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Research Article

Ethanolic Purslane (*Portulaca oleracea*) Leaf Extract Improves the Quality of Cryopreserved Goat Semen

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Portulaca oleracea contains a wide range of chemical constituents, including flavonoids, alkaloids, polysaccharides, fatty acids, terpenoids, sterols, proteins, vitamins, and minerals. These chemical constituents are linked to the plant's diverse pharmacological properties, such as antimicrobial and antioxidant activities. Therefore, this study is aimed at evaluating the effect of adding two concentrations (50 or 100 μg/mL) of *Portulaca oleracea* leaf ethanolic extracts in a native form (POLE) or nanoformulation (POLENF) on the antioxidant status of sperm medium to improve goat sperm freezability. Semen was evaluated postequilibration (5°C for 2 h), postthawing, and 2 h postincubation (37°C and 5% CO_2). Phytochemical analysis showed the presence of 30 compounds in POLE, including phenolic acids and flavonoids and their glucosides. Total flavonoids were higher in POLE than in POLENF. Both extracts showed strong free radical scavenging capacity by the 2,2-diphenyl-1-picryl-hydrazyl-hydrate assay, which was higher for POLENF than POLE, whereas the antioxidant activity by the ferric reducing antioxidant power assay had no differences. In comparison to free extender, the addition of POLE (100 μg) or POLENF (50 and 100 μg) significantly improved sperm motility and viability postequilibration and postincubation and antioxidant status of sperm medium postthawing. All POLE and POLENF concentrations significantly improved motility, viability, membrane integrity, and apoptosis of spermatozoa postthawing. Enzyme activities of AST, ALT, and LDH in sperm medium postthawing were not affected by treatments. In comparing different types and concentrations of POLE with free extender, the addition of POLENF (100 μg/mL) to goat semen extender gave the most valuable results as an effective free radical scavenger and antiapoptotic during cryopreservation.

1. Introduction

Sperm cryopreservation, with a focus on freezing, is a vital technique for animal production, especially goat farming. This reduces breeding expenses by preventing the need to buy and retain breeders while increasing revenues through genetic improvement by employing high-quality germplasm, managerial ease, and even the sale of sperm. Despite this, after artificial insemination with frozen goat sperm, the fertility rate is lower than intended, stressing the need to improve the sperm quality. Supplementing antioxidant compounds to semen extenders before cryopreservation is one strategy for preventing oxidative stress [1–3].

Most antioxidants are phenolic compounds that can reduce oxidative stress as scavengers of different free radical species (reactive oxygen species (ROS)). Thus, the phenolic compounds in a plant have antioxidative actions. Antioxidants can neutralize or scavenge ROS and are potential for optimal health protection [4]. Plants comprise a great variety of compounds, such as flavonoids (flavones, anthocyanins, etc.),

and phenolic components, such as nonflavonoids (phenolic acids, lignins, stilbenes, terpenoids, etc.). These compounds differ in their structures (number and position of phenolic hydroxyl groups), resulting in marked variation in their antioxidative actions [5]. Several reports have indicated a positive correlation between the phenolic compound contents in plants with the antioxidative capacity [6, 7]. *Portulaca oleracea* L. (PO), Portulacaceae family, genus *Portulaca*, commonly named purslane, is widely used as a potherb in different countries (the Mediterranean, Central Europe, and Asia). The World Health Organization (WHO) listed PO as a medicinal plant; it has been given the term "Global Panacea" due to its high distribution in various environmental conditions [8, 9] and exhibition of pharmacological impacts, including antioxidant [5, 10–12], anti-inflammatory [13], and antibacterial [14] properties. Also, PO is taken to improve the male reproductive functions and fetal development in females [15]. The antioxidant capacity of PO involves different compounds (i.e., alkaloids [16–20], flavonoids [21, 22], phenolic acids [5], terpenoids [23], coumarins [24], α-linolenic acid, β-carotene [25], and trace elements [26]). PO is rich in polyunsaturated fatty acids, including ω-3 (α-linolenic acid; C18:3) and ω-6 (linoleic acid; C18:2) [27, 28]. Compared to other vegetables, PO contains the highest content of ω-3 fatty acids [27]. Also, PO contained glutathione [29], gallotannins [30], terpenoids [31], and volatile oils [31]. Moreover, shoots of PO included high levels of vitamins, such as A, E, C, B1, B2, B3, B6, and B9, and minerals, such as Ca, Fe, Mg, Mn, K, and Zn [27, 32]. PO leaves (POL) have a protective ability against oxidative stress caused by vitamin A deficiency [33]. Also, polysaccharides in PO act as scavengers of superoxide anion, 2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH), nitric oxide, and hydroxyl radicals in a dose-dependent manner [34]. PO also increases the antioxidant enzyme activities in hepatic, renal, and testicular tissues [35]. As observed in other vegetables, there are marked differences in antioxidant contents in different parts of PO, which may depend on environmental conditions, and POL has higher antioxidant properties through the phosphor-molybdenum assay [36, 37]. Several reports have mentioned the free radical scavenging property of PO extract in terms of the presence of phenolic compounds, ascorbic acid, α-tocopherol, and pigments [5, 38, 39]. *Portulaca oleracea* leaf extract (POLE) can act as a donor of electrons by reacting with free radicals to convert these radicals to stable products [40] and also block the radical chain reaction [41].

Recent nanotechnologies reflect new prospects for developing novel and noninvasive techniques for sperm cryopreservation [42]. The key factor of nanoparticle (NP) activity is the characteristics of their surface, such as size, charge density, and hydrophobicity. Different types of NPs are new forms of materials with promising biological properties and low toxicity and seem to have a high potential for passing through physiological barriers and accessing specific target tissues [43]. Furthermore, the antioxidant properties of herbal NPs recently contributed to optimizing the cryopreservation protocols [44, 45].

Information on the effect of POLE in semen extenders on improving the quality of preserved semen is rare. In this study, ethanolic POLE is preferred because it is a proper solvent used in polyphenolic compound extraction from plant tissues. Ethanol, like methanol, can inhibit the polyphenol oxidase action that causes polyphenol oxidation, and it is easier to evaporate than water [46]. This study is aimed at evaluating the effect of adding two concentrations of ethanolic POLE, as a natural antioxidant, in a native form or nanoformulation on increasing the antioxidant status of sperm medium to improve the sperm freezing ability of goat semen.

2. Materials and Methods

2.1. Extract Preparation and Characteristics

2.1.1. Harvest and Preparation of Ethanolic POLE. POL was harvested from a local region in Dakahlia Governorate, Egypt, dried, and grounded, and ~100 g of leaves was soaked thrice in 100% ethanol (500 mL) at ambient temperature. After extraction, POLE was first filtered through a fresh cotton plug and finally with Whatman filter papers (No. 1) and then dried under a vacuum (40°C).

2.1.2. Nanoformulation of POL (POLENF). POLENF was prepared from POL by hydrothermal squeezing according to Shin et al. [47]. A sample of dried POL (10 g) was placed in analytical grade ethanol (60 mL) in a Teflon beaker (100 mL). The mixture was placed for ~10 h in an autoclave (70 psi at 110°C without stirring). The extract was squeezed from the leaves under the temperature and pressure in the autoclave. The obtained extract was collected, centrifuged at 6000 rpm for 10 min, and stored in a cold dark place until dried under a vacuum (40°C).

2.1.3. Ultramorphology of POLENF. In the Electron Microscope Unit at the Central Lab of Mansoura University, transmission electron microscopy (TEM; JEOL-JEM-2100; JEOL Ltd., Tokyo, Japan) was used to evaluate POLENF morphologically at 160 kV. A mixture of 1 mL POLENF sample and double-deionized water was sonicated by an ultrasonic bath for 2 min. By adding one drop of the diluted POLENF to a carbon-coated copper grid, a thin film stretched over the holes was done, the excess materials were removed, and the film was dried at room temperature. Using the image captured by Gatan version 2.11.1404.0, the average diameters of POLENF were estimated in Nano Measurer 1.2.5 in 250 particles for the extract against the scale bar provided with the image capture system.

2.1.4. Phytochemical Analysis of Ethanolic POLE. Phytochemical analysis was carried out using high-performance liquid chromatography-photodiode array detector-tandem mass spectrometry (HPLC-PDA-MS/MS). The LC system was Shimadzu LC-MS 8050 (Shimadzu, Japan) coupled with a triple-quadruple spectrometer with an electrospray ionization (ESI) source. The separation was achieved using a C18 reverse-phase column (Zorbax Eclipse XDB-C18; rapid resolution, 4.6 × 150 mm, 3.5 μm; Agilent, USA). A gradient of water and acetonitrile (ACN; 0.1% formic acid each) was applied from 5% to 60% ACN over 36 min with a flow rate of 1 mL/min. The samples were injected automatically using Autosampler SIL-40C xs. The instrument was controlled by

LC solution software (Shimadzu). MS operated in the negative mode. The collision energy of 35% was used in MS/MS fragmentation. The ions were detected in a full-scan mode and a mass range of 100 to 1500 m/*z*.

