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ORIGINAL ARTICLE

Human sperm DNA damage inhibition and antioxidant activity of *T. arjuna* bark: an in vitro study

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Abstract Complimentary or natural antioxidant type of alternative medicine is developed worldwide to treat male infertility. The aim of this study is to the extraction *of T. arjuna bark and activity against* human sperm DNA damage in asthenoteratospermic smoker's subjects—an in vitro *study*. All preliminary and antioxidant assays (DPPH, H_2O_2, and total antioxidant, reducing power activity) were done. *T. arjuna* bark metal analysis was done with AAS. On the other hand, patients were asked to fill a direct questionnaire about smoking history; 25 infertile smokers were identified as asthenoteratospermic; 34 fertile non-smokers (control) were assessed for semen parameters by CASA, seminal plasma Zinc analysis by AAS, DNA fragmentation by colorimetric method and semen genomic DNA damage inhibition by modified non-enzymatic salting out extraction method. Most of the antioxidants are highly present in the aqueous extract; meanwhile, the major content in this extract is zinc 16 µg/g (Ca = 0.5 µg/g; Se = 2.2 µg/g and Mg = 1.6 µg/g) along with FT-IR peaks which also confirmed the metal presence. The semen parameters in smokers that were noticed are low sperm count and morphological changes. Meanwhile, in the seminal plasma of smokers, zinc and DNA fragmentation results were positively correlated with sperm morphology ($p < 0.001$). Repaired DNA bands were noticed in the in vitro study of aqueous *T. arjuna* bark, in smokers' semen. *T. arjuna* bark will act as cryo protector as well as great zinc supplementary to maintain sperm motility and morphology in smokers.

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Introduction

Cigarettes encompassing tobacco, nicotine, marijuana, caffeine, and illegal drugs impair the body mechanism by manifesting as stress, hypertension, blood pressure, cholesterol, diabetes mellitus, obesity, zinc deficiency, etc (Mishra et al. 2016). Adding to the mentioned health problems, it also impairs the reproductive potential of both men and women. Approximately 10–15% of healthy couples are identified as clinically infertile due to smoking and also, 50% of these problems are caused by defects in the male reproductive system (Harlev et al. 2015). The fact that cigarette smoke is a carcinogen and a somatic cell mutagen provides strong evidence that it is the direct cause of active problems like cancer, lung, and CVD disorders. However, the passive effects of smoking, like reproductive health problems, are yet to be confirmed. More than 100 different types of diseases (like liver disorder, hepatitis, diabetes, lung disorder, brain disorder, and degenerative disease) have associated oxidative stress etiology; on consumption, cigarette oxidants increase the reactive oxygen species (ROS) that might degrade semen quality and decrease the integrity of the DNA (Dai et al. 2015). Overproduction of ROS can cause in vivo antioxidant reduction, thereby resulting in imbalance between free radicals and antioxidants in semen (Taha et al. 2013). Antioxidants are natural defenses and consist of flavonoids, carotenoids, vitamin C (spermatogenesis) and E (steroidogenesis), poly phenols, trace elements like Zn (sperm motility and morphology) and Mg; all of these act as scavengers against ROS which donates its electron to the free radical of oxygen species.

However, the endogenous antioxidants are not enough to cause homeostasis during oxidative stress; thus, supplementation of antioxidants is needed for body defense (Lukmanul et al. 2008; Mojab et al. 2003; Vasu et al. 2009; Yadav and Agrawala 2011).

