

# Effects of hydroethanolic extracts of *Terminalia chebula* and *Thymbra spicata* on ram fresh semen under normal and oxidative stress conditions

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## Abstract

The objective of this study was to evaluate protective effects of hydroethanolic extracts of *Terminalia chebula* and *Thymbra spicata* on viability, lipid peroxidation (LPO) and DNA integrity of ram fresh semen under normal and oxidative stress (OS) conditions. Antioxidant activities of different concentrations of *Terminalia chebula* and *Thymbra spicata* extracts were evaluated with DPPH assay. Semen samples were taken from three fertile adult rams. After diluting semen with Tris-base extender, different concentrations of *Terminalia chebula* and *Thymbra spicata* (30, 300, and 3000  $\mu\text{g/ml}$ ) extracts were used under normal and induced OS conditions. The group not receiving any supplements was considered as control group. A total of 50  $\mu\text{M}$  hydrogen peroxide was used to induce OS. MTT solution was added to each of treatment groups which were kept in an incubator at 37°C for 2 h. After incubation, readings were obtained by ELISA reader. DNA integrity and LPO were determined with acridine orange (AO) staining and malondialdehyde (MDA) assay. Higher concentrations of *Terminalia chebula* and *Thymbra spicata* extracts preserved viability and DNA integrity while reducing MDA concentrations compared to other treatment groups. Also, under induced OS, higher concentrations of both extracts reduced detrimental effects of  $\text{H}_2\text{O}_2$ . In conclusion, it seems that addition of *Terminalia chebula* and *Thymbra spicata* extracts can reduce induced OS in spermatozoa.

## KEYWORDS

oxidative stress, ram, spermatozoa, *Terminalia chebula*, *Thymbra spicata*

## 1 | INTRODUCTION

Preservation of spermatozoa extends fertility potential of endangered species by including valuable genetic material of animals that failed to reproduce naturally or died prematurely. Biobanking allows bridging time and space in breeding programmes. Frozen sperm can be easily transported for artificial insemination and can be used years after orig-

inal collection (Howard & Wildt, 2009). Sperms are cells sensitive to reactive oxygen species (ROS) attacks. When manipulated in vitro by the assisted reproductive techniques, these cells run the risk of producing and being exposed to high levels of ROS (du Plessis et al., 2008). All cellular components are potential targets of oxidative stress (OS). Also, OS considerably damages spermatozoa actions due to lipid peroxidation (LPO) induced by ROS. LPO happens readily in the tissues rich in

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highly oxidizable polyunsaturated fatty acids (PUFA) (Chatterjee et al., 2001; Gandeshmin et al., 2020). Spermatozoa have high levels of PUFA; thus, they are highly sensitive to LPO (Tuncer et al., 2010).

The extent of OS-induced damages depends on the nature and amount of ROS involved, duration of exposure to ROS and extracellular factors including oxygen tension, temperature and composition of the surrounding environment (e.g. ions, proteins and ROS scavengers) (Agarwal et al., 2008). As internal repair mechanisms are limited in spermatozoa and as the contribution of seminal plasma antioxidants is less effective due to semen dilution for the storage, an exogenous supplement of antioxidants is presumably substantial for preserving the fertilizing potentials (La Falci et al., 2011). Many researchers stated that addition of antioxidants to semen diluents has protected semen quality against damages caused by peroxidation (Hegazy et al., 2020).

It is now known that many medicinal plants, including *Terminalia chebula* and *Thymbra spicata*, have remarkable values of antioxidant compounds, being used to produce natural antioxidant formulation in the fields of nutrition and medicine (Nigam et al., 2020).

*Terminalia chebula* (family of Combretaceae) is a native plant of India, dried fruit of which is widely used to make various kinds of home-made treatments (Rathinamoorthy & Thilagavathi, 2014). Their considered health benefits may be attributed to the presence of different phytochemicals including terpenes, anthocyanins, polyphenols, glycosides alkaloids and flavonoids (Bag et al., 2013). These also generally used to remedy different diseases such as paralysis, leprosy, cardiovascular diseases, ulcers, cancers, cough, fever, arthritis, epilepsy, gout, skin problems, urinary tract infections, diarrhea, gastroenteritis and wound infections (Rathinamoorthy & Thilagavathi, 2014; Manosroi et al., 2013).