2.1.5. Total Flavonoid Content (TFC) and Total Phenolic Content (TPC) in POLE. The FLUOstar Omega (BMG Labtech, Ortenberg, Germany) microplate reader was used for measuring TPC and TFC in POLE and POLENF at 630 and 510 nm (UV spectrophotometer), respectively [48, 49]. TPC and TFC were expressed in terms of mg gallic acid equivalent (GAE)/g extract and mg/g extract, respectively. The experiment was repeated thrice at each concentration.

2.1.6. DPPH Free Radical Scavenging Assay. The free radical scavenging capacity of POLE and POLENF was determined by the DPPH assay, as described previously [50]. The hydrogen atom-donating ability of the plant extractives was determined by the decolonization of the methanol solution of DPPH. DPPH produces a violet/purple color in methanol solution and fades to shades of yellow color in the presence of antioxidants. Absorbance was estimated using the FLUOstar Omega microplate reader at 540 nm. Trolox standard preparation for DPPH is as follows: a stock solution of 100 μM concentration of Trolox was prepared in methanol from which 7 concentrations were prepared including 50, 40, 30, 20, 15, 10, and 5 μM. The measurement was repeated thrice.

2.1.7. Ferric Reducing Antioxidant Power (FRAP) Assay. The FRAP assay was used to determine the power of antioxidants based on the reduction at low pH value of ferric-tripyridyltriazine (TPTZ) to an intense blue color ferric-TPTZ complex with an absorption maximum at wavelength of 593 nm. Trolox standard for FRAP assay was prepared as follows: Trolox stock solution of 2 mM in methanol was prepared, and 8 serial dilutions were prepared in the concentrations of 1500, 1000, 800, 600, 400, 200, 100, and 50 μM. The measurement was repeated thrice. In POLE or POLENF, the ferric reducing antioxidant capacity (FRAC) assay was performed by the FLUOstar Omega microplate reader [51].

2.2. Experiment of Semen Cryopreservation

2.2.1. Animals. In this study, five sexually mature Damascus bucks with a body weight of 60 to 70 kg and age of 2 to 4 years with acceptable semen quality were used for semen collection. Animals were kept under the same management and feeding systems. The ration presented to animals consisted of 1 kg concentrate/buck (14% CP) and 0.750 kg berseem (*Trifolium alexandrinum*) hay/buck. Free access to trace-mineralized salt lick blocks and drinking water was available at all times. This study was approved by the Scientific Research Ethics Committee of Mansoura University in accordance with Animal Research: Reporting of *In Vivo* Experiments guidelines.

2.2.2. Semen Collection. A conventional artificial vagina for goat bucks was used before feeding (7:00–8:00 a.m.) for semen ejaculation. Bucks were used as semen donors weekly for 6 semen collection weeks (30 ejaculates). After ejaculation, individual semen was placed in a water bath at 37°C and immediately transported to the laboratory. Only semen ejaculates with mass motility of ≥70%, viability and membrane integrity ≥ 80%, and sperm concentration ≥ 2.4×10^9 /mL were pooled in each run.

2.2.3. Semen Extender Preparation. The basal extender used for semen dilution in this study was Tris-citric-lecithin, including Tris (3.028 g), citric acid monohydrate (1.675 g), glucose (1.25 g), 1% soybean lecithin (L-phosphatidylcholine, LAB, product no. MC041), 5% glycerol, penicillin (100 μg/mL), and streptomycin (100 μg/mL; Sigma, Chemical Co., St. Louis, MO, USA).

Before the addition of cryoprotectants, the extender was assessed to 280 to 300 mOsmol using an osmometer (Micro-Osmometer, Loser Type 6; Loser Messtechnik, Berlin, Germany) and a pH value of 6.8 to 6.9 using a pH meter (pH/mV Temperature Meter, Jenway 3510; Jenway, Staffordshire, UK). Before semen dilution, the extender was gently shaken and warmed up to 37°C in a water bath.

2.2.4. Experimental Design. In this study, semen was diluted with five extender types as follows: E1 = control extender, E2 = control extender supplemented with POLE at 50 μg/mL extender, E3 = control extender supplemented with POLE at 100 μg/mL extender, E4 = control extender supplemented with POLENF at 50 μg/mL extender, and E5 = control extender supplemented with POLENF at 100 μg/mL extender (see Figure 1). The dilution rate of the semen with different extenders was at 1 : 20 (semen/extender) to reach an initial concentration of 2.4×10^9 sperm/mL in the pooled semen.

2.2.5. Freezing and Thawing. The pooled semen was divided into five tubes, and each tube was extended with each type of extender. After dilution, semen was shaken gently and placed in a water bath (37°C) and equilibrated at a cool temperature (5°C) for 2 h (equilibration period) before loading into straws (0.25 mL). Over 4 cm of vapor of liquid nitrogen (LN) vapor, the straws were cooled for 10 min and placed deeply in LN. Semen was cryopreserved (4 weeks) until thawing at 37°C for 30 s in a water bath for further semen evaluations.

2.2.6. Semen Evaluation. The same professional investigator performed the blind analysis conducted in three replicates. Semen was evaluated for progressive motility, vitality, membrane integrity, and abnormality after equilibration at 5°C for 2 h, thawing at 37°C for 30 s, and incubation at 37°C and 5% CO_2 for 2 h.

Sperm progressive motility was determined by a phase-contrast microscope (DM 500; Leica, Switzerland) in a diluted semen sample (10 μL aliquot) on a warm slide (37°C). In five microscopic fields, the sperm count was determined for sperm cells showing forward movement in a long semiarc pattern.

Sperm vitality and abnormality were estimated according to Moskovtsev and Librach [52] in a mixture of diluted semen samples with dual stain (5% eosin and 10% nigrosine) smeared on a glass slide. A light microscope (Leica DM 500;

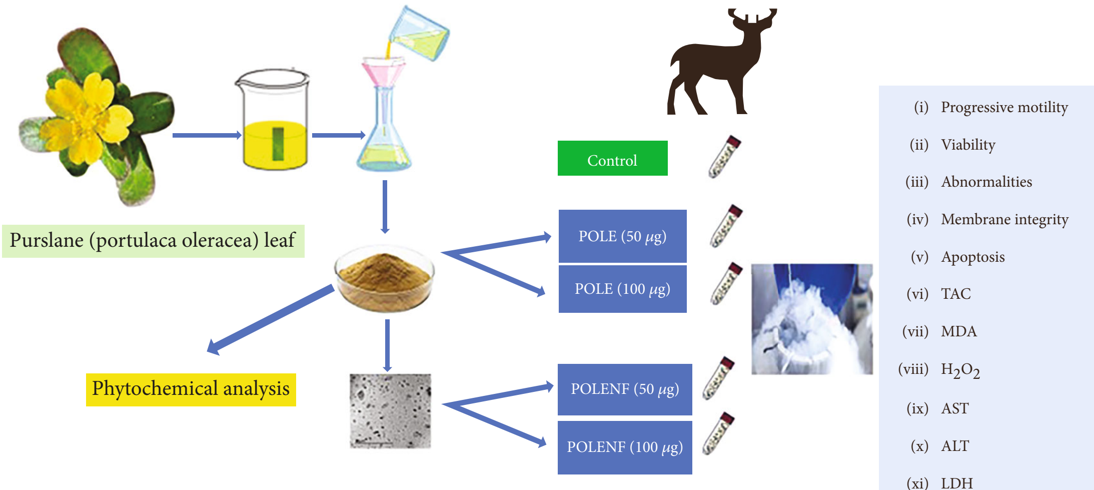

Figure 1: Experimental design.

Leica Mikrosysteme Vertrieb GmbH, Wetzlar, Germany) examined five fields at ×400 magnification. Stained (dead), unstained (live), and the total number of sperm cells in five microscopic fields were counted as sperm vitality percentages. On the same slides, the sperm morphological abnormalities concerning head, middle piece, main piece, and cytoplasmic droplets were counted to calculate the sperm abnormality percentage [53].

Sperm membrane integrity was determined by the hypoosmotic swelling test according to Neild et al. [54]. A solution of 75 mOsmol/L, including fructose (6.75 g/L) and sodium citrate (3.67 g/L), was used as a hypoosmotic solution. About 100 μL of this solution was added to 10 μL diluted semen and incubated at 37°C for 30 min. In five microscopy fields, sperm cells showing swollen or/and curling were determined using phase-contrast microscopy (×400).