Recent innovative studies have reported that plants are natural sources of antioxidants, with various physiological activities, that are non-toxic, eco-friendly, and cost-effective (Chatterjee 1994; Biswas et al. 2011; Das et al. 2010; Mandal et al. 2010; Nema et al. 2012). In this aspect, a plant species called *T. arjuna* is identified as a source of several natural antioxidants. This tree is nick named as 'guardian of the heart' and considered as a perfect tonic for heart diseases worldwide. *T. arjuna* tree (family Combretaceae) is a wide spectrum, versatile medicinal plant which grows during the hot season, mainly from February to April. The bark of *T. arjuna* has anti-diuretic, cardio tonic, hypolipidemic, antimicrobial, antioxidant, lithotropic, and anti-uremic activity (Trivedi et al. 2015). Most useful constituents isolated from *T. arjuna* include terpenoids and triterpenoids; studies reported in recent pharmacology explained its cardiovascular, anti-liver cirrhosis, hypertension relief, and anti-cancer activity (Buduru and Vedantam 2016). *T. arjuna* in vivo studies of mice model reported anti-inflammatory activity (Biswas et al. 2011). Most of the pharmacological studies involve active components from plant roots, tips, flowers, leaves, and seeds; barks are rarely reported; hence, the aim of this study is to investigate the antioxidant and DNA damage inhibition activity of aqueous *T. arjuna* bark—An in vitro *study in* infertile male smokers.

Materials and methods

Chemicals

Sulphuric acid (H_2SO_4), methanol, ferric chloride ($FeCl_3$), trichloroacetic acid, potassium ferricynade ($K_3Fe(CN)_6$), hydrogen peroxide (H_2O_2), sodium chloride (NaCl), gallic Acid, sodium phosphate, ammonium molybdate, ascorbic acid, aluminium chloride, disodium hydrogen phosphate, potassium dihydrogen phosphate, ethanol, sodium bi carbonate, Folin- Ciocalteau reagent, ninhydrin, chloroform, nitroblue tetrazolium (NBT), dimethyl sulfoxide (DMSO), and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were used in this study.

Plant collection, extraction, preparation, and yield

The bark of *T. arjuna* was collected from the naturally growing forest of Javvathu hills (12.5996°N, 78.8871°E), located in Thiruvannamali (dt), Tamilnadu, India, and was confirmed with G. Kothandam, Professor, Plant Bio technology, VIT University, Vellore-14. The collected barks of *T. arjuna* tree were sun dried for a period of 2 weeks, after which the barks were cut into small pieces and crushed into fine powder using an electrical grinder. After obtaining the powdered form, it was diluted in two different solvents (aqueous and methanol) and extracts were obtained. These extracts were used for the analysis of various qualitative tests, quantitative tests, and phytochemical analysis.

Phytochemical analysis of *T. arjuna* by qualitative methods

Phytochemical analysis of the test sample was carried out by standard methods (Patil and Gaikwad 2010). TLC analysis for antioxidant constituents was followed by the standard Kannan et al. (2010). TLC analysis for flavonoid constituents was followed by the standard technique from Raj and Radhamany (2010).

Antioxidant and metal analysis

The hydrogen peroxide scavenging activity of *T. arjuna* was followed by standard technique from Ruch et al. (1989) along with few modifications (Chen et al. 2013). The DPPH radical scavenging activity of the aqueous and methanol extract was compared and followed by the Guha et al. (2010). The reducing power activity of the extract was examined by potassium ferricyanide–ferric chloride method (Tundis et al. 2013). The metal (Zinc) content in aqueous extract of 1.5 ml *T. arjuna* bark was checked with the respective standard using atomic absorption spectrophotometer. The detection range is 540 nm and the metal analysis of the bark extract was confirmed by FT-IR peaks. Meanwhile, magnesium and selenium are also present, but in low concentrations, hence, it cannot be detected.

Research ethics

This study is a part of a major research project for which human ethical approval and clearance has been obtained from VIT University institutional human ethical committee (Ref.No. VIT/UHEC-3/NO.11). To participate in this clinical study, written consent was obtained from the patients. Sample donor name, address, and their background have been documented and maintained confidentially.