*Thymbra spicata* L. (Labiaceae) is distributed in the East Mediterranean to central Asia. *Thymbra spicata* has small blue flowers emerging on the upper part of branches up to 60-cm tall (Yousefzadeh & Naghdi Badi, 2017). Essential oil of *Thymbra spicata* is chiefly used by perfumery, beverages, cosmetics, food and flavouring industries (Tonutti & Liddle, 2010) and extracts from aerial parts are used as cough relieving, carminative, expectorant, simulative and antispasmodic agent while being used to cure certain skin diseases and chronic bronchitis (Chopra et al., 1956; Inan et al., 2011). *Thymbra spicata* contains different polyphenolic compounds. Fathiazad & Hamedeyazdan (2011) reported different flavonoids including apigenin, luteolin quercetin, diosmin, and their glucosides and phenolic compounds such as syringic, p-hydroxybenzoic, protocatechuic, ferulic, chlorogenic and caffeic acids.

The sperm quality can be better maintained by addition of various antioxidants to semen extender compared to control (Zanganeh et al., 2013). There is no document studying the effect of medicinal plant extracts, such as *Terminalia chebula* and *Thymbra spicata* extracts, as a pool of antioxidants on sperm quality during different conditions.

Therefore, present study was conducted to determine the effects of different concentrations of hydroethanolic extracts of *Terminalia chebula* and *Thymbra spicata*, as antioxidant agents, on ram fresh semen under normal and OS conditions.

## 2 | MATERIALS AND METHODS

### 2.1 | Chemicals

All chemicals were obtained from Sigma-Aldrich (USA); otherwise, they are specified within the text.

### 2.2 | Preparation of *Terminalia chebula* and *Thymbra spicata* extracts

To prepare *Terminalia chebula* and *Thymbra spicata* extracts, plants were purchased from herbs market. In brief, dried plants of *Terminalia chebula* and *Thymbra spicata* (50 g) were powdered and soaked in 250 ml of 70% ethanol for 72 h. Then, the mixture was filtered through Whatman no. 1 filter paper and the filtrate was evaporated to be dried under reduced pressure at 40°C using a rotary evaporator. The extracts were freeze-dried and stored at 4°C to be used later.