2.2.7. Antioxidant Capacity and Enzyme Activities. In post-thawed semen, the antioxidant capacity parameters, including total antioxidant capacity (TAC), malondialdehyde (MDA), and hydrogen peroxide (H_2O_2) levels, were determined according to Aebi, Koracevic et al., and Ohkawa et al. [55–57], respectively. The activity of lactic dehydrogenase (LDH) was assayed after the method of Bais and Philcox [58], whereas aspartate transaminase (AST) and alanine transaminase (ALT) activities were measured as described by Reitman [59]. All assays were achieved using a spectrophotometer (Spectro UV-Vis Auto, UV-2602; Labomed, Los Angeles, CA, USA) and commercial kits (Biodiagnostic, Giza, Egypt) according to the manufacturer's instructions.

2.2.8. Annexin V/Propidium Iodide (PI) Assay (Sperm Apoptosis and Necrosis). Three samples of semen extended with each type of supplementation were assessed for processing Annexin V staining according to Chaveiro et al. [60]. Briefly, sperm cells (1 mL) were suspended in a 2 mL binding buffer and well mixed. About 100 μL of this suspension was mixed in Annexin V (5 μL; fluorescein isothiocyanate label) and PI (5 μL; phycoerythrin label) and incubated in a dark place at room temperature for at least 15 min. The incubated sperm cells were suspended in a binding buffer (200 μL). For acquisition and analyzing the data, flow cytometry analysis using an Accuri C6 Cytometer (BD Biosciences, San Jose, CA, USA) and software (Becton Dickinson) was performed [61].

The percentages of negative or positive Annexin V (A-/A+) and PI (PI-/PI+) and the double-positive cells were determined. According to Peña et al. [62], spermatozoa were classified into four categories: (1) viable cells: without fluorescence signal and membrane dysfunction (A-/PI-); (2) sperm cells with apoptosis: viable cells labeled with Annexin V but without PI (A+/PI-); (3) sperm cells with early apoptosis: dead cells labeled with Annexin V and PI and with damaged permeable membranes (A+/PI+); and (4) sperm cells with necrosis: dead cells labeled with PI without Annexin V and with complete membrane loss (A-/PI+). The number of cells of interest was determined based on the anterior and lateral scattering characteristics.

2.2.9. Statistical Analysis. All numerical data have been checked for homogeneity of variance using Levene's test and normality of distribution using the Shapiro-Wilk test. Data were statistically analyzed as one-way analysis of variance using SAS (2008) to study the effect of treatment (supplementation 1–5). The significant differences were separated by Tukey's test at $P < 0.05$.

3. Results

3.1. Characteristics of the Extracts

3.1.1. Phytochemical Analysis. Altogether, 30 compounds were annotated at the ethanolic extract of purslane. They included a series of phenolic acids and flavonoids and their glucosides. The compounds were annotated according to their molecular weights, retention times, and fragmentation pattern (see Figure 2) and are shown in Table 1.

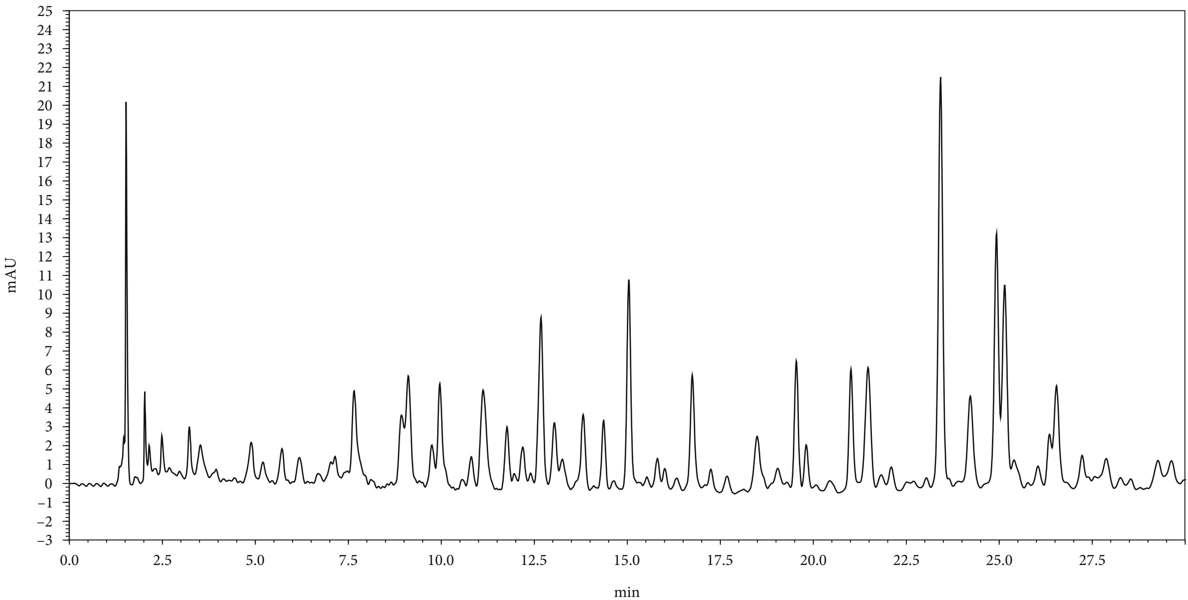

Figure 2: LC-MS profile of purslane.

3.1.2. TFC and TPC of POLE and POLENF. TFC and TPC in POLE and POLENF are presented in Table 2. TFC was higher in POLE than in POLENF; however, there were no differences between both extracts regarding TPC.

3.1.3. Antioxidant Activities of POLE and POLENF. In DPPH and FRAP assays, POLE and POLENF showed higher antioxidant activity (Table 3). Both extracts showed strong free radical scavenging capacity (DPPH) compared to standard Trolox, which was higher in POLENF than POLE. Meanwhile, the antioxidant activity by the FRAP assay showed no differences between both extracts.

3.1.4. Ultramorphological Characteristics of POLENF. Ultramorphological examination of POLENF by TEM revealed a mean particle size of 30 nm and a Z-potential value was 6.25 mV (Figure 3).

3.2. Sperm Parameters in Goat Semen

3.2.1. After Equilibration (5°C for 2 h). After equilibration, semen extended by POLE (100 μg) or POLENF (50 or 100 μg) significantly increased the percentages of progressive motility and vitality of spermatozoa compared to the free extender. Meanwhile, the percentages of sperm membrane integrity and abnormality were not affected significantly by POLE or POLENF supplementation. Extender supplemented with POLENF (100 μg/mL) resulted in the best progressive motility and vitality of spermatozoa in postequilibrated semen (Table 4).

3.2.2. After Thawing. In postthawed semen, the percentages of sperm progressive motility, vitality, and membrane integrity significantly improved by all POLE and POLENF supplements compared to the free extender. However, the sperm abnormality percentage was not affected significantly by POLE or POLENF supplementation. The highest percentages of sperm motility, vitality, and membrane integrity were obtained after freezing/thawing in semen extended with POLENF (100 μg/mL) extender (Table 5).

3.2.3. After Incubation (37°C and 5% CO_2 for 2 h) of Postthawed Semen. After semen incubation for 2 h at 37°C and 5% CO_2, the percentages of sperm motility, vitality, and plasma membrane integrity showed significant improvements by POLE (100 μg) and POLENF (50 or 100 μg) extenders by comparing the control and POLE (50 μg) extenders. The sperm abnormality percentage was not affected by treatments. The highest percentages of progressive motility, vitality, and plasma membrane integrity of sperm cells were obtained by a higher concentration of POLENF (Table 6).

3.2.4. Oxidative Biomarkers in Postthawed Semen. The TAC level in postthawed semen was higher ($P < 0.05$) in POLE (100 μg/mL) and both POLENF concentrations than in the control extender. However, MDA and H_2O_2 levels were significantly reduced by all POLE and POLENF supplements compared to the control extender. Supplementation of Tris-extender with POLENF (100 μg) showed the highest improvement of antioxidant status in semen after thawing (Table 7).

3.2.5. Effect of Treatment on Enzyme Activity in Postthawed Semen. The effect of treatment was significant only on AST activity in postthawed semen, but no significant differences were recorded between each treated extender and the control one. However, ALT and LDH activities were not affected significantly by POLE or POLENF supplements (Table 8).

3.2.6. Apoptotic and Necrotic Spermatozoa in Postthawed Semen (Annexin V/PI Assay). The viable sperm percentage

Table 1: Chemical constituents of purslane.