Patient's selection criteria and human semen collection analysis

Male infertile partners with fertile female partners were only targeted for the study. Subjects with a history of

genital examination (testis and scrotum), family inheritance, medication allergy, toxins, radiotherapy, and chemotherapy were excluded from the study. In the current study, subjects who have had a history of smoking for at least 8–11 years (before enrollment in the study) were categorized as asthenoteratospermic (AST) smokers ($n = 25$) based on smoking index unit (SI); non-smokers, or fertile subjects ($n = 34$) are selected as control. The selected smokers as well as non-smokers are aged in 27–39 years. The selected subjects were instructed to collect semen samples after 48–72 h of abstinence through masturbation at Bangalore Assisted Conception Centre (BACC), (MoU with BACC, Bangalore) and it was allowed to stand at room temperature (30 min) for liquefaction. After liquefaction, the automated computer-assisted semen analysis (CASA) assessed the semen quality (pH, volume, morphology, sperm count, and total and progressive motility) according to WHO 2010 guidelines. After CASA, the semen samples were carefully transported to Gene Cloning and Technology Lab, VIT University with the help of Bio-cane cryogenic storage containing liquid nitrogen and semen stability was maintained under −196 °C according to WHO protocol.

Seminal plasma zinc analysis

About 1.5 ml of the semen sample was taken and centrifuged at 3500 rpm for 15 min. The isolated pellets were used for sperm DNA extraction, and the supernatant was used to assess the seminal plasma zinc concentration in infertile subjects as a comparison with fertile subjects (Vickram et al. 2013).

In vitro sperm DNA damage inhibition activity of *T. arjuna* bark

DNA damage inhibition activity of aqueous extract of *T. arjuna* bark was checked in cigarette smoke exposed irradiated infertile human semen DNA sample with control pBR322 plasmid DNA using simple modified non-enzymatic salting out DNA extraction method (Selit et al. 2013). Three micro centrifuge tubes were added with a total of 1 µl of aliquots of smoke irradiated extracted sperm DNA sample. About 50 µg of aqueous extract of *T. arjuna* bark was added in two micro tubes out of three, where the third one was used as a negative control in smoker subjects. For positive control, pBR322 was taken without adding extract. The samples in all the tubes were run in agarose gel electrophoresis. Modified colorimetric method was used to measure sperm DNA damage or fragmentation in the selected subjects (El-Melegy and Ali 2011).

NBT staining

NBT staining was performed as described in Parkhey et al. (2012). A smear of NBT stained cells was made on a glass slide and the cells were viewed under a microscope.

Statistics

All the calculations were done with Graph pad prism version 6.0 and represented here as mean ± standard error of mean (SEM). Spearman correlation was used to analyze statistical significance with $p < 0.001$ and $p < 0.01$. Sperm DNA integrity or damage was calculated with help Gel-Pro preprogram and represented here in % (SBST, VIT University, Vellore). The percentage of sperm DNA fragmentation was measured by the following formula:

$$\%\,\text{DNA fragmentation} = \frac{\text{OD}_{575}\ \text{of seminal plasma} \times 100}{\text{OD}_{575}\ \text{of seminal plasma} + \text{OD}_{575}\ \text{of sperm cells}}.$$

Results

Qualitative results of *T. arjuna* bark

In the initial process with aqueous and methanol extract of *T. arjuna* bark, most of active compounds like phytosterols were obtained in high concentration; along with these, active compounds like triperpenoids, alkaloids and carbohydrates are also present and are shown in Table 1. The *T. arjuna bark*, with DPPH free radical antioxidant scavenging activity, is shown as yellow spot in TLC plate (Fig. 1), implying its strong antioxidant activity.

In the TLC plate, blue color formation indicates the presence of flavonoid in *T. arjuna* bark, which is shown in Fig. 2 (chloroform: toluene: methanol, in the ratio of 4:4:1). Both the antioxidant and flavonoid content travelling points, Rf, are 0.2 and 0.4 (Chloroform: Toluene: Methanol (4:4:1, v/v/v), with the anisaldehyde–sulfuric acid as a revealing reagent). The total phenol content was found to be 190 mg quercetin equivalent/g of dried aqueous extract in *T. arjuna* bark. The total flavonoid content in *T. arjuna* bark was found to be 120 mg/g of dried aqueous extract.