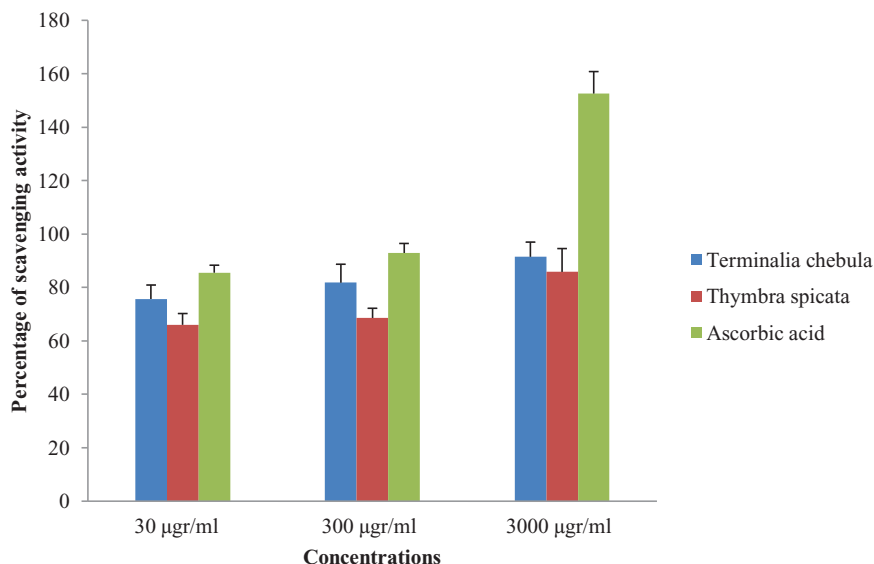
### 2.3 | DPPH radical scavenging assay

Fresh methanolic solution of DPPH (0.2 mM) was prepared and incubated in the dark for 2 h prior to the analysis. Extracts and the ascorbic acid powder, as the standard, were dissolved individually in their corresponding extracting solvent. Total free radical scavenging capacity of the extracts from two plant samples was estimated according to the method of Liyana-Pathiranan and Shahidi (2005). Solution of 0.135 mM DPPH in methanol was prepared, 1.0 ml of which was mixed with 1.0 ml of different concentrations (30, 300 and 3000 µg) of two extracts in methanol. The reaction mixture was vortexed thoroughly and left in the dark at room temperature for 30 min. Mixture absorbance was measured at 517 nm spectrophotometrically. Synthetic antioxidant ascorbic acid was included in experiments as a positive control. Inhibition of free radical DPPH was calculated in percent by the following formula:

$$\text{Inhibition \%} = \frac{A_{\text{Blank}} - A_{\text{Sample}}}{A_{\text{Blank}}} \times 100.$$

### 2.4 | Animals and semen collection

Ejaculates from three adult fertile rams were used in this study. Ejaculates were collected from rams with the help of an artificial vagina twice a week according to AI standard procedures. Semen samples were pooled in order to obtain sufficient sperm for the experiment and also to eliminate the ram effect. Then, they were incubated in a water bath at 34°C to be evaluated for semen quality microscopically in the laboratory. Ejaculates were assessed within 20 min after they were collected. Only ejaculates containing minimally 80% motile and morphologically 90% normal spermatozoa were used in this research.



**FIGURE 1** DPPH radical scavenging activity of *Terminalia chebula* and *Thymbra spicata* extracts

## 2.5 | Diluent preparation

A Tris-based extender (tris 297.58 mM, fructose 82.59 mM, citric acid 105.35 mM, 20% egg yolk, 6% glycerol) was used as extender. Each pooled ejaculate sample was divided into eight equal aliquots and diluted (37°C) with the base extender containing different concentrations of hydroethanolic extracts of *Terminalia chebula* and *Thymbra spicata* (30, 300 and 3000 µg/ml) under normal and induced OS conditions. A total of 50 µM hydrogen peroxide was used to induce OS. The group not receiving any supplements was considered as control group.

## 2.6 | MTT reduction assay

MTT assay was carried out routinely (Aitken et al., 2020). Two hundred microliters of diluted semen in tris-base extender plus 20 µl of MTT stock solution (5 mg of MTT/ml of PBS) were placed in a 1.5-ml microtube. Concentration of sperm cells was adjusted to about  $15 \times 10^6$  cells/ml. Reaction vials were put in an incubator at 37°C for 2 h. Then, they were left open to allow for oxidative phosphorylation. MTT reduction rates were recorded immediately and after incubation using an ELISA reader at a wavelength of 570 nm. MTT reduction rate of each sample was calculated by determining the difference between the first and second readings of the spectrophotometer.

## 2.7 | Acridine orange (AO) staining

Spermatozoa from each subject were smeared separately on glass slides, air-dried and fixed overnight in Carnoy's solution (methanol/acetic acid, 3:1). Once rinsed and air dried, slides were stained for 5 min with freshly prepared AO stain as follows: 10 ml of 1% AO in distilled water was added to a mixture of 40 ml of 0.1 M citric acid and 2.5 ml of 0.3 M Na<sub>2</sub>HPO<sub>4</sub>·7H<sub>2</sub>O. Then, they were washed

with distilled water and examined under fluorescent microscope at the excitation wavelength of 450–490 nm.

