($P<0.0101$) increased after 48 hours (252.09 U/L), compared to the other chilling time (2, 24 and 72 hours). This coincided within treatment (within columns) (1.6 and 2.4 mg MOLE / 5 ml tris extender).

Concerning the enrichments of 5 ml tris extender with different concentrations of MOLE (within rows) (Table 7), the concentrations of 2.0 and 2.8 mg were the best MOLE enrichments that maintained highest CAT activity from 2 to 72 hours compared to control and the concentrations of 1.6 and 2.4 mg MOLE. This was analogous to the overall means with their respective concentrations (P<0.0001).

3.2.5. GSH concentration (mg/dl):

Data output in table (8) showed that the overall mean of GSH concentration was significantly (P<0.0012) increased from 3.14 mg/dl after 2 hours to 4.35 mg/dl after 72 hours. This corresponded to the treatment (within columns) for the concentrations of 2.4 and 2.8 mg MOLE / 5 ml tris extender, while the control and the concentration of 1.6 mg showed significant (P<0.0007, P<0.0050) irregular decrease in GSH concentration from 2 to 72 hours.

Table (1): Sperm motility percentages (Mean ±SE) of rabbit semen after chilled storage in TCGY extenders enriched with different concentrations of MOLE.

Concentration (Mg/5ml)	Chilling Duration (hours)			
	2 hours	24 hours	48 hours	72 hours
Control	88.33 ^{AB} ± 1.67	73.33 ^{D} ± 1.67	61.67 ^{C} ± 1.67	33.33 ^{E} ± 1.67
0.4	87.50 ^{B} ± 1.45	72.50 ^{D} ± 1.45	57.50 ^{D} ± 1.45	32.50 ^{E} ± 1.45
0.8	87.50 ^{B} ± 1.45	75.00 ^{CD} ± 0.00	62.50 ^{C} ± 1.45	37.50 ^{D} ± 1.45
1.2	87.50 ^{B} ± 1.45	82.50 ^{A} ± 1.45	67.50 ^{B} ± 1.45	42.50 ^{C} ± 1.45
1.6*	92.50 ^{A} ± 1.45	82.50 ^{A} ± 1.45	72.50 ^{A} ± 1.45	45.00 ^{BC} ± 0.00
2.0*	92.50 ^{A} ± 1.45	82.50 ^{A} ± 1.45	72.50 ^{A} ± 1.45	52.50 ^{A} ± 1.45
2.4*	92.50 ^{A} ± 1.45	80.00 ^{AB} ± 0.00	67.50 ^{B} ± 1.45	47.50 ^{B} ± 1.45
2.8*	92.50 ^{A} ± 1.45	77.50 ^{BC} ± 1.45	67.50 ^{B} ± 1.45	42.50 ^{C} ± 1.45
3.2	87.50 ^{B} ± 1.45	75.00 ^{CD} ± 0.00	62.50 ^{C} ± 1.45	37.50 ^{D} ± 1.45
3.6	87.50 ^{B} ± 1.45	72.50 ^{D} ± 1.45	57.50 ^{D} ± 1.45	37.50 ^{D} ± 1.45
4.0	87.50 ^{B} ± 1.45	67.50 ^{E} ± 1.45	52.50 ^{E} ± 1.45	32.50 ^{E} ± 1.45
P<	0.0193	0.0001	0.0001	0.0001

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

Table (2): Total phenolics, total flavonoids and Antioxidant activity of MOLE as determined by the ABTS, DPPH and FRAP assays.

Total phenols (mg GAE/g)	Total flavonoids (mg CE/g)	Antioxidant activity (mM TE/g extract)		
		ABTS	DPPH	FRAP
19.78 ± 0.53	11.94 ± 0.49	53.47 ± 0.99	34.37 ± 0.99	64.59 ± 2.36

Table (3): HPLC analysis of polyphenolic compounds MOLE.

