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ORIGINAL ARTICLE

RNA-sequencing attest increased sperm motility in bovine spermatozoa treated with ethanolic extract of *Putranjiva roxburghii*

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Abstract

In the course of time, scientific communities have a growing interest in understanding ethano medicines. The *Putranjiva roxburghii*, a native plant of the Indian Subcontinent is described as a "Child amulet tree" in Ayurveda. Based on the fact that this herbal medicine has an indispensable component of integrative medicine, the present study was planned to assess the effect of ethanolic dried extract of *Putranjiva* seeds on the motility of X and Y-bearing bovine spermatozoa. The in-vitro effect of seed extract diluted in S-TALP medium on bull semen has been evaluated by Computer Assisted Semen Analysis (CASA) shows a marked increase in the motility of spermatozoa. Motile and non-motile spermatozoa have been separated by glass wool column from the control as well as treated group. The X and Y-bearing sperm quantification have been carried out by droplet digital polymerase chain reaction (ddPCR). The extract didn't exert any differential effect on the motility and viability of X and Y chromosome-bearing spermatozoa. The transcriptome profiling (RNA-Seq) identified 93 differentially expressed genes between the extract treated and control group. It unveils the up-regulation of CATSPER, AKAP3, SPAG, ADAM1B, ADAM2 and ADAM32 genes that are involved in increasing sperm motility. Transcriptome profile also unveil the expression of ZAR1, CYP17A1, APPL2, HOXB4 and SP9 genes involved with embryonic development processes in *Putranjiva* extract-treated motile spermatozoa. The results envisaged the medicinal value of *Putranjiva* herb on increased fertility due to combinatory effect like increased sperm motility and favourableness on embryogenesis. The study ruled out the possibility of herbs having any biased effect on the selection of either male or female-bearing spermatozoa in the bull.

Keywords *Putranjiva roxburghii* · CASA · ddPCR · RNA-sequence profiling · Infertility

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Abbreviations

P. roxburghii	*Putranjiva roxburghii*
CASA	Computer assisted semen analysis
KEGG	Kyoto Encyclopedia of Genes and Genomes
MAPK	Mitogen-activated protein kinase
FPKM	Fragments per kilobase million
ddPCR	Digital droplet polymerase chain reaction
S-TALP	Sterile-Tyrode's albumin lactate pyruvate
VCL	Curvilinear velocity
VAP	Average path velocity
VSL	Straight line velocity
LIN	Linearity
STR	Straightness
WOB	Wobbling Index
BCF	Beat-cross frequency
ALH	Amplitude of lateral head displacement
DNC	Dancing
DNM	Dancing mean
MAPK	Mitogen-activated protein kinase

Introduction

A semen examination is a first-level diagnostic test for male infertility, as it can provide information about sperm fertility potential. However, the implementation of several assisted reproductive techniques has led to the observation that normal sperm parameters are not always capable of inducing pregnancy in a healthy woman. Therefore, the growing interest in ethno-medicine-based therapeutic drug development to resolve the infertility problem of a male is promising. The foundation of ethno medicine is racialized; therefore, the principle of epigenetics is a well-established feature that is essential in the treatment of any disease.

Semen quality is generally assessed by sperm motility, morphology, and concentration via a semen analysis, and it is the first step in diagnosing male factor infertility. The ability of sperm to migrate appropriately through the female reproductive tract or through the water to reach the egg is referred to as sperm motility. It is well understood that sperm motility is an essential element in determining the quality of sperm. One of the most common causes of male infertility is a dearth of sperm motility. Many studies have shown that treating subnormal semen samples with certain chemicals prior to artificial insemination improves their fertilizing capacity such as stimulants, caffeine, pentoxifylline, relaxin, prostaglandin E, diltiazem, flunaoine, captopril, trifluoperazine, bromocriptine, etc. (Fenichel et al. 1996; Liu et al. 2004).