Rt	[M-H]$^-$	MS/MS	Assigned compounds
1.45	191	111	Quinic acid
1.57	133		Malic acid
3.61	315	153	Protocatechuic acid glucoside
3.68	331	125, 169	Galloyl glucose
5.01	477	135, 153, 315	Protocatechuic acid caffeoyl-glucoside
7.52	341	135, 179	Caffeoyl glucose
9.03	137	119	*p*-Hydroxybenzoic acid
10.47	353	191	Chlorogenic acid
10.57	341	135, 179	Caffeic acid glucoside
11.31	325	163	Coumaric acid glucoside
13.54	305	197	Gallocatechin
13.89	355	193	Feruloyl glucose
14.13	341	135, 179	Caffeic acid glucoside
14.17	385	223	Sinapic acid glucoside
15.88	431	315	Coumaric acid galloyl-malate
15.94	421	313	Benzyl-*O*-galloyl glucose
16.24	353	191	Neochlorogenic acid
16.36	437	313	Hydroxybenzyl-*O*-galloyl glucose
16.84	467	313	Protocatechoyl-*O*-galloyl glucose
17.68	325	179	Caffeic acid glucoside
18.40	337	163	*p*-Coumaroylquinic acid
20.26	197	163	Syringic acid
22.31	563	357	Apigenin 6-C-glucosyl-8-C-xyloside
22.79	289	245	Catechin
23.99	399	197	Syringic acid derivative
24.30	449	269	Apigenin derivative
28.50	447	301	Quercetin rhamnoside
28.62	447	285	Kaempferol glucoside
31.14	462	285	Kaempferol glucuronide
31.80	417	271	Naringenin rhamnoside

Rt: retention time; M-H: pseudomolecular ion in the negative ion mode; MS/MS: mass fragmentation pattern.

Table 2: TFC and TPC in POLE and POLENF (mean ± standard deviation (SD), $n = 3$).

Sample	TFC (mg/g extract)	TPC (mg GAE/g extract)
POLE	28.46 ± 1.15	38.35 ± 1.81
POLENF	21.72 ± 1.42	39.84 ± 2.83

TFC: total flavonoid content; TPC: total phenolic content; POLE: *Portulaca oleracea* leaf extract; POLENF: *Portulaca oleracea* leaf extract nanoformulation.

Table 3: Antioxidant activities of POLE and POLENF as measured by DPPH and FRAP assays (mean ± SD, $n = 3$).

Sample	DPPH (EC_{50} μg/mL)	FRAP (μM TE/mg extract)
POLE	244.4 ± 16.06	137.52 ± 7.11
POLENF	332.4 ± 16.37	147.48 ± 11.95
Trolox (μM)	24.42 ± 0.87	—

DPPH: 2,2-diphenyl-1-picryl-hydrazyl-hydrate; FRAP: ferric reducing antioxidant power assay; TE: Trolox equivalence; POLE: *Portulaca oleracea* leaf extract; POLENF: *Portulaca oleracea* leaf extract nanoformulation.

significantly increased, whereas the early apoptotic sperm percentages significantly decreased by all POLE or POLENF supplements compared to control. However, no significant differences were found in necrotic sperm percentage between treatments. Semen extended with 100 μg POLENF showed the highest viable sperm percentage and the lowest sperm percentage with early apoptosis and apoptosis after cryopreservation (Table 9).

4. Discussion

Several reports have indicated a positive correlation between TPC and TFC in plants with an antioxidative capacity [6, 7, 63], especially in POL [64, 65]. Mature plants of PO have higher TPC and antioxidant activities [5, 38, 39]. Also, the contents of polyunsaturated fatty

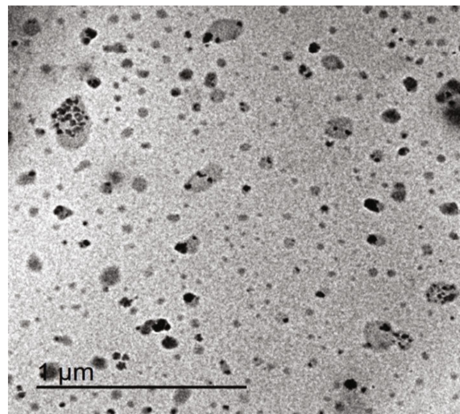

Figure 3: Ultramorphology of POLENF by TEM.

acids (PUFAs; α-linolenic acid and linoleic acid) are highly and positively correlated with antioxidant activity in POL [25, 27, 28, 63] because PUFAs, especially ω-3, play a role in inhibiting superoxide [66]. Purslane also contains antioxidant vitamins, such as α-tocopherol, ascorbic acid, β-carotene, and glutathione, as well as ω-3 fatty acids [67]. Moreover, polysaccharides in PO could act as scavengers of superoxide anion, DPPH, nitric oxide, and hydroxyl radicals in a dose-dependent manner [34]. The DPPH free radical scavenging assay is an accepted mechanism for screening the antioxidant activity of plant extracts [34]. The methanol extract possessed the highest antioxidant activity among purslane extracts, which is associated with TPC [68]. Methanol gave the highest extraction efficiency in POL, and this was mainly due to the ability of the solvent to inhibit the action of polyphenol oxidase that caused the phenolic oxidation [69]. The fresh hydroalcoholic purslane extract exhibited a promising radical scavenging activity [12].

In this study, both extracts of POL have considerable amounts of TFC and TPC and have shown strong free radical scavenging capacities in terms of DPPH and FRAP assays compared to standard Trolox. Previous findings and those proven in this study indicated the potential antioxidant capacity of purslane leaves. In this respect, Rahman et al. [70] reported that the free radical scavenging capacity (DPPH) is positively correlated ($P < 0.001$) with TPC to inhibit lipid peroxidation. Scavenging DPPH radicals by the hydrogen-donating ability of polyphenol contents and tocopherols were reported [71, 72]. This study indicated a higher free radical scavenging ability of both POLEs, but the stronger activity was recorded for POLENF than POLE. The reducing capacity on ferrous ion was higher for POLENF than POLE. TPC was nearly similar in both extracts, but TFC was higher in POLENF than in POLE.

This study evaluated the effect of adding two concentrations of POLE (native form) or POLENF on increasing the antioxidant status of sperm medium to improve the sperm quality in cryopreserved goat semen. Results indicated that only sperm motility and vitality percentages increased in equilibrated semen by Tris-extender supplemented with a higher concentration of POLE (100 μg) and both POLENF concentrations. Sperm motility is the most important sperm variable for normal fertilization. Sperm motility is an indirect indicator of the metabolism and viability of sperm cells [73], and sperm motility is a good predictor of fertilization in *in vitro* embryo production [74]. These results indicated more impact of POLE (100 μg) or both POLENF concentrations on maintaining sperm motility, vitality, and membrane integrity without affecting sperm abnormality in postthawed semen after incubation for 2 h at 37°C. The improvements in sperm characteristics in postthawed semen after incubation were associated with increased POLE (100 μg) or both POLENF concentrations (50 and 100 μg) due to the increased antioxidant status of sperm medium during cryopreservation or after semen incubation at 37°C for 2 h in terms of elevating the TAC level and decreasing the H_2O_2 level. The mentioned supplements also increased the viable sperm percentage but reduced early apoptotic and apoptotic sperm percentages. Enhancement of sperm medium by a higher concentration of POLE or both POLENF concentrations may mainly be attributed to ROS formation that could be neutralized by phenolic compounds (e.g., flavonoids) in POLE; the enhancement in their content leads to increasing antioxidant capacities [75]. An antioxidant that reduces oxidative stress and improves sperm motility could be useful in managing male infertility [76]. Antioxidants, in general, are scavengers that restrain ROS formation. The essential normal function of sperm cells is the balancing benefits and risks of ROS and antioxidants. Although seminal plasma is considered fluid with powerful antioxidants, adding antioxidants to semen extenders was reported to enhance techniques in semen cryopreservation [77]. PO has displayed free radical scavenging activity [38, 69, 78] and the ability to inhibit lipid oxidation [79]. Purslane can be used in improving SOD levels and reducing MDA levels in the brains of mice treated with D-galactosamine [80]. Although all POLE supplements did not affect AST, ALT, and LDH activities in cryopreserved semen, purslane reduced liver enzyme activity (ALT, AST, γ-glutamyl transferase, and alkaline phosphatase), indicating its role in the protection against liver damage [10].

In this study, viable sperm percentage increased, whereas early apoptosis and apoptosis percentages decreased without significantly affecting necrotic sperm cells by all extender supplements. These findings may suggest that POLE reduced the apoptosis that is a common form of sperm death in eukaryotes and appears in fragmented DNA, activated caspase, mitochondrial membrane dysfunction, and increased permeability of sperm membrane [81, 82]. Programmed cellular death (apoptosis) affects single cells but does not cause inflammatory effects on adjacent tissues, but necrosis is another form of cell death resulting from injury and affects numerous cells, causing cell swelling and membrane rupture [83]. Many reports indicated that sperm cells with fragmented DNA have a fertilizing ability [84] but with abnormal embryos, late embryonic development, and lower pregnancy rates [85]. Several antioxidants positively impact apoptosis in sperm cells of different species [86–89].

Table 4: Sperm variable percentage as affected by supplementing Tris-extender with POLE or POLENF in goat semen after equilibration at 5°C for 2 h (mean ± SE, $n = 6$).