## 2.8 | Malondialdehyde measurement

LPO was detected using thiobarbituric acid (TBA). Using appropriate kits (TPR INNOVATIVE), malondialdehyde (MDA) concentrations were measured according to the manufacturer's instructions. The absorbance was measured at 532 nm. Concentrations of MDA were expressed as µM.

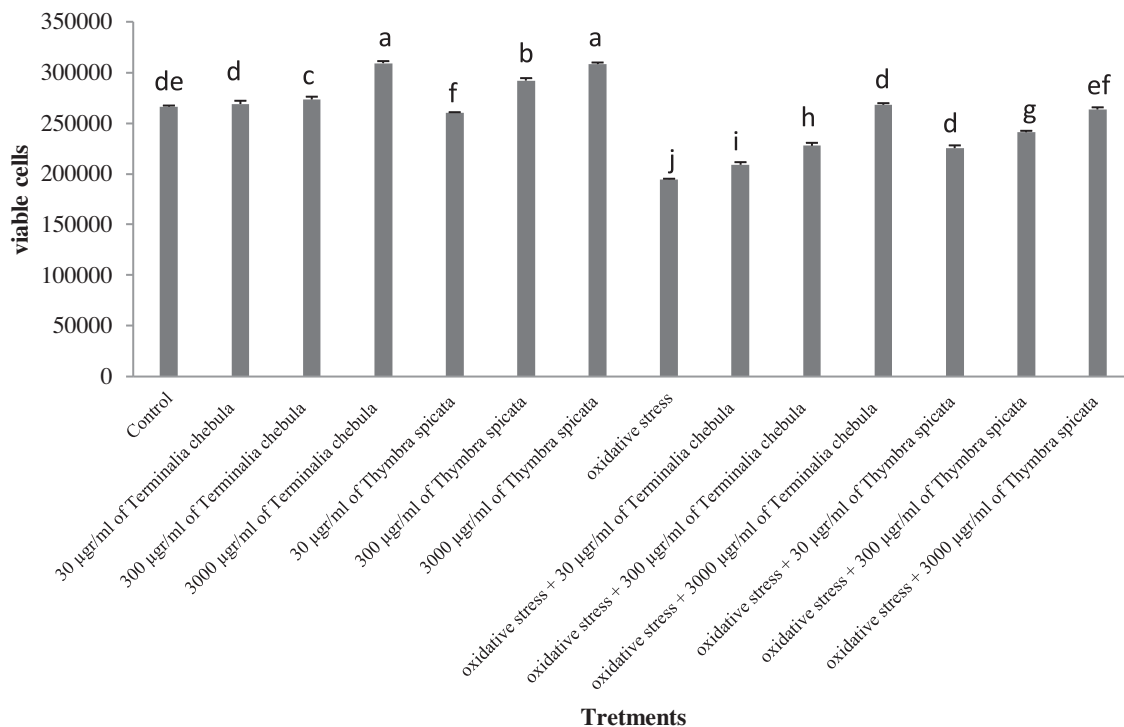
## 2.9 | Statistical analyses

Results are presented as the mean ± standard deviation (SD). Data were analysed by statistical variance (ANOVA) analysis using SPSS16.0 software;  $p < 0.05$  was considered as statistically significant.

## 3 | RESULTS

In our study, after the preparation of both hydro-ethanolic extracts (*Terminalia chebula* and *Thymbra spicata*), their antioxidant activities were evaluated by DPPH. DPPH functions are reduced by a hydrogen-donating compound which results in DPPH dis-coloration from dark violet to light yellow, which can be recorded by a spectrophotometer. Results for DPPH radical scavenging activity of both extracts are as shown in Figure 1. Based on the results, there were no significant differences in DPPH reduction between two extracts ( $p > 0.05$ ).