Putranjiva roxburghii is a well-known plant assigned an important role in Ayurvedic texts as an antipyretic, anti-inflammatory and anti-rheumatic herb useful in gynaecology and fertility ailments (Gupta 2016). The "*Putranjiva*" is a small genus of trees distributed in the Indo-Malaysian region. The species commonly seen in India is *P. roxburghii*, known as a child's amulet tree or child-life tree. *P. roxburghii* has been the centre of controversy owing to its nomenclature and ability to beget a male child. The concept of changing the sex of a baby post-conceptionally has been documented in ancient Ayurvedic texts. Pumsavana is one of the Shodasha Karmas (Sixteen rituals) performed in ancient India for begetting the desired progeny. In the Samhitas certain herbs and methods are mentioned for Pumsavana karma (Vidhya and Nishteswar 2015). The phytochemical analysis of *Putranjiva* ethanol extract shows the presence of alkaloids, carbohydrates, fixed oil, saponins, caumarins, sterols, aminoacid and terpenoids (Emasushan and Britto 2018).

Mammalian ejaculated spermatozoa are highly differentiated terminal cells with a haploid genomic nucleus that is strongly compressed. These sperm RNAs are still necessary for spermiogenesis, spermatogenesis, and embryo development and these RNA transcripts may play important roles in spermatozoa development, chromatin repackaging, genomic imprinting, and possibly zygote development, according to recent research (Miller et al. 2005). Spermatozoal transcripts also affect the offspring's phenotype, which could aid in the diagnosis and treatment of male infertility. Because species-specific discrepancies are to be expected, it is necessary to document the composition of spermatozoal transcripts for each species to understand the factors involved in spermatogenesis, failed fertilisation, embryonic mortality, failures in interspecies hybrid reproductive processes, somatic cell cloning, and other reproduction-related manipulations (Selvaraju et al. 2017). To decipher male fertility, the whole transcriptome sequencing technique has been used. The analysis of transcript profiles could help to assess male fertility (Raval et al. 2019).

There are no data concerning the In vitro effect of ethanolic extract of *Putranjiva* seed on bull spermatozoa parameters and its transcriptome profiling. Therefore, the present study was aimed to investigate the In-vitro effect of *Putranjiva* seed ethanolic extract on bull spermatozoa parameters and unveils transcriptome profile of mature spermatozoa treated with herbal preparation compared to normal matured spermatozoa.

Material and methods

Preparation of S-TALP media

A Tyrode's albumin lactate pyruvate (TALP) media is typically used as a base for in-vitro study of capacitation and fertilization (Table S1). S-TALP was prepared as described by Parrish (2014), with slight modifications and a pH was

adjusted to 7.6. For minimisation of contamination, we added 4 µl Gentamicin (50 mg/ml, Sigma-aldrich, USA) to media and storage at—4 °C. for further use.

Preparation of *Putranjiva* seed ethanolic extract

Five grams of the selected mature seeds of *P. roxburghii* (*Putranjiva*) were weighed and ground manually using mortar and pestle. For defatting, the fine powder of *Putranjiva* seeds was soaked in n-Hexane in a ratio of 1:10 (powder:*n*-hexane) for 90 min at room temperature in a static condition. The supernatant was aspirated, and the residual powder was allowed to dry for 2 h on a hot plate at 40 °C, followed by soaking the residual powder in absolute ethanol for 24 h at room temperature in a ratio of 1:10 (powder:ethanol) with continuous shaking on the orbital rocker. After incubation, the supernatant was collected in a 50 ml glass beaker and kept at 40 °C on a hot plate until the complete evaporation of ethanol. Remaining dried extract was diluted in 10 ml S-TALP media and stored at 4 °C temperature for further use and was considered as stock seed extract.