Extender	Sperm characteristics (%)			
	Progressive motility	Vitality	Membrane integrity	Abnormality
Control	75.0 ± 1.83^{b}	77.3 ± 1.78^{b}	80.2 ± 2.64	6.7 ± 0.84
POLE (50 μg)	79.2 ± 1.54^{ab}	81.5 ± 1.86^{ab}	83.0 ± 2.22	6.2 ± 0.95
POLE (100 μg)	82.5 ± 1.12^{a}	83.5 ± 1.06^{a}	84.8 ± 0.70	5.7 ± 0.88
POLENF (50 μg)	83.3 ± 1.05^{a}	84.2 ± 1.19^{a}	85.0 ± 1.26	6.8 ± 0.60
POLENF (100 μg)	84.2 ± 1.54^{a}	86.3 ± 1.26^{a}	86.7 ± 1.05	5.0 ± 0.58

[a,b]Means denoted with different superscripts in each column are significantly different at $P < 0.05$. POLE: Portulaca oleracea leaf extract; POLENF: Portulaca oleracea leaf extract nanoformulation.

Table 5: Sperm variable percentage as affected by supplementing Tris-extender with POLE or POLENF in thawed goat semen (mean ± SE, $n = 6$).

Extender	Sperm characteristics (%)			
	Progressive motility	Vitality	Membrane integrity	Abnormality
Control	26.7 ± 1.05^{b}	28.0 ± 1.15^{b}	26.3 ± 0.92^{b}	9.2 ± 0.75
POLE (50 μg)	32.5 ± 1.12^{a}	34.0 ± 1.46^{a}	34.3 ± 1.86^{a}	9.2 ± 1.25
POLE (100 μg)	33.3 ± 1.05^{a}	35.0 ± 1.15^{a}	35.5 ± 1.45^{a}	8.8 ± 1.01
POLENF (50 μg)	34.2 ± 2.01^{a}	36.3 ± 1.82^{a}	34.2 ± 1.72^{a}	9.3 ± 0.76
POLENF (100 μg)	36.7 ± 1.05^{a}	38.5 ± 1.26^{a}	37.2 ± 1.62^{a}	8.2 ± 0.48

[a,b]Means denoted with different superscripts in each column are significantly different at $P < 0.05$. POLE: *Portulaca oleracea* leaf extract; POLENF: *Portulaca oleracea* leaf extract nanoformulation.

Table 6: Sperm variable percentage as affected by supplementing Tris-extender with POLE or POLENF in postthawed goat semen incubated at 37°C and 5% CO_2 for 2 h (mean ± SE, $n = 6$).

Extender	Sperm characteristics (%)			
	Progressive motility	Vitality	Membrane integrity	Abnormality
Control	22.5 ± 1.12^{b}	23.5 ± 0.72^{b}	23.3 ± 0.80^{b}	11.3 ± 0.56
POLE (50 μg)	27.5 ± 1.12^{ab}	28.3 ± 1.31^{ab}	27.5 ± 1.28^{ab}	10.7 ± 1.20
POLE (100 μg)	29.2 ± 1.54^{a}	31.2 ± 1.78^{a}	30.2 ± 1.42^{a}	10.3 ± 0.99
POLENF (50 μg)	30.8 ± 1.54^{a}	32.3 ± 1.78^{a}	30.5 ± 1.67^{a}	11.3 ± 0.71
POLENF (100 μg)	32.5 ± 1.12^{a}	33.3 ± 0.88^{a}	31.3 ± 0.99^{a}	10.2 ± 0.48

[a,b]Means denoted with different superscripts in each column are significantly different at $P < 0.05$. POLE: Portulaca oleracea leaf extract; POLENF: Portulaca oleracea leaf extract nanoformulation.

Table 7: Antioxidant biomarkers in goat semen after thawing as affected by supplementing Tris-extender with POLE or POLENF (mean ± SE, $n = 6$).

Extender	Seminal plasma antioxidant capacity		
	TAC (mM/L)	H_2O_2 (mM/L)	MDA (nmol/mL)
Control	0.178 ± 0.013^{b}	0.106 ± 0.009^{a}	2.57 ± 0.143^{a}
POLE (50 μg)	0.215 ± 0.011^{ab}	0.055 ± 0.004^{b}	0.67 ± 0.049^{b}
POLE (100 μg)	0.230 ± 0.020^{a}	0.048 ± 0.007^{b}	0.66 ± 0.052^{b}
POLENF (50 μg)	0.222 ± 0.005^{a}	0.046 ± 0.006^{b}	0.64 ± 0.056^{b}
POLENF (100 μg)	0.232 ± 0.005^{a}	0.044 ± 0.004^{b}	0.67 ± 0.042^{b}

[a,b]Means denoted with different superscripts in each column are significantly different at $P < 0.05$. POLE: *Portulaca oleracea* leaf extract; POLENF: *Portulaca oleracea* leaf extract nanoformulation.

Table 8: Enzyme activity of AST, ALT, and LDH in goat semen after thawing as affected by supplementing Tris-extender with POLE or POLENF (mean ± SE, $n = 6$).

Extender	Enzyme activity AST (U/mL)	ALT (U/mL)	LDH (U/L)
Control	39.2 ± 1.01^{ab}	14.5 ± 0.50	177.6 ± 4.61
POLE (50 μg)	36.8 ± 1.08^{b}	12.0 ± 1.44	160.3 ± 4.64
POLE (100 μg)	39.8 ± 0.91^{ab}	12.5 ± 1.06	162.8 ± 3.13
POLENF (50 μg)	41.8 ± 1.14^{a}	12.0 ± 1.39	165.5 ± 4.63
POLENF (100 μg)	36.0 ± 1.15^{b}	13.0 ± 0.58	163.1 ± 6.38

^{a,b}Means denoted with different superscripts in each column are significantly different at $P < 0.05$. POLE: *Portulaca oleracea* leaf extract; POLENF: *Portulaca oleracea* leaf extract nanoformulation.

Table 9: Percentages of viable, early apoptotic, apoptotic, and necrotic spermatozoa in goat semen after thawing as affected by supplementing Tris-extender with POLE or POLENF (mean ± SE, $n = 3$).

Extender	Sperm variable (%) Viable (A−/PI−)	Early apoptotic (A+/PI−)	Apoptotic (A+/PI+)	Necrotic (A−/PI+)
Control	28.8 ± 0.06^{e}	0.5 ± 0.06^{a}	54.0 ± 1.30^{a}	16.8 ± 1.30^{abc}
POLE (50 μg)	31.2 ± 0.00^{d}	0.2 ± 0.03^{bc}	50.7 ± 2.14^{b}	18.0 ± 2.17^{ab}
POLE (100 μg)	39.8 ± 0.49^{c}	0.1 ± 0.03^{c}	40.7 ± 0.00^{c}	19.5 ± 0.52^{a}
POLENF (50 μg)	53.6 ± 0.43^{b}	0.3 ± 0.06^{b}	34.5 ± 0.69^{d}	11.7 ± 0.20^{c}
POLENF (100 μg)	69.2 ± 0.78^{a}	0.0 ± 0.00^{c}	17.0 ± 0.17^{e}	13.9 ± 0.61^{bc}

$^{a-e}$Means denoted with different superscripts in each column are significantly different at $P < 0.05$. POLE: *Portulaca oleracea* leaf extract; POLENF: *Portulaca oleracea* leaf extract nanoformulation.

Interestingly, all POLE types showed beneficial effects on cryopreserved semen after equilibration, thawing, and incubation, but the best results were recorded for POLENF (100 μg). Such results are associated with decreasing the mean particle size to 30 nm and a Z-potential value of 6.25 mV as estimated by TEM. Also, a stronger DPPH free radical scavenging capacity (332.4 ± 16.37 vs. 244.4 ± 16.06 $EC_{50}\mu$g/mL) and increased total flavonoids (28.46 ± 1.15 vs. 21.72 ± 1.42 mg/g extract) were proven for POLENF than POLE. Flavonoids with a chromanol ring system have a stronger antioxidant activity that lacks the hydroxyl group [90]. The antioxidant activity of the compounds was determined by the DPPH radical scavenging assay, which was improved to evaluate the four lignans isolated from POLE [91]. Flavonoids have an antioxidative action and show protective effects against aluminum chloride, which causes testicular dysfunction, poor semen quality, and reduced testosterone level [92]. In contrast, phenolic compounds were nearly similar in both extracts (POLE and POLENF), whereas the individual phenolic contents measured in a plant crude extract can be considered quite low [78, 93] to biologically explain a sufficient antioxidant potential.

5. Conclusions

Ethanolic POLE is an essential source of natural antioxidants and can be used effectively to scavenge free radicals and/or inhibit ROS. Hence, POLE in a native form or nanoformulation at 50 or 100 μg/mL is considered a natural antioxidant in Tris-extender of goat cryopreserved semen for improving sperm freezing ability and protecting spermatozoa from cryodamage by increasing the semen antioxidant capacity. Ethanolic POLENF at 100 μg/mL gave valuable results as an effective free radical scavenger during the cryopreservation of goat semen.

Data Availability

The data that support the findings of this study are available from the corresponding author, W.A.K., upon reasonable request.