Antioxidant activity

Electron giving potential is directly proportional to major antioxidant activity. In this study, we found that *T. arjuna* bark aqueous extract in a dose-dependent manner (200 µg/ml) exhibits high hydrogen peroxide scavenging activity which was found to be IC_{50} 92 µg/ml. Hydrogen peroxide radical scavenging activity compared with methanol

Table 1 Preliminary phytochemical analysis of *T. arjuna* bark

Phyto constituents	Test	Aqueous	Methanol
Phytosterols	Salkowski reaction	+ ++	+
Triterpenoids	Liebermann–Burchard's test	++	+
Saponins	Foam test	+	+
Alkaloids	Dragndroff's test	++	+
Carbohydrates	Molisch's test	++	+
Flavonoids	Lead Acetate test	+++	+
Lactones	Legal's test	+++	+
Phenolic compounds and Tannins	5% feel test	+++	−
Proteins	Ninhydrin test	+	−
Glycosides	Keller–Killiani test	+	−

+ low concentration, ++ high concentration, +++ present in very high concentration, − very low concentration

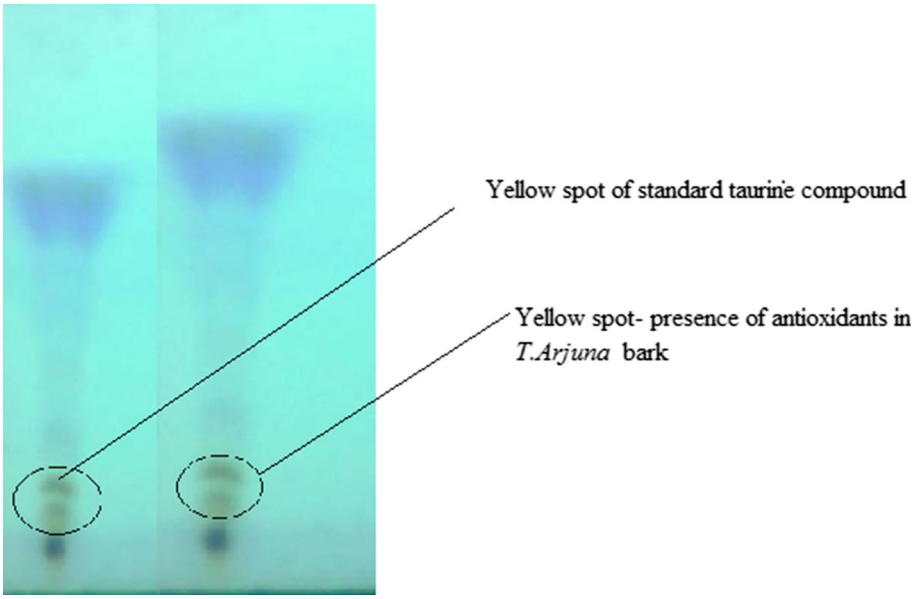

Fig. 1 TLC antioxidant activity analysis of *T. arjuna* bark extract constituents

extract of *T. arjuna* bark using ascorbic acid as the standard is shown in Fig. 3. In *T. arjuna* bark aqueous extract, DPPH radical scavenging was found in a dose–dependent manner (200 µg/ml) with IC_{50} 95.38 µg/ml. The results are expressed in mean ± standard deviation with standard ascorbic acid and methanolic *T. arjuna bark* extract which is shown in Fig. 4.

Reducing power activity

The aqueous extract *T. arjuna* bark showed good reducing power activity based on the increasing dosage of extract and it was observed as 1.77 ± 0.08 at 1000 µg/ml. The best reducing activity against standard ascorbic acid and methanolic extract is shown in Fig. 5.

Aqueous *T. arjuna* bark extracts metal analysis and confirmation

The initial FT- IR peaks (Fig. 6) showed the presence of metal constituents in the extract; in the same aqueous *T. arjuna* bark, zinc was found to be more when compared to other metals like (Ca = 0.5 µg/g; Se = 2.2 µg/g and Mg = 1.6 µg/g). About 16 µg/ml of zinc was found in AAS detection with respect to zinc chloride standard. Detection range was 550–580 nm.