Compound	Retention Time (min)	Concentration (µg / g extract)	Compound	Retention Time (min)	Concentration (µg / g extract)
Pyrogallol	4.90	1104.16	Rutin	36.18	8278.57
Gallic acid	5.90	0.00	*P*-coumaric acid	36.95	0.00
Protochatechuic acid	10.03	0.00	Naringeen	38.07	186.80
P-hydroxybenzoic acid	15.22	671.18	Hisperdin	38.60	544.57
Catachine	18.37	0.00	Apeginin-7-glucoside	38.96	214.01
Chlorogenic acid	20.28	36.06	Myrcetin	40.24	190.40
Caffeic acid	21.08	218.44	Rosmarinic acid	40.95	1364.81
Syringic acid	22.52	0.00	Cinnamic acid	41.52	32.95
Vanillic acid	24.82	39.28	Quercetin	43.01	0.00
Scoplatine	31.07	0.00	Apegnin	43.72	0.00
Ferulic acid	32.17	0.00	Kaempferol	46.22	37.31
Sinapic acid	33.56	0.00	Chyrsin	52.24	0.00

Table (4): MDA concentrations (Mean ± SE, nmol/ml) of diluted rabbit buck semen in TCG extender enriched with different concentrations (mg/5 ml extender) of MLME after chilled storage.

Chilling Duration (hours)	Control (TCGY)	Concentrations of MLME (mg) / tris-extender (5 ml)				*P*<	Overall mean*	*P*<
		1.6	2.0	2.4	2.8			
2	2.69 ^{ABb} ± 0.19	2.73 ^{ABa} ±0.21	2.23 ^{Ba} ±0.11	2.87 ^{ABa} ±0.11	2.93 ^{Aa} ±0.23	0.1291	2.69 ^{k}	0.6215
24	2.77 ^{ABb} ± 0.17	3.24 ^{Aa} ±0.27	2.45 ^{Ba} ±0.09	2.81 ^{ABa} ±0.26	2.48 ^{Ba} ±0.13	0.1117	2.74 ^{k}	
48	3.28 ^{Ab} ± 0.30	2.61 ^{BCa} ±0.13	2.08 ^{Ca} ±0.11	2.96 ^{ABa} ±0.11	2.68 ^{Ba} ±0.16	0.0099	2.58 ^{k}	
72	4.16 ^{Aa} ± 0.31	2.71 ^{BCa} ±0.18	2.40 ^{Ca} ±0.14	3.15 ^{Ba} ±0.16	2.41 ^{Ca} ±0.19	0.0007	2.66 ^{k}	
P<	0.0125	0.2188	0.2158	0.5516	0.2687	Interaction: time × concentration = 0.1050		
Overall mean*		2.82 $^{K L}$	2.29 ^{M}	2.95 ^{K}	2.62 ^{L}			
P<	0.0001							

Overall mean concerns 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): H_2O_2 concentrations (Mean±SE, mmol/l) of diluted rabbit buck semen in TCG extender enriched with different concentrations (mg/5 ml extender) of MLME after chilled storage.

Chilling Duration (hours)	Control (TCGY)	Concentrations of MLME (mg) / tris-extender (5 ml)				*P*<	Overall mean*	*P*<
		1.6	2.0	2.4	2.8			
2	0.10 ^{Ab} ± 0.003	0.08 ^{Aa} ±0.007	0.05 ^{Bb} ±0.009	0.04 ^{Bb} ±0.01	0.07 ^{ABa} ±0.01	0.0081	0.061 ^{m}	0.0002
24	0.11 ^{Ab} ± 0.008	0.07 ^{Ba} ±0.01	0.05 ^{Bb} ±0.01	0.11 ^{Aa} ±0.002	0.08 ^{ABa} ±0.01	0.0070	0.076 ^{l}	
48	0.12 ^{Aab} ± 0.006	0.07 ^{BCa} ±0.01	0.04 ^{Cb} ±0.01	0.10 ^{ABa} ±0.003	0.08 ^{Ba} ±0.008	0.0026	0.076 ^{l}	
72	0.14 ^{Aa} ± 0.009	0.10 ^{BCa} ±0.01	0.11 ^{Ba} ±0.003	0.09 ^{Ca} ±0.007	0.09 ^{Ca} ±0.006	0.0024	0.098 ^{k}	
P<	0.0262	0.3416	0.0030	0.0026	0.6241	Interaction: time × concentration = 0.0005		
Overall mean*		0.083 ^{K}	0.063 ^{L}	0.084 ^{K}	0.081 ^{K}			
P<	0.0186							