Semen collection and evaluation

Semen utilized in this study was acquired from the artificial vaginal ejaculate of Gir bulls (*Bos indicus*) as per Indian Animal Ethics Guidelines and with approval from the Institutional Animal Ethics Committee, Anand Agricultural University. Semen was collected from the Department of Veterinary Gynecology (CVASH, AAU, Anand, India) during the morning hours between 7.30 a.m. to 8.30 a.m. and transported to the research facility. The ejaculates were collected in the artificial vagina over the dummy buffalo bull at 42–43 °C temperature, as described by Salisbury and VanDemark (1961). Immediately after collection, the tubes containing semen were placed in a water bath maintained at 34 °C and the samples were soon evaluated for various gross and microscopic seminal characteristics.

Somatic cell removal

The semen samples were transferred to a 15 ml sterile centrifuge tube and centrifuged at 1500 rpm for 5 min at room temperature. The resultant sperm pellet was re-suspended in 3 ml S-TALP media. The sperm count was carried out using a haemocytometer.

Treatment of seed extract to sperm:

The five different concentrations (2.5, 5.0, 10.0, 20.0 and 40.0 mg/ml) from stock seed extract were made in S-TALP media. Along with above mentioned five concentrations of seed extract, 1 ml of S-TALP and 1 ml of the stock seed extract (50.0 mg/ml) were taken as a control during all the experiments. A total of 0.1 million spermatozoa from semen samples were treated with each different seed extract in a 2 ml microfuge tube and incubated in a CO_2 incubator for 1 h at 37 °C. After treatment of spermatozoa with the aforementioned conditions, sperm motility and viability were observed under a trinocular compound microscope. Based on the initial evaluation of treated spermatozoa under a microscope, the different concentrations were selected for CASA system evaluation, where sperm motility and live sperm concentration were higher. The Statistical analysis was performed using SPSS 14.0 statistical software for Windows (SPSS Inc., Chicago, IL, USA). All the data were normally distributed. The paired sample *t*-tests were used to evaluate the variable under the study. The $p < 0.05$ were considered statistically significant.

Separation of motile and non-motile sperm

The motile spermatozoa were separated with the aid of the glass-wool column by following the method of Van der Ven et al. (1988). Motile sperm that passed through glass wool was collected in a 1.5 ml microfuge tube for ddPCR and transcriptome profiling. Dead and non-motile spermatozoa trapped within the column were also collected for ddPCR and transcriptome profiling.

DNA extraction from motile and non-motile spermatozoa

The DNA was extracted from separated motile and non-motile spermatozoa using a protocol of Aravindakshan et al. (1998). The quality of DNA was checked by NanoDrop 1000 Spectrophotometer.

Quantification of X and Y spermatozoa

The quantification of X and Y spermatozoa was carried out from motile and non-motile spermatozoa with ddPCR. For ddPCR amplification of SRY (Y-chromosome specific) and F9 (X-chromosome specific) gene, target-specific primers and TaqMan hydrolysis probes were designed as per the manufacturer's guidelines. For absolute quantification and duplex assay development, FAM and VIC fluorescent dye combinations were used.

Transcriptome profiling of *Putranjiva* seed extract-treated spermatozoa

The concentration at which the higher motility of spermatozoa recorded was selected for transcriptome profiling. Four different samples have been selected for transcriptome profiling, which includes a control motile spermatozoon, control

non-motile spermatozoa, seed extract treated motile spermatozoa and non-motile spermatozoa. All four samples were centrifuged at 2500 rpm for 6 min at 34 °C temperature, and pellets were formed. Each pellet was diluted in 1 × PBS (each 7 µl sample containing 600 sperms) for further use. Total RNA and WTA (Whole Transcriptome Amplification) from a single cell, its enzymatic fragmentation and sequencing library preparation were performed using QIAseq FX Single Cell RNA Library Kit (Qiagen, Germany) according to the manufacturer's protocol. All the prepared transcriptome libraries were sequenced on Illumina MiSeq (Illumina, USA) according to recommended protocols.

Bioinformatics analysis

Sequences were mapped against *Bos taurus* genome build 4.6.1 using STARaligner v2.6. Transcriptome assembly and differential gene expression analysis were performed using Cufflinks. DEGs between gropus were identified using the 'Cuffdiff' 'module with absolute $\log_2$ fold change > 2 between different groups and a false discovery rate (FDR) of $p < 0.05$. After differential gene expression analysis, gene ontology was carried out from commonly present highly abundant genes with the help of DAVID v6.8.