Semen parameter analysis

Semen parameters assessed using CASA are given in Table 2. When compared to non-smokers, smokers have

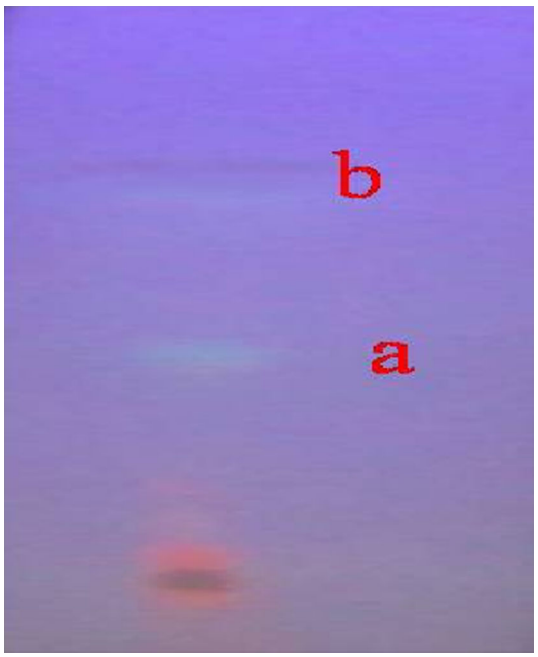

Fig. 2 TLC analysis for flavonoid compounds in *T. arjuna* bark aqueous extract

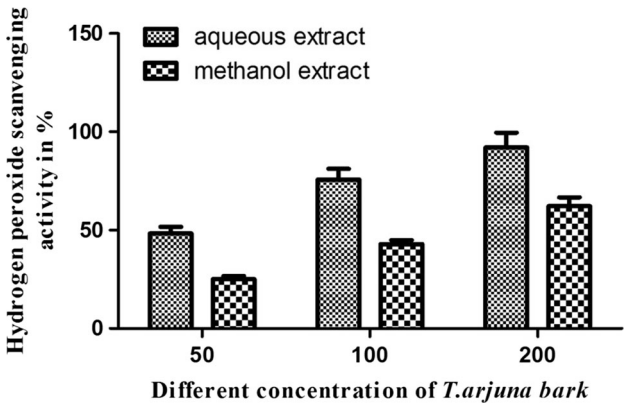

Fig. 3 Scavenging of hydrogen peroxide of *T. arjuna* bark extract

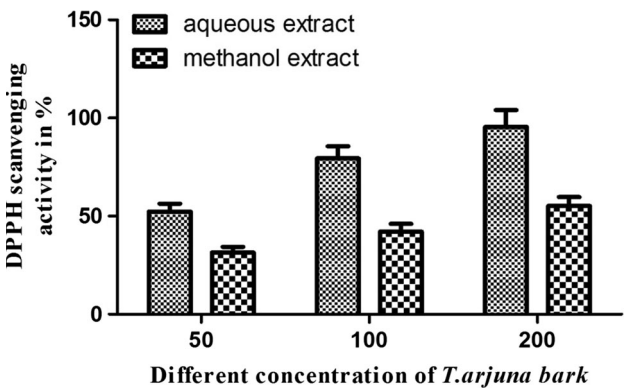

Fig. 4 DPPH radical scavenging activity of *T. arjuna* bark extract

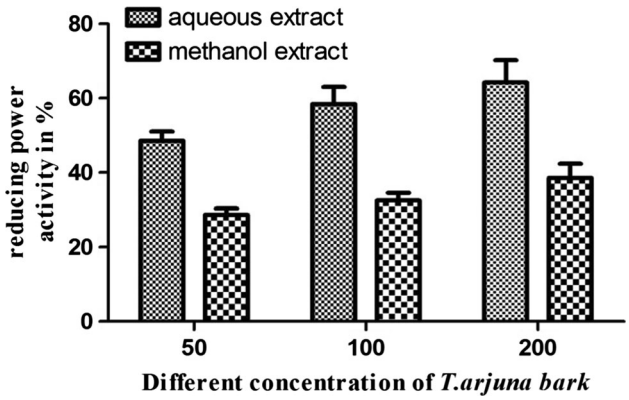

Fig. 5 Reducing power activity of *T. arjuna* bark extract

lower progressive motility as well as lower normal morphology of sperms. Sperm morphology of head to tail piece connection was altered in smokers due to Cadmium toxicity. Seminal zinc values and smoking altered sperm DNA fragment in smokers and non-smokers are given in Table 3.