Table (6): SOD activity (Mean±SE, U/ml) of diluted rabbit buck semen in TCG extender enriched with different concentrations (mg/5 ml extender) of MLME after chilled storage.

Chilling Duration (hours)	Control (TCGY)	Concentrations of MLME (mg) / tris-extender (5 ml)				*P*<	Overall mean*	*P*<
		1.6	2.0	2.4	2.8			
2	87.03 ^{Ab} ± 1.48	63.06 ^{Ba} ±6.06	85.02 ^{Aa} ±5.50	78.35 ^{Aa} ±3.72	80.52 ^{Aa} ±5.83	0.0391	76.74 ^{k}	0.2001
24	95.41 ^{Ab} ± 0.47	72.07 ^{Aa} ±9.74	84.46 ^{Aa} ±9.75	79.95 ^{Aa} ±3.50	100.79 ^{Aa} ±10.66	0.1516	84.31 ^{k}	
48	97.54 ^{ABb} ± 0.58	60.80 ^{Ca} ±7.74	94.59 ^{ABa} ±5.27	85.02 ^{Ba} ±7.24	111.48 ^{Aa} ±6.54	0.0018	87.97 ^{k}	
72	113.94 ^{Aa} ±6.38	69.25 ^{Ca} ±6.20	85.02 ^{BCa} ±8.67	68.13 ^{Ca} ±6.89	105.29 ^{ABa} ±9.57	0.0050	81.92 ^{k}	
P<	0.0027	0.7057	0.7449	0.2662	0.1292	Interaction: time × concentration = 0.3380		
Overall mean*		66.29 ^{M}	87.27 ^{L}	77.86 ^{L}	99.52 ^{K}			
P<	0.0001							

Table (7): CAT activity (Mean±SE, U/L) of diluted rabbit buck semen in TCG extender enriched with different concentrations (mg/5 ml extender) of MLME after chilled storage.

Chilling Duration (hours)	Control (TCGY)	Concentrations of MLME (mg) / tris-extender (5 ml)				*P*<	Overall mean*	*P*<
		1.6	2.0	2.4	2.8			
2	99.64 ^{Cc} ± 2.27	116.35 ^{Cc} ±4.2	306.16 ^{Aa} ±21.7	185.26 ^{Bc} ±13.28	271.89 ^{Aa} ±17.39	0.0001	219.92 ^{l}	0.0101
24	150.04^{Bb}±11.14	144.42 ^{Bb} ±6.9	251.02 ^{Ab} ±5.2	253.17 ^{Ab} ±7.3	230.24 ^{Aa} ±8.28	0.0001	219.72 ^{l}	
48	241.76^{Ba}±11.61	244.48 ^{Ba} ±7.22	209.4 ^{Bb} ±10.86	327.75 ^{Aa} ±20.5	226.69 ^{Ba} ±19.72	0.0021	252.09 ^{k}	
72	248.88^{Ba}±11.61	126.81 ^{Dbc} ±9.25	321.53 ^{Aa} ±18.59	178.94 ^{Cc} ±15.71	288.37^{ABa}±22.74	0.0001	228.92 ^{l}	
P<	0.0001	0.0001	0.0032	0.0003	0.0994			
Overall mean*		158.02 ^{M}	272.03 ^{K}	236.28 ^{L}	254.30 ^{KL}	Interaction: time × concentration = 0.0001		
P<	0.0001							

Table (8): GSH concentration (Mean ±SE, mg/dl) of diluted rabbit buck semen in TCG extender enriched with different concentrations (mg/5 ml extender) of MLME after chilled storage.