Under normal and/or induced OS conditions, the percentage of sperm viability in higher concentrations of both extracts (3 mg/ml) increased significantly in comparison with other treatment groups ( $p < 0.05$ ; Figure 2). Also, positive effects of both *Terminalia chebula* and *Thymbra spicata* extracts on viability were observed at intermediate



**FIGURE 2** Analysis of ram semen samples using MTT test after addition of *Terminalia chebula* and *Thymbra spicata* extracts under normal or induced oxidative stress conditions

concentrations (300 µg/ml) compared to the control group. Higher concentrations of *Terminalia chebula* and *Thymbra spicata* extracts (3 mg/ml) preserved viability significantly ( $p < 0.05$ ) compared to lower concentrations (30 and 300 µg/ml) under induced OS conditions (Figure 2).

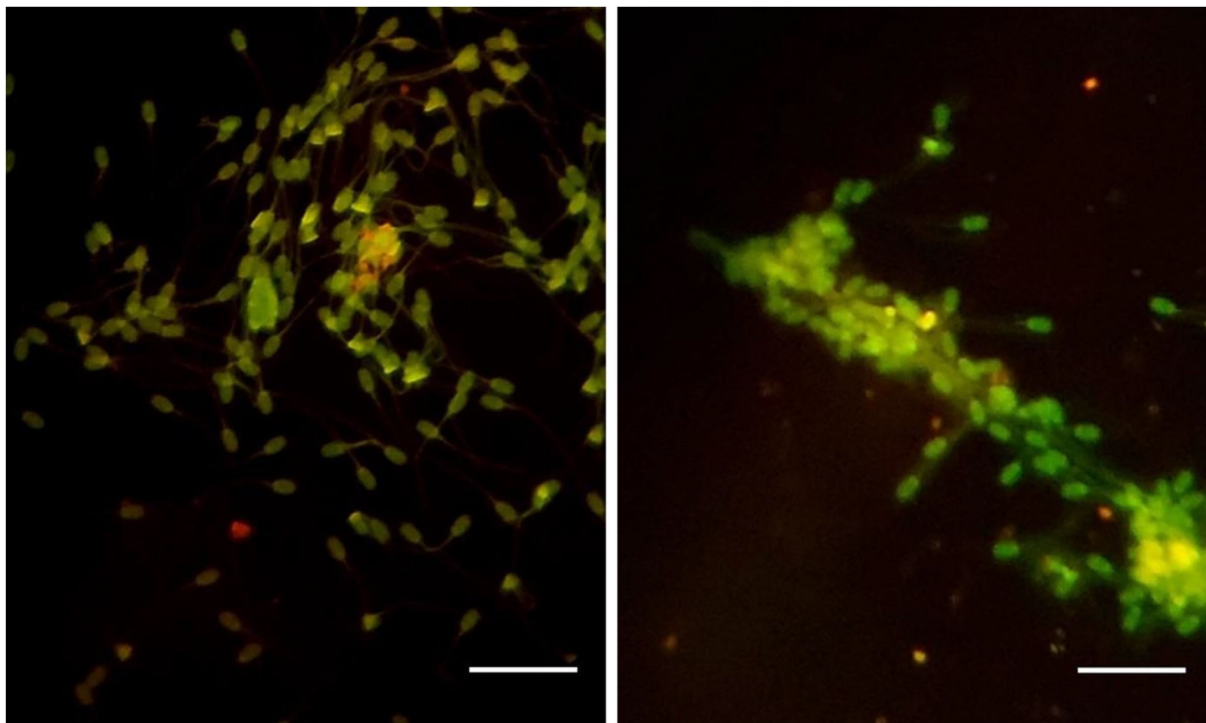
Detection of DNA damages in ram spermatozoa by Acridine orange test provides unfragmented DNA green fluorescence against yellow-orange fluorescence for fragmented DNA (Figure 3). Addition of higher concentration (3 mg/ml) of *Terminalia chebula* to semen extender preserved DNA compared to 30 and 300 µg/ml of *Terminalia chebula* and *Thymbra spicata* and control group under normal and induced oxidative stress conditions, but there was no difference between 3 mg/ml of *Terminalia chebula* and 3 mg/ml *Thymbra spicata* although there was a numerical increase (Figure 4). There was a significant decrease in percentage of intact DNA when sperm cells were treated with H<sub>2</sub>O<sub>2</sub> (to induce oxidative stress) compared to the control and other treatment groups (Figure 4). Sperm DNA fragmentation was changed significantly by acridine orange staining as a result of treatments with *Terminalia chebula* and *Thymbra spicata* extracts under induced oxidative stress conditions. The highest rate of DNA preservation was observed after treatments with higher concentrations of both herbal extracts (Figure 4).