Sperm DNA damage inhibition results

Most of the active components were high when compared to methanol, and hence, we chosen aqueous extract for sperm DNA damage inhibition analysis. The major evaluation of this in vitro study proved that the aqueous extract of *T. arjuna* bark has potential to inhibit sperm DNA damage against smoking released reproductive metal toxicants like cadmium and lead which is shown in Fig. 7. Meanwhile, the change in DNA fragmentation and integrity before and after treatment with *T. arjuna* bark is listed in Tables 4 and 5. In future, this plant can be expected to act as a source of spermatogenesis boosters or zinc supplementary cryo protectors for cryo injuries in environmental or occupational released sperm disorders. NBT staining of sperms shown in Fig. 8 shows that further DNA fragmentation was arrested after incubating with *T. arjuna* bark.

Discussion

Compromised fertility/infertility is a major health issue both males and females (Sofowara et al. 1993). Amalraj and Gopi (2017) and Momin and Satardekar et al. (2017) reported that *T. arjuna* is rich in phytochemicals (carbohydrates, proteins, phenols, terpenoids, triterpinoids, flavonoid, saponins, glycosides, and alkaloids), antioxidants, and antimicrobial agents. These compounds have higher medicinal efficacy and elicit many physiological activities. All over the world, different parts of this tree have been reported to possess antioxidants and various medicinal

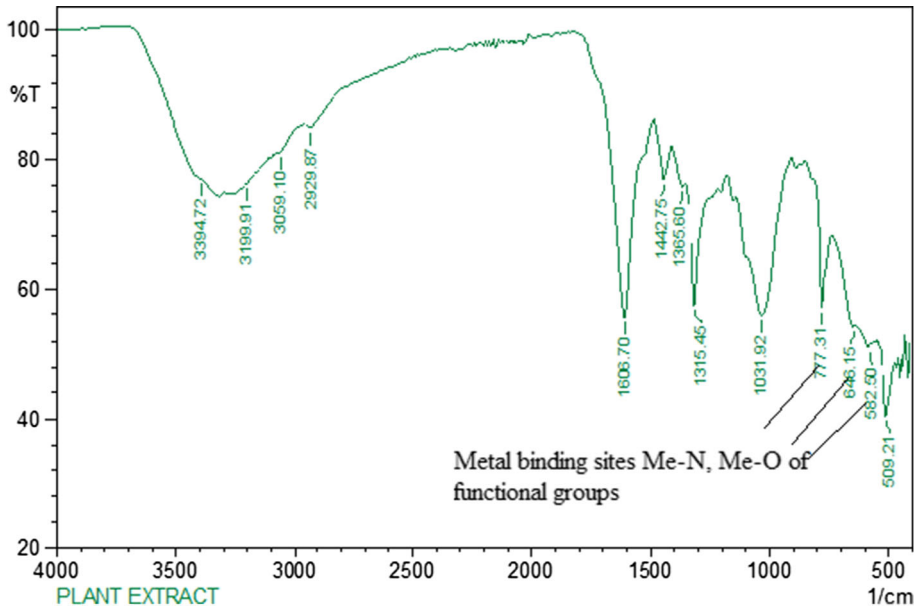

Fig. 6 *T. arjuna bark* aqueous extract of Aqueous Fourier Transform and Infrared (AQ FT-IR) showing the functional groups