Conflicts of Interest

The authors declare no conflict of interest.

Authors' Contributions

Conceptualization was participated by W.A.K., A.A.I., R.A.S., A-K.E.A-K., A.A.S., and M.A.E. Methodology was performed by W.A.K., A.A.I., and A.I.Y. Software was contributed by W.A.K., A.A.I., and A-K.E.A-K. Formal analysis was carried out by W.A.K., R.A.S., and A.A.S. Investigation was performed by W.A.K., A.A.I., A-K.E.A-K., and M.A.E. Data curation was assisted by W.A.K., A.A.I., A-K.E.A-K., and M.A.E. Writing of original draft preparation was contributed by W.A.K., A.A.I., and A.I.Y. Writing, reviewing, and editing were performed by W.A.K., R.A.S., A.A.S., A-K.E.A-K., and M.A.E. Visualization was carried out by W.A.K. and A.A.I. Supervision was participated by W.A.K.,

A-K.E.A-K., and M.A.E. All authors have read and agreed to the published version of the manuscript. Wael A. Khalil and Aya A. Ismail contributed equally to this work and share first authorship.

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References

[1] D. El-Badry, A. Azab, M. Karima Gh, and T. Scholkamy, “The effect of extender type on freezability of one-humped camel spermatozoa with special reference to their fine structure,” in *Paper Presented at the Proceedings of the 25th Annual Conf*, Taba, Egypt, 2016.

[2] Z. Nur, B. Zik, B. Ustuner, H. Sagirkaya, and C. Ozguden, “Effects of different cryoprotective agents on ram sperm morphology and DNAintegrity,” *Theriogenology*, vol. 73, no. 9, pp. 1267–1275, 2010.

[3] M. Sardoy, M. Carretero, and D. Neild, “Evaluation of stallion sperm DNA alterations during cryopreservation using toluidine blue,” *Animal Reproduction Science*, vol. 107, no. 3-4, pp. 349-350, 2008.

[4] M. Percival, “Antioxidants clinical nutrition insights,” *Advances in Nutrition*, vol. 31, 1998.

[5] N. Erkan, “Antioxidant activity and phenolic compounds of fractions from *Portulaca oleracea* L,” *Food Chemistry*, vol. 133, no. 3, pp. 775–781, 2012.

[6] V. Katalinic, M. Milos, T. Kulisic, and M. Jukic, “Screening of 70 medicinal plant extracts for antioxidant capacity and total phenols,” *Food Chemistry*, vol. 94, no. 4, pp. 550–557, 2006.

[7] A. Lugasi and J. Hóvári, “Antioxidant properties of commercial alcoholic and nonalcoholic beverages,” *Food/Nahrung*, vol. 47, no. 2, pp. 79–86, 2003.

[8] M. Heydari, M. H. Hashempur, B. Daneshfard, and S. H. Mosavat, “Bioactive foods as dietary intervention for diabetes from the perspective of Persian medicine,” in *Bioactive Food as Dietary Interventions for Diabetes*, pp. 49–68, Elsevier, 2019.

[9] B. Wu, L. Yu, X. Wu, and J. Chen, “New $CuCl_2$-induced glucoside esters and other constituents from Portucala oleracea,” *Carbohydrate Research*, vol. 351, pp. 68–73, 2012.

[10] M. A. Dkhil, A. E. A. Moniem, S. Al-Quraishy, and R. A. Saleh, “Antioxidant effect of purslane (*Portulaca oleracea*) and its mechanism of action,” *Journal of Medicinal Plant Research*, vol. 5, no. 9, pp. 1589–1593, 2011.

[11] M. G. Melilli, A. Pagliaro, R. Bognanni, S. Scandurra, and V. Di Stefano, “Antioxidant activity and fatty acids quantification in Sicilian purslane germplasm,” *Natural Product Research*, vol. 34, no. 1, pp. 26–33, 2020.

[12] V. Sicari, M. R. Loizzo, R. Tundis, A. Mincione, and T. M. Pellicano, “*Portulaca oleracea* L.(purslane) extracts display antioxidant and hypoglycaemic effects,” *Journal of Applied Botany and Food Quality*, vol. 91, no. 1, pp. 39–46, 2018.

[13] Y. Meng, Z. Ying, Z. Xiang et al., “The anti-inflammation and pharmacokinetics of a novel alkaloid from *Portulaca oleracea* L,” *The Journal of Pharmacy and Pharmacology*, vol. 68, no. 3, pp. 397–405, 2016.

[14] B. C. Chan, X. Q. Han, S. L. Lui et al., “Combating against methicillin-resistant Staphylococcus aureus–two fatty acids from purslane (*Portulaca oleracea* L.) exhibit synergistic effects with erythromycin,” *The Journal of Pharmacy and Pharmacology*, vol. 67, no. 1, pp. 107–116, 2015.

[15] I. A. Okafor, M. B. Ayalokunrin, and L. A. Orachu, “A review on *Portulaca oleracea* (purslane) plant – its nature and biomedical benefits,” *International Journal of Biomedical Research*, vol. 5, no. 2, pp. 75–80, 2014.

[16] M. Jiang, W. Zhang, X. Yang et al., “An isoindole alkaloid from *Portulaca oleracea* L,” *Natural Product Research*, vol. 32, no. 20, pp. 2431–2436, 2018.

[17] C. Li, Z. Ying, M. Gao et al., “Two new similar alkaloids from *Portulaca oleracea* L,” *Natural Product Research*, vol. 31, no. 15, pp. 1792–1798, 2017.

[18] F. Xiu, X. Li, W. Zhang, F. He, X. Ying, and D. Stien, “A new alkaloid from *Portulaca oleracea* L. and its antiacetylcholinesterase activity,” *Natural Product Research*, vol. 33, no. 18, pp. 2583–2590, 2019.

[19] L. Xu, Z. Ying, W. Wei et al., “A novel alkaloid from *Portulaca oleracea* L,” *Natural Product Research*, vol. 31, no. 8, pp. 902–908, 2017.

[20] C. Zhao, Z. Ying, X. Tao, M. Jiang, X. Ying, and G. Yang, “A new lactam alkaloid from *Portulaca oleracea* L. and its cytotoxity,” *Natural Product Research*, vol. 32, no. 13, pp. 1548–1553, 2018.

[21] A. Hassan, “Chemical and remedial effects of purslane (*Portulaca oleracea*) plant,” *Life Science Journal*, vol. 11, pp. 31–42, 2014.

[22] X. Yang, Z. Ying, H. Liu, X. Ying, and G. Yang, “A new homoisoflavone from *Portulaca oleracea* L. and its antioxidant activity,” *Natural Product Research*, vol. 33, no. 24, pp. 3500–3506, 2019.

[23] J. Z. Acedo, C. T. Reyes, and E. B. Rodriguez, “Chemical composition of purslane (*Portulaca oleracea* L.) and anti-inflammatory activity of its lipid components,” *Philippine Journal of Crop Science*, vol. 37, no. 2, pp. 13–19, 2012.

[24] N. Awad, “Lipid content and antimicrobial activity of phenolic constituents of cultivated *Portulaca oleracea* L,” *Bulletin of Faculty of Pharmacy, Cairo University*, vol. 32, no. 1, pp. 137–142, 1994.

[25] L. Liu, P. Howe, Y.-F. Zhou, Z.-Q. Xu, C. Hocart, and R. Zhang, “Fatty acids and β-carotene in Australian purslane (*Portulaca oleracea*) varieties,” *Journal of Chromatography A*, vol. 893, no. 1, pp. 207–213, 2000.

[26] S. A. Petropoulos, A. Karkanis, Â. Fernandes et al., “Chemical composition and yield of six genotypes of common purslane (*Portulaca oleracea* L.): an alternative source of omega-3 fatty acids,” *Plant Foods for Human Nutrition*, vol. 70, no. 4, pp. 420–426, 2015.

[27] O. Azuka, M. Ayalokunrin, and L. Orachu, “Portulaca oleracea (purslane) plant–its nature and biomedical benefits,” *Internasional Jurnal of Boimedical Research, Nigeria*, vol. 46, 2014.

[28] S. Eljach Mosquera, *Purslane (Portulaca oleracea L.) an Excellent Source of Omega-3 and Omega-6 Fatty Acids with Abatement of Risk Factors, [M.S. thesis]*, McGill University, 2014.

[29] A. S. Devi, U. D. Meetei, and R. B. Devi, “Evaluation of *Portulaca oleracea* Linn. for hypoglycaemic effects,” *International Journal of Pharmaceutical Sciences and Research*, vol. 5, no. 5, p. 1908, 2014.

[30] Y.-X. Zhou, H.-L. Xin, K. Rahman, S.-J. Wang, C. Peng, and H. Zhang, “*Portulaca oleracea* L.: a review of phytochemistry

and pharmacological effects," *BioMed Research International*, vol. 2015, Article ID 925631, 11 pages, 2015.