Lipid peroxides are derived from the peroxidation of polyunsaturated fatty acids, the most abundant of which is MDA (Rao et al., 1989). MDA concentrations in sperm groups treated with higher concentrations of *Terminalia chebula* and *Thymbra spicata* extracts decreased significantly ( $p < 0.05$ ) in comparison with the control and other treatment groups. MDA levels are shown in Figure 5. Under induced OS conditions, mean MDA concentration was significantly higher compared to

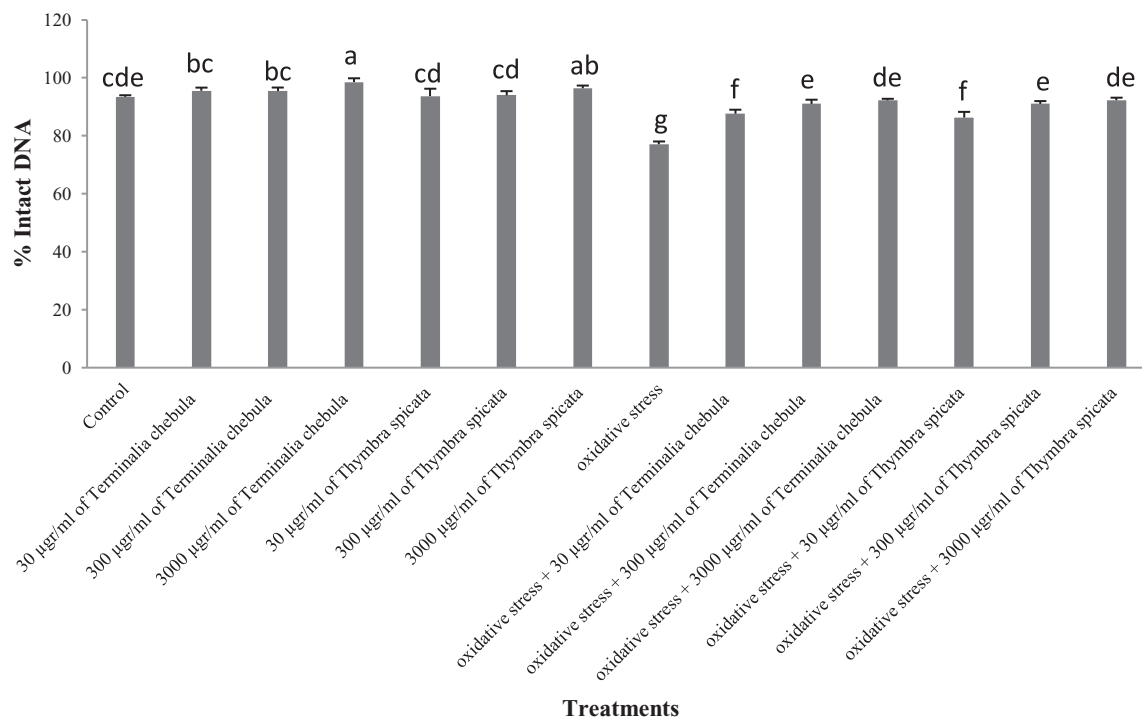
other treatment groups (Figure 5;  $p < 0.05$ ). Results obtained by the present study showed that seminal plasma concentration of MDA was significantly lower when higher concentrations of *Terminalia chebula* and *Thymbra spicata* extracts (3 mg/ml) were added under OS condition compared with other concentrations under the same conditions (Figure 5).