Table 2 Statistical values of semen parameters in selected subjects

Semen parameters	Fertile ($n = 34$)	AST infertile smokers ($n = 25$)
pH	7.411 ± 0.025	7.990 ± 0.032
Volume (mL)	2.839 ± 0.073	1.120 ± 0.092
Count × 10^6	49.46 ± 0.746	9.20 ± 3.267
Morphology (%)	27.71 ± 0.966	3.33 ± 0.366***
Total motility (%)	49.750 ± 0.603	32.50 ± 2.535
Rapid progressive motility (%)	43.21 ± 0.906	2.31 ± 0.7551**
Slow progressive motility (%)	0.3036 ± 0.026	29.54 ± 6.242
No. of cigarettes/day (SI)	–	12.78 ± 0.97
Age (years)	28.32 ± 1.09	32.45 ± 3.56

Values are presented here Mean ± SEM

Significance: ** $p < 0.01$ and *** $p < 0.001$ (0.865 & 0.798)

AST asthenoteratospermic

Table 3 Statistics of seminal biochemical and DNA damage markers

Semen parameters	Fertile ($n = 34$)	AST infertile smokers ($n = 25$)
Zinc (mg/ml)	8.79 ± 1.02	0.25 ± 0.001**
DNA fragmentation in (%)	9.85 ± 0.73^{NS}	17.40 ± 1.77***

Values are presented here: Mean ± Standard error of mean (SEM)

Significance ** $p > 0.01$ and *** $p > 0.001$, non-significant (NS)

agents (which have anti-arthritis, anti-lipid, and anti-diabetic activity) in different in vivo *and* in vitro studies (Hancock et al. 2001; Jain et al. 2013; Kumar et al. 2013; Stangeland et al. 2009). *T. arjuna is* one of the many epidemic medicinal plants used for various medicinal and pharmacological studies for degenerative disorder. In this present study, preliminary phyto chemical analysis showed that *T. arjuna* bark aqueous extract contains higher amount of flavonoid and phenolic contents; this implies that the natural antioxidants were higher, irrespective of high phenolic and flavonoid content. Here, *T. arjuna* bark extract showed higher amount of metals like zinc and

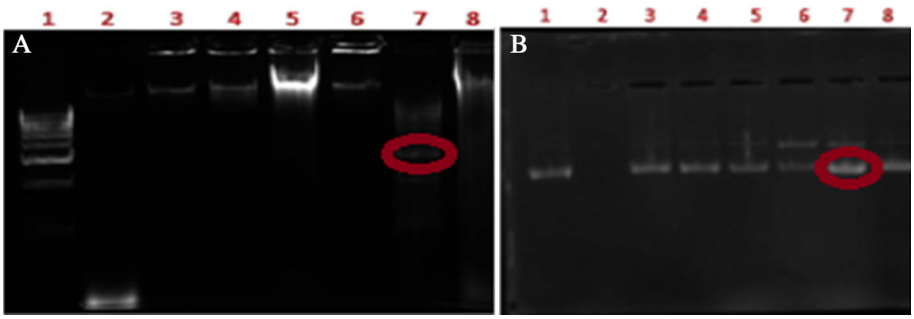

Fig. 7 Sperm DNA damage inhibition assay in smokers infertile and fertile non-smokers by aqueous *T. arjuna Bark extract:* an in vitro study. The *rounded bands* are showing higher intensity with highly protected by *T. arjuna bark*

Table 4 Statistical values of seminal plasma % of DNA fragmentation results in series of before and after *T. arjuna* bark incubation

Incubation (mins)	Before addition of *T. bark* to AST infertile ($n = 25$) negative control	With *T. bark* AST infertile ($n = 25$)	Without incubation with *T. bark* control samples (fertile) ($n = 34$)
0 mts	17.40 ± 1.77	17.40 ± 1.77	9.12 ± 0.05
1 mts	19.83 ± 0.71	17.40 ± 1.75	9.14 ± 0.09
10 mts	19.92 ± 0.70	17.38 ± 1.75	9.15 ± 0.11
15 mts	21.79 ± 0.68	17.20 ± 1.72	9.18 ± 0.15
30 mts	22.75 ± 0.68	17.1 ± 1.71	9.21 ± 0.18
45 mts	24.72 ± 0.63	16.90 ± 1.23	9.24 ± 0.21
60 mts	25.60 ± 0.60	16.92 ± 0.98	9.28 ± 0.25