Results

Gross and microscopic seminal characteristics of semen

The mean ejaculate volume of semen recorded in the Gir bull understudy was 6.73 ± 0.12 (range 5–8) ml. The colour of semen was found to be milky white to yellowish, which is the normal colour of cattle bull semen. The normal colour of semen found in all bulls suggested that the genital tracts of all the bulls were healthy without any infection or trauma and the ejaculates were free from any contaminants. The mean sperm concentration of semen recorded in bull was 1111.57 ± 29.46 MM/ml. The mean mass activity scores (0–5 scale) and the percentages of initially motile and live sperms in the semen of bull were 3.35 ± 0.08 and 81.50 ± 1.30% to 83.70 ± 1.04%, respectively.

Microscopic evaluation of spermatozoa after treatment of seed extract

On the detailed microscopic observation, the high live sperm count, and highest mortality were found in the sperm treated with the 10 mg/ml and 20 mg/ml concentration of seed extract. The live sperm percentages were 78.75 ± 4.7 and 55 ± 10.8, and the motility of sperm was 3.12 ± 0.2 and 2.75 ± 0.5 for 10 mg/ml and 20 mg/ml treated samples, respectively (Table 1). These effective concentrations (range 10–20 mg/ml) were chosen for a further detailed evaluation of the effects of seed extract on spermatozoa.

Table 1 Sperm motility and live sperm percentage under effects of *Putranjiva* seed extract with comparison of other control group

Sr no	Putranjiva seed extract treated group (mg/ml)	Live sperm (%) ($n = 5$)	Motility (0–5 scale) ($n = 5$)
1	S-TALP (1 ml)	66.25 ± 4.7	2.75 ± 0.2
2	2.5	63.75 ± 7.5	1.87 ± 0.6
3	5.0	65 ± 4.08	1.75 ± 0.5
4	10.0	78.75 ± 4.7	3.12 ± 0.2
5	20.0	55 ± 10.8	2.75 ± 0.5
6	40.0	38.75 ± 6.29	2.12 ± 0.2
7	50.0 (stock)	10 ± 4.08	1.37 ± 0.4
8	1X PBS (1 ml)	68.75 ± 2.5	2.5 ± 0.5

Evaluation of spermatozoa after treatment of seed extract by CASA

The sperm motility profile of seed extract-treated spermatozoa was observed soon after giving the plant extract treatment by the Biovis CASA system. The analysis set up appropriate for treated spermatozoa was mentioned in table S2. The evaluation of CASA results had shown a significant ($p < 0.05$) increase in sperm motility with 15 and 17.5 mg/ml concentration of seed extract in the control group (Fig. 1, Table S3). Along with increased spermatozoa motility, the percentage of non-progressive and slow-progressive spermatozoa was significantly ($p < 0.05$) higher in the seed extract-treated group. However, the rapid progressive spermatozoa percentage was found higher in 15 mg/ml concentration than in control, 1X PBS treated as well as 17.5 mg/ml concentration (Table S3). Due to the higher sperm motility percentage in sperm treated with 15 mg/ml, this dose is selected for further experiments.

Sperm velocity/kinematics assessment

The CASA analysis of freshly treated spermatozoa depicted the overall mean sperm velocity and kinematics parameters based on total motile and progressively motile sperms. The velocity curvilinear (VCL), velocity average path (VAP), and velocity straight line (VSL) of progressive motile sperms observed in bull semen were 69.45 ± 33.1, 33.44 ± 15.9 and 30.62 ± 14.9 µm/s, respectively in sperm treated with 15 mg/ml of seed extract and was 81.58 ± 13.9, 39.97 ± 17.4 and 37.77 ± 18.1 µm/s, respectively in sperm treated with 17.5 mg/ml extract. The mean values of another kinematics