Chilling Duration (hours)	Control (TCGY)	Concentrations of MLME (mg) / tris-extender (5 ml)				*P*<	Overall mean*	*P*<
		1.6	2.0	2.4	2.8			
2	4.91 ^{Aa} ± 0.36	2.60 ^{Cab} ±0.20	3.17 ^{BCa} ±0.33	3.71 ^{Bb} ±0.17	3.04 ^{BCb} ±0.29	0.0018	3.14 ^{l}	0.0012
24	4.35 ^{Aa} ± 0.55	3.11 ^{BCa} ±0.19	2.93 ^{BCa} ±0.10	3.64 ^{ABb} ±0.35	2.46 ^{Cb} ±0.13	0.0155	3.04 ^{l}	
48	4.89 ^{Aa} ± 0.13	3.04 ^{Bbc} ±0.21	3.49 ^{Ba} ±0.32	4.13 ^{Aa} ±0.33	4.60 ^{Aa} ±0.24	0.0001	3.82 ^{k}	
72	1.92 ^{Cb} ± 0.09	1.86 ^{Cc} ±0.35	3.02 ^{Ba} ±0.19	3.95 ^{Aa} ±0.11	5.58 ^{Aa} ±0.54	0.0001	4.35 ^{k}	
P<	0.0007	0.0050	0.4810	0.0026	0.0007			
Overall mean*		3.41 ^{L}	3.15 ^{L}	3.86 ^{K}	3.92 ^{K}	Interaction: time × concentration = 0.0001		
P<	0.0001							

Concerning the enrichments of 5 ml tris extender with different concentrations of MOLE (within rows) (Table 8), the concentrations of 2.0, 2.4 and 2.8 mg MOLE / 5 ml tris extender showed significant (*P*<0.0001) increase in GSH concentration compared to the control after 72 hours, where the concentrations of 2.4 and 2.8 mg were the best MOLE enrichments that maintained highest GSH concentration after 72 hours. This was approved by the analogous overall means (*P*<0.0001).

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 (Capucho et al., 2012). The chilling is one of the detrimental factors that induce the production of ROS in extended semen of rabbits (Iaffaldano et al., 2010; Rosato and Iaffaldano, 2011). The feed on natural products as prescribed in folk medicine improved the motility and fertilizing capability of sperm, through facing up 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 (Burdock, 1998).

Moringa leaves was used in this study as a natural additive to semen extender owing to its high contents of vitamins, amino acids, minerals and various antioxidant compounds (Khalafalla et al., 2010) including phenols, flavonoids, proanthocyanidins, flavonols, vitamin C, vitamin E, β-carotene, zinc and selenium which have been

documented to possess strong antioxidant potential (Aqil et al., 2006). The phenolic content of MOLE observed in this study corroborated with the findings of Frum and Viljoen (2006) and Sreelatha and Padma (2009) and Atawodi et al. (2010) on different fractions of this plant. The synergistic effect of phenolic compounds may contribute significantly to the ability of the extracts to adsorb and neutralize free radicals or decompose peroxides (Adedapo et al., 2008). Their ability as free radical scavengers could be due to their redox properties, the presence of conjugated ring structures and carboxylic group which have been reported to inhibit lipid peroxidation (Oyedemi et al., 2010).