Table 5 Semen genomic DNA protection activity of sperm DNA integrity checking with *T. arjuna* bark extract of in vitro study

Lane	Contents	Sperm DNA integrity in AST infertile smokers (%) ($n = 25$)	Fertile (%) ($n = 34$)
1	Untreated infertile smokers semen	12.21	13.054
2	Control semen + H_2O_2 (2 ng/µl)	11.58	11.81
3	Control semen + H_2O_2 + UV treatment	11.60	8.981
4	Semen + H_2O_2 + *T. arjuna Bark* treated	11.66	16.53
5	IF smokers semen + *T. arjuna bark* (5 ng/µl)	11.65	16.62
6	IF smokers semen + *T. arjuna bark* (25 ng/µl)	11.72	16.98
7	IF smokers semen *T. arjuna bark* (50 ng/µl)	11.89	17.21
8	Known control plasmid pBR 322	12.68	12.68

IF infertile

Fig. 8 NBT staining of sperms, before and after aqueous *T. arjuna bark* incubation

selenium. The aqueous extract of *T. arjuna* bark showed good antioxidant activity due to higher flavonoid content; this will be helpful to therapeutic and traditional medicine (Nema et al. 2012; Biswas et al. 2011). Oxygen species are able to initiate or increase the severity of different diseases. ROS is a well-known causative agent for any type of DNA damage in the early (spermatogenesis) or developed stages of human sperms in cigarette smokers (Taymour 2010). Partial damage in sperm DNA will cause cancer in younger generations who smoke. Only limited medicinal plants are reported to have the ability to inhibit DNA damage and to stop or cure the free radical induced damages (Wang and Jiao 2000; Yadav and Agrawala 2011; Devasagayam et al. 2004). Hence, in this study, the capability of aqueous extract of *T. arjuna* bark to inhibit sperm DNA damage was assessed. Results show that it possesses great inhibition activity against cigarette smoking induced sperm DNA damage. DNA fragmentation was observed in semen samples (without incubation with *T. bark* from both) of fertile non-smokers and infertile smokers. DNA fragmentation in these samples increased with time. However, in the semen samples treated with *T. bark,* DNA fragmentation was arrested after incubation with *T. bark*. Release of reproductive toxicant Cadmium from smoke is antagonistic to seminal plasma zinc which affects the sperm motility and morphology which is seen in NBT stained sperms of smokers. Smoking leads to zinc deficiency in semen (Vickram et al. 2013). Based on our unpublished report, this *T. arjuna* bark has the highest Zinc content and hence able to function as a Zn supplement in body defense as smokers are majorly deficient in seminal Zinc at the time of semen ejaculation. Further studies are needed to prove the ability of *T. arjuna* bark extract to act as a nutrient cryo protector of in vitro or in vivo studies of spermatogenesis. Aqueous *T. arjuna* bark extract had higher zinc potential which reduces reproductive metal toxicants like cadmium and lead toxicity in smokers. Many studies reported its free radical induced DNA damage inhibition efficacy in blood and tissue (Guha et al. 2011; Kalita et al. 2012; Priya et al. 2012). Our results evaluated that aqueous *T. arjuna* bark can inhibit the sperm DNA damage in smoker's semen due to its high zinc content. From our earlier studies, it is proved that *T. arjuna* bark is apt to use as semen extender—cryo medium which supplements Zinc nutrition to sperm cells (Parameswari et al. 2017).

Conclusion

Aqueous *T. arjuna bark* extracts possess different kinds of phytochemical activity, which can comfortably defend against free radicals induced sperm DNA damage in smoker semen subjects; it also had higher natural antioxidants. This is the first report on the use of this tree to assess DNA damage inhibition in AST smoker's semen, and hence, this can be used as a cryo protect medium in future.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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