[31] C. Choudhary, A. Meruva, N. Ranjith, and K. Elumalai, "A review on phytochemical and pharmacological profile of Portulaca oleracea Linn. (purslane)," *International Journal of Research in Ayurveda and Pharmacy*, vol. 4, no. 1, pp. 34–37, 2013.

[32] I.-Y. Kim, M.-H. Lee, S.-B. Shim, and Y.-J. Chun, "Skin lightening and wrinkle improving efficacy of organic Portulaca oleracea extract in skin care cosmetic," *International Journal of Biological Sciences*, vol. 5, no. 5, pp. 75–84, 2013.

[33] S. F. Arruda, E. M. Siqueira, and E. M. Souza, "Malanga (*Xanthosoma sagittifolium*) and purslane (*Portulaca oleracea*) leaves reduce oxidative stress in vitamin A-deficient rats," *Annals of Nutrition & Metabolism*, vol. 48, no. 4, pp. 288–295, 2004.

[34] C. YouGuo, S. ZongJi, and C. XiaoPing, "Evaluation of free radicals scavenging and immunity-modulatory activities of purslane polysaccharides," *International Journal of Biological Macromolecules*, vol. 45, no. 5, pp. 448–452, 2009.

[35] K. Oyedeji and A. Bolarinwa, "Effects of crude extracts of Portulaca oleracea on male reproductive functions in albino rats," *Journal of Pharmacy Biological Sciences*, vol. 4, no. 6, pp. 71–79, 2013.

[36] K. Lahbib, S. Dabbou, S. E. Bok, G. Pandino, S. Lombardo, and M. E. Gazzah, "Variation of biochemical and antioxidant activity with respect to the part of Capsicum annuum fruit from Tunisian autochthonous cultivars," *Industrial Crops and Products*, vol. 104, pp. 164–170, 2017.

[37] G. Pandino, M. Meneghini, R. Tavazza, S. Lombardo, G. Mauromicale, and O. Culture, "Phytochemicals accumulation and antioxidant activity in callus and suspension cultures of *Cynara scolymus* L," *Plant Cell, Tissue and Organ Culture*, vol. 128, no. 1, pp. 223–230, 2017.

[38] I. Oliveira, P. Valentão, R. Lopes, P. B. Andrade, A. Bento, and J. A. Pereira, "Phytochemical characterization and radical scavenging activity of Portulaca oleraceae L. leaves and stems," *Microchemical Journal*, vol. 92, no. 2, pp. 129–134, 2009.

[39] M. Uddin, A. S. Juraimi, M. Ali, and M. R. Ismail, "Evaluation of antioxidant properties and mineral composition of purslane (*Portulaca oleracea* L.) at different growth stages," *International Journal of Molecular Sciences*, vol. 13, no. 8, pp. 10257–10267, 2012.

[40] S. Dabbou, K. Lahbib, G. Pandino, S. Dabbou, and S. Lombardo, "Evaluation of pigments, phenolic and volatile compounds, and antioxidant activity of a spontaneous population of *Portulaca oleracea* L. grown in Tunisia," *Agriculture*, vol. 10, no. 8, p. 353, 2020.

[41] İ. Gulcin, "Antioxidants and antioxidant methods: an updated overview," *Archives of Toxicology*, vol. 94, no. 3, pp. 651–715, 2020.

[42] I. M. Saadeldin, W. A. Khalil, M. G. Alharbi, and S. H. Lee, "The current trends in using nanoparticles, liposomes, and exosomes for semen cryopreservation," *Animals*, vol. 10, no. 12, p. 2281, 2020.

[43] S. S. Suri, H. Fenniri, and B. Singh, "Nanotechnology-based drug delivery systems," *Journal of Occupational Medicine Toxicology*, vol. 2, no. 1, pp. 1–6, 2007.

[44] S. A. Abdelnour, M. A. E. Hassan, A. K. Mohammed et al., "The effect of adding different levels of curcumin and its nanoparticles to extender on post-thaw quality of cryopreserved rabbit sperm," *Animals*, vol. 10, no. 9, pp. 1508–1513, 2020.

[45] A. A. Ismail, A.-K. E. Abdel-Khalek, W. A. Khalil et al., "Effects of mint, thyme, and curcumin extract nanoformulations on the sperm quality, apoptosis, chromatin decondensation, enzyme activity, and oxidative status of cryopreserved goat semen," *Cryobiology*, vol. 97, pp. 144–152, 2020.

[46] L. Yao, Y. Jiang, N. Datta et al., "HPLC analyses of flavanols and phenolic acids in the fresh young shoots of tea (*Camellia sinensis*) grown in Australia," *Food Chemistry*, vol. 84, no. 2, pp. 253–263, 2004.

[47] Y. Shin, J. M. Blackwood, I.-T. Bae, B. W. Arey, and G. J. Exarhos, "Synthesis and stabilization of selenium nanoparticles on cellulose nanocrystal," *Materials Letters*, vol. 61, no. 21, pp. 4297–4300, 2007.

[48] E. Attard, "A rapid microtitre plate Folin-Ciocalteu method for the assessment of polyphenols," *Open Life Sciences*, vol. 8, no. 1, pp. 48–53, 2013.

[49] T. J. Herald, P. Gadgil, and M. Tilley, "High-throughput micro plate assays for screening flavonoid content and DPPH-scavenging activity in sorghum bran and flour," *Journal of the Science of Food*, vol. 92, no. 11, pp. 2326–2331, 2012.

[50] R. Boly, T. Lamkami, M. Lompo, J. Dubois, and I. Guissou, "DPPH free radical scavenging activity of two extracts from *Agelanthus dodoneifolius* (Loranthaceae) leaves," *International Journal of Toxicological Pharmacological Research*, vol. 8, no. 1, pp. 29–34, 2016.

[51] R. Benzi, L. Biferale, S. Ciliberto, M. Struglia, and R. Tripiccione, "Generalized scaling in fully developed turbulence," *Physica D: Nonlinear Phenomena*, vol. 96, no. 1-4, pp. 162–181, 1996.

[52] S. I. Moskovtsev and C. L. Librach, "Methods of sperm vitality assessment," in *Spermatogenesis (Vol. 927, pp. 13-19)*, Springer, 2013.

[53] A. G. Menon, J. C. Thundathil, R. Wilde, J. P. Kastelic, and H. W. Barkema, "Validating the assessment of bull sperm morphology by veterinary practitioners," *The Canadian Veterinary Journal*, vol. 52, no. 4, pp. 407-408, 2011.

[54] D. Neild, G. Chaves, M. Flores, N. Mora, M. Beconi, and A. Agüero, "Hypoosmotic test in equine spermatozoa," *Theriogenology*, vol. 51, no. 4, pp. 721–727, 1999.

[55] H. Aebi, "Catalase in vitro," in *Methods in Enzymology (Vol. 105, pp. 121-126)*, Elsevier, 1984.

[56] D. Koracevic, G. Koracevic, V. Djordjevic, S. Andrejevic, and V. Cosic, "Method for the measurement of antioxidant activity in human fluids," *Journal of Clinical Pathology*, vol. 54, no. 5, pp. 356–361, 2001.

[57] H. Ohkawa, N. Ohishi, and K. Yagi, "Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction," *Analytical Biochemistry*, vol. 95, no. 2, pp. 351–358, 1979.

[58] R. Bais and M. Philcox, "Approved recommendation on IFCC methods for the measurement of catalytic concentration of enzymes. Part 8. IFCC method for lactate dehydrogenase (l-lactate: NAD+oxidoreductase, EC 1.1.1.27). International Federation of Clinical Chemistry (IFCC)," *European Journal of Clinical Chemistry and Clinical Biochemistry*, vol. 32, no. 8, pp. 639–655, 1994.

[59] S. Reitman and S. Frankel, "A colorimetric method for the determination of serum glutamic oxalacetic and glutamic pyruvic transaminases," *American Journal of Clinical Pathology*, vol. 28, no. 1, pp. 56–63, 1957.

[60] A. Chaveiro, P. Santos, and F. Da Silva, “Assessment of sperm apoptosis in cryopreserved bull semen after swim-up treatment: a flow cytometric study,” *Reproduction in Domestic Animals*, vol. 42, no. 1, pp. 17–21, 2007.

[61] A. Masters and P. Harrison, “Platelet counting with the BD AccuriTMC6 flow cytometer,” *Platelets*, vol. 25, no. 3, pp. 175–180, 2014.

[62] F. J. Peña, A. Johannisson, M. Wallgren, and H. Rodriguez-Martinez, “Assessment of fresh and frozen-thawed boar semen using an Annexin-V assay: a new method of evaluating sperm membrane integrity,” *Theriogenology*, vol. 60, no. 4, pp. 677–689, 2003.