#### 4 | DISCUSSION

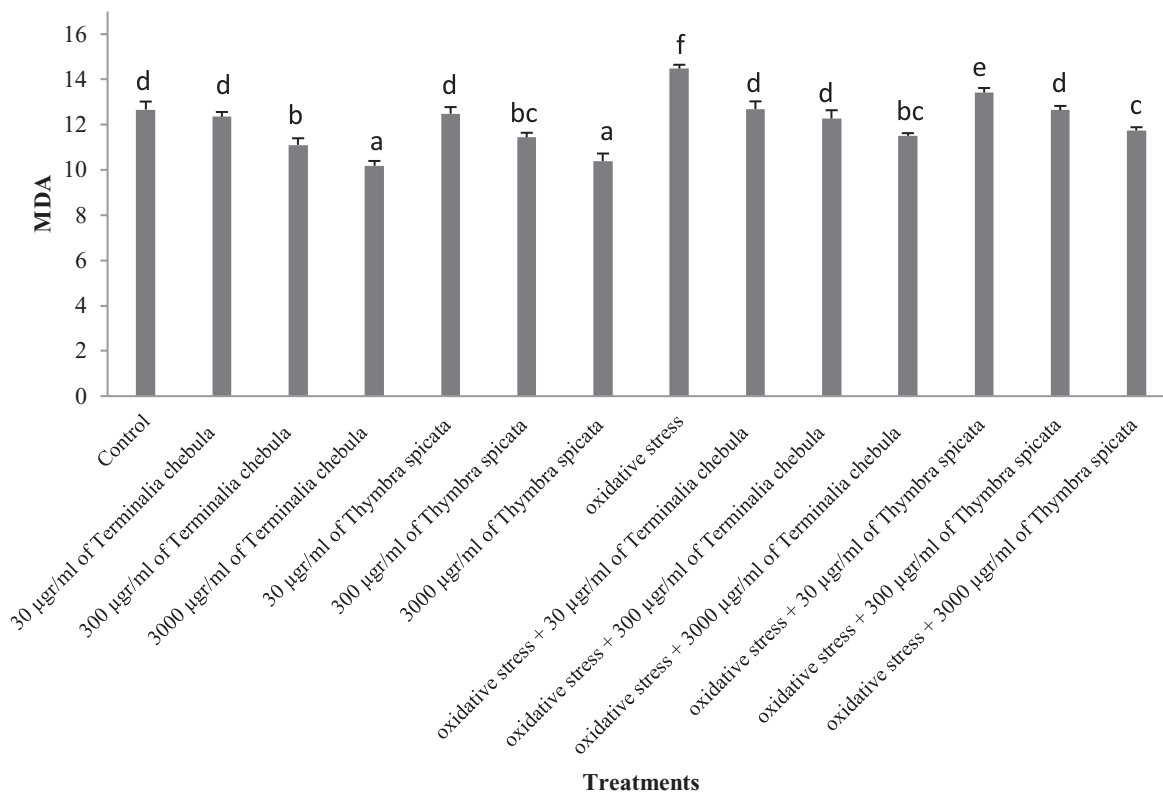
Spermatozoa generate low amounts of ROS that take a considerable part in plenty of the sperm physiological processes including capacitation, hyper-activation and sperm-oocyte fusion (Gupta et al., 2021; Wu et al., 2020). However, ROS must be continuously inactivated so that its lower concentration, which is essential for preserving normal cell functions, can be kept constant. Over-production of ROS in sperm cells can cause damages to them due to its structurally particular composition (Celik-Ozenci et al., 2020). Residual cytoplasm includes high level of specified cytoplasmic enzymes (G6PDH, SOD), which are also a source of ROS (Gomez et al., 1996). Cytoplasm deficiency results in reduced antioxidant defence. This process is associated with low sperm quality and increased ROS. In vitro studies have shown that antioxidants eliminated free radicals including hydrogen peroxide, hydroxyl radicals, superoxide ion and peroxy (Ashrafi et al., 2013). Because of acting as chelating agents in being the hydroxyl group to metal ions, phenolic compounds inhibit free radical activities (Osawa, 1994). Incorporation of antioxidants into semen diluents can solve this problem. A variety of antioxidants have been evaluated to either scavenge ROS directly