The reaction of antioxidant compounds presents in MOLE with DPPH and ABTS radical discoloured the visible colour by measuring the changes in absorbance. The degree of discolouration indicates the scavenging potential of the extract due to hydrogen proton donation (Verma et al., 2009). Sreelatha and Padma (2009) demonstrated that methanol extract of *M. oleifera* leaves significantly reduced DPPH radicals though lower than our observed results. Variation could be due to difference in polarity of the solvents and geographical location of the plant (Sreelatha and Padma, 2009). Siddhuraju and Becker (2003) reported that the reducing power of bioactive compounds was associated with antioxidant activity. The reducing power of the plant extracts was found to be concentration dependent. The antioxidant activity of MOLE is correlated with its total polyphenolic contents (Pourmorad et al., 2006). The findings obtained from this study agreed with Siddhuraju and Becker (2003) who showed that antioxidant properties were concomitant with the development of reducing power. Therefore, phenolic compounds present in MOLE are good electron donors and could terminate the radical chain reaction by converting free radicals to stable product.

In this consent, the present results showed that the addition of 2.0 – 2.8 mg MOLE/ 5 ml Tris extender had significantly minimized sperm lipid peroxidation and H_2O_2 concentration, SOD activity and increased CAT activity and GSH concentration during chilling till 72 hours compared to the control treatment. This was attributed mainly to the high content of rutin (quercetin-3-rhamnosyl glucoside) (8.278 mg/g extract), which has a potent reducing power against the lipid peroxidation and exhibit strong DPPH, hydroxyl radical and superoxide radical scavenging activity (Abarikwu et al., 2016; Afolabi et al., 2013; Moretti et al., 2012; Moyo et al., 2012; Sadek, 2014). Whereas, the presence of some other phenolic compounds as rosmarinic acid, pyrogallol, *P*-hydroxybenzoic acid, Hisperdin, Caffeic acid, Apeginin-7-glucoside, Myrcetin and Naringeen that have scavenging antioxidant activity against free radicals interpreted the benefit beyond the enrichment of rabbit semen tris-based extender to overcome the lipid peroxidation process induced through the chilling period (Gibb et al., 2013; Johinke et al., 2014, 2015; Li et al., 2010; Moretti et al., 2012). This may elongate the period of chilling in rabbit extended semen above 72 hours in agreement with Di lorio et al. (2014) and Johinke et al. (2014). On the contrary, El-Nattat et al. (2011) had used an antioxidant (L-carnitine) in rabbit semen tris-extender that doesn't exceed the 48 hours chilling.

Superoxide dismutase (SOD) has been reported as one of the most important antioxidant defence enzymes that scavenge superoxide anion in order to reduce toxic effect caused by this radical (Liyana-Pathiranan et al., 2006). The present study revealed the high activity of SOD in the extender supplemented with MOLE than the control. This observation implies an efficient protective mechanism of MOLE against superoxide anion radical relative to the high concentration of phenolics and flavonoids contents (Robak and Gryglewski, 1998). Catalase (CAT) is another antioxidant enzyme widely distributed in the animal tissues (Oyedemi et al., 2010). The enzyme is reported to protect the system from highly reactive hydroxyl radicals through hydrogen peroxide decomposition (Chance et al., 1952). Reduction of this enzyme activity may promote the cellular damage caused by the assimilation of superoxide and hydrogen peroxide. In the present study, the enrichment of semen extender with MOLE increased CAT activity. The elevated activity of SOD and CAT may suggest an induction of the enzymes by MOLE supplementation. Reduced glutathione (GSH) is a non-enzymatic biological antioxidant. It protects cells against reactive oxygen species in the body (Arivazhagan et al., 2000). The activity of GSH was significantly increased in extender supplemented with MOLE which is associated with a decrease in the level of lipid peroxidation. The effect of antioxidant potential of plant extracts orally administered into rats on GSH activity has been observed (Choi et al., 2010; Oyedemi et al., 2010). Generally, high phenolic contents of the extract correspond with the GSH antioxidant activity due to the combined effect of these compounds.

In conclusion, the enrichment of rabbit semen tris-basic extender with 2.0 – 2.8 mg MOLE / 5 ml tris-extender (as the best and safe concentrations) minimize the sperm lipid peroxidation and H_2O_2

concentration, in addition to the elevation of SOD, CAT activity and GSH concentration.

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