[63] A. Saffaryazdi, A. Ganjeali, R. Farhoosh, and M. Cheniany, “Variation in phenolic compounds, α-linolenic acid and linoleic acid contents and antioxidant activity of purslane (*Portulaca oleracea* L.) during phenological growth stages,” *Journal of Physiology Molecular Biology of Plants*, vol. 26, no. 7, pp. 1519–1529, 2020.

[64] M. A. Alam, A. Juraimi, M. Rafii, A. Hamid, F. Aslani, and M. Alam, “Effects of salinity and salinity-induced augmented bioactive compounds in purslane (*Portulaca oleracea* L.) for possible economical use,” *Food Chemistry*, vol. 169, pp. 439–447, 2015.

[65] G. Nagarani, A. Abirami, P. Nikitha, and P. Siddhuraju, “Effect of hydrothermal processing on total polyphenolics and antioxidant potential of underutilized leafy vegetables, Boerhaavia diffusa and *Portulaca oleracea*,” *Asian Pacific Journal of Tropical Biomedicine*, vol. 4, Suppl 1, pp. S468–S477, 2014.

[66] E. R. Grela, W. Samolińska, B. Kiczorowska, R. Klebaniuk, and P. Kiczorowski, “Content of minerals and fatty acids and their correlation with phytochemical compounds and antioxidant activity of leguminous seeds,” *Biological Trace Element Research*, vol. 180, no. 2, pp. 338–348, 2017.

[67] A. I. Mohamed and A. S. Hussein, “Chemical composition of purslane (*Portulaca oleracea*),” *Plant Foods for Human Nutrition*, vol. 45, no. 1, pp. 1–9, 1994.

[68] R. El Kashef, A. S. Soliman, H. Hassan, and N. A. Abd-Elhak, “Evaluation of total phenolic content and antioxidant activity of different solvent extracts of Egyptian purslane leaves,” *Current Science*, vol. 7, pp. 616–623, 2018.

[69] Y. Y. Lim and E. P. Quah, “Antioxidant properties of different cultivars of *Portulaca oleracea*,” *Food Chemistry*, vol. 103, no. 3, pp. 734–740, 2007.

[70] M. Rahman, M. Islam, M. Biswas, and A. Khurshid Alam, “*In vitro* antioxidant and free radical scavenging activity of different parts of Tabebuia pallida growing in Bangladesh,” *BMC Research Notes*, vol. 8, no. 1, pp. 1–9, 2015.

[71] J. Baumann, F. V. Bruchhausen, and G. Wurm, “A structure-activity study on the influence of phenolic compounds and bioflavonoids on rat renal prostaglandin synthetase,” *Naunyn-Schmiedeberg's Archives of Pharmacology*, vol. 307, no. 1, pp. 73–78, 1979.

[72] X. Huang, H. D. Halicka, F. Traganos, T. Tanaka, A. Kurose, and Z. Darzynkiewicz, “Cytometric assessment of DNA damage in relation to cell cycle phase and apoptosis,” *Cell Proliferation*, vol. 38, no. 4, pp. 223–243, 2005.

[73] N. Marcus-Braun, G. Braun, G. Potashnik, and I. Har-Vardi, “Effect of cryopreservation on quality and fertilization capacity of human sperm,” *European Journal of Obstetrics & Gynecology and Reproductive Biology*, vol. 116, no. 1, pp. 63–66, 2004.

[74] M. R. Freeman, A. E. Archibong, J. J. Mrotek, C. M. Whitworth, G. A. Weitzman, and G. A. Hill, “Male partner screening before *in vitro* fertilization: preselecting patients who require intracytoplasmic sperm injection with the sperm penetration assay,” *Fertility and Sterility*, vol. 76, no. 6, pp. 1113–1118, 2001.

[75] A. Noriham, F. Dian-Nashiela, B. Kherni Hafifi, H. Nooraain, and A. Azizah, “Influences of maturity stages and extraction solvents on antioxidant activity of Cosmos caudatus leaves,” *International Journal of Research Studies in Biosciences*, vol. 3, no. 12, pp. 1–10, 2015.

[76] A. Bansal and G. Bilaspuri, “Effect of ferrous sulphate and ascorbic acid on motility, viability and lipid peroxidation of crossbred cattle bull spermatozoa,” *Animal*, vol. 2, no. 1, pp. 100–104, 2008.

[77] C. Budai, I. Egerszegi, J. Olah, A. Javor, and A. Kovacs, “The protective effect of antioxidants on liquid and frozen stored ram semen-review,” *Animal Science Biotechnologies*, vol. 47, no. 1, pp. 46–52, 2014.

[78] S. Siriamornpun and M. Suttajit, “Microchemical components and antioxidant activity of different morphological parts of Thai wild purslane (Portulaca oleracea),” *Weed Science*, vol. 58, no. 3, pp. 182–188, 2010.

[79] F. Abas, N. H. Lajis, D. Israf, S. Khozirah, and Y. U. Kalsom, “Antioxidant and nitric oxide inhibition activities of selected Malay traditional vegetables,” *Food Chemistry*, vol. 95, no. 4, pp. 566–573, 2006.

[80] H. Hao, Y. Nancai, F. Lei et al., “Retracted: Antiaging effect of purslane herb aqueous extracts and its mechanism of action,” *Phytotherapy Research*, vol. 23, no. 9, pp. i–vii, 2009.

[81] G. Martin, O. Sabido, P. Durand, and R. Levy, “Cryopreservation induces an apoptosis-like mechanism in bull sperm,” *Biology of Reproduction*, vol. 71, no. 1, pp. 28–37, 2004.

[82] K. Nagata, K. Obata, M. Odashima et al., “Nicorandil inhibits oxidative stress-induced apoptosis in cardiac myocytes through activation of mitochondrial ATP-sensitive potassium channels and a nitrate-like effect,” *Journal of Molecular and Cellular Cardiology*, vol. 35, no. 12, pp. 1505–1512, 2003.

[83] A. H. Wyllie, J. R. Kerr, and A. Currie, “Cell death: the significance of apoptosis,” *International Review of Cytology*, vol. 68, pp. 251–306, 1980.

[84] L. Gandini, F. Lombardo, D. Paoli et al., “Full-term pregnancies achieved with ICSI despite high levels of sperm chromatin damage,” *Human Reproduction*, vol. 19, no. 6, pp. 1409–1417, 2004.

[85] L. Muriel, N. Garrido, J. L. Fernández et al., “Value of the sperm deoxyribonucleic acid fragmentation level, as measured by the sperm chromatin dispersion test, in the outcome of in vitro fertilization and intracytoplasmic sperm injection,” *Fertility and Sterility*, vol. 85, no. 2, pp. 371–383, 2006.

[86] S. A. Hassan, W. A. Khalil, M. A. Hassan et al., “Antioxidant and Antiapoptotic effects of a Turraea fischeri leaf extract on cryopreserved goat sperm,” *Animals*, vol. 11, no. 10, p. 2840, 2021.

[87] M. A. Shahin, W. A. Khalil, I. M. Saadeldin, A. A. Swelum, and M. A. El-Harairy, “Comparison between the effects of adding vitamins, trace elements, and nanoparticles to shotor extender on the cryopreservation of dromedary camel epididymal spermatozoa,” *Animals*, vol. 10, no. 1, p. 78, 2020.

[88] M. Sobeh, S. A. Hassan, M. A. El Raey, W. A. Khalil, M. A. Hassan, and M. Wink, "Polyphenolics from *Albizia harveyi* exhibit antioxidant activities and counteract oxidative damage and ultra-structural changes of cryopreserved bull semen," *Molecules*, vol. 22, no. 11, p. 1993, 2017.

[89] M. Sobeh, S. A. Hassan, M. A. E. Hassan et al., "A polyphenol-rich extract from *Entada abyssinica* reduces oxidative damage in cryopreserved ram semen," *Frontiers in Veterinary Science*, vol. 7, pp. 1–9, 2020.

[90] J. Yu, L. Wang, R. L. Walzem, E. G. Miller, L. M. Pike, and B. S. Patil, "Antioxidant activity of citrus limonoids, flavonoids, and coumarins," *Journal of Agricultural and Food Chemistry*, vol. 53, no. 6, pp. 2009–2014, 2005.

[91] Q. Xiong, S. Kadota, T. Tani, and T. Namba, "Antioxidative effects of phenylethanoids from *Cistanche deserticola*," *Biological & Pharmaceutical Bulletin*, vol. 19, no. 12, pp. 1580–1585, 1996.

[92] M. I. Yousef and A. F. Salama, "Propolis protection from reproductive toxicity caused by aluminium chloride in male rats," *Food and Chemical Toxicology*, vol. 47, no. 6, pp. 1168–1175, 2009.

[93] Z. Huang, B. Wang, D. H. Eaves, J. M. Shikany, and R. D. Pace, "Phenolic compound profile of selected vegetables frequently consumed by African Americans in the Southeast United States," *Food Chemistry*, vol. 103, no. 4, pp. 1395–1402, 2007.