**FIGURE 3** Fluorescent microscopy of acridine orange-stained cells displays that spermatozoa showing green fluorescence were found to have normal DNA content, whereas spermatozoa displaying a spectrum of yellow–orange to red fluorescence were considered to have damaged DNA



**FIGURE 4** DNA integrity of fresh ovine spermatozoa after addition of *Terminalia chebula* and *Thymbra spicata* extracts under normal or induced oxidative stress conditions using acridine orange method



**FIGURE 5** Comparison of MDA concentrations after addition of *Terminalia chebula* and *Thymbra spicata* extracts under normal or induced oxidative stress conditions

or effects of counter ROS toxicity in semen of a variety of mammalian species (Asadpour et al., 2011). In the present study, as alternatives to these antioxidant additives, we tested effects of *Terminalia chebula* and *Thymbra spicata* extracts added directly to ram semen, obtaining positive effects on sperm viability, DNA integrity and LPO status under normal and induced OS conditions. In this study, antioxidant effects of *Terminalia chebula* and *Thymbra spicata* extracts were investigated using DPPH assay. Then, OS conditions were induced by hydrogen peroxide to investigate beneficial antioxidant effects of *Terminalia chebula* and *Thymbra spicata* extracts. We found that addition of *Terminalia chebula* and *Thymbra spicata* extracts to ram semen extenders enhanced sperm viability and preserved DNA integrity while reducing LPO under normal and induced OS conditions. There is no study evaluating the antioxidant effects of any of these extracts on sperms (under different normal and OS conditions) as well as on fresh or frozen semen. For this purpose, some comparison was made on effects of other herbal extracts.

*Ceratonia siliqua* extract reduced detrimental effects of vitrification on sperm parameters and chromatin quality of normozoospermic aged men (Faramarzi et al., 2020). In the study by AL-Helal et al. (2018), the effects of different concentrations of *Opuntia ficus-indica* hydroalcoholic extract (0, 0.5, 1, 1.5, 3%) on the characteristics of sperm after freezing-thawing processes were evaluated. They showed that 1% concentration of *Opuntia ficus-indica* extract outperformed other concentrations in freezing sperms of Awassi rams, which is in agreement with our results (AL-Helal & Hobi, 2018; Faramarzi et al., 2020). Supple-

mentation of bull semen extenders with 10% and 20% pomegranate juice provided good chilling and improved frozen-thawed semen quality (Reda et al., 2016). Addition of 100 µg/ml of oregano extract to sperm-freezing extenders increased total motility and viability and decreased the percentage of 2',7'-dichlorofluorescein-positive cells and MDA levels (Shiri et al., 2020). Supplementation of human semen extenders with 40 and 50 mg/ml of *Tribulus terrestris* extract significantly enhanced total sperm motility, number of progressive motile spermatozoa and curvilinear velocity over 60–120 min of holding time (Khaleghi et al., 2017).

## 5 | CONCLUSIONS

Further studies are needed to identify the role of *Terminalia chebula* and *Thymbra spicata* extracts during sperm storage or cryopreservation. Also, it is suggested that the *Terminalia chebula* and *Thymbra spicata* extracts should be purified to discover new effective antioxidants for improving sperm cryopreservation processes.

## ETHICS

Animal husbandry and handling were conducted in accordance with the guidelines of Animal Ethics Committee (Permission number: IR.RAZI.REC.1399.027) of Razi University, Kermanshah, Iran.



## AUTHOR CONTRIBUTIONS

Hereby we declare that all individuals contributed equally to this research in study design, data analysis, and paper drafting.

## DATA AVAILABILITY STATEMENT

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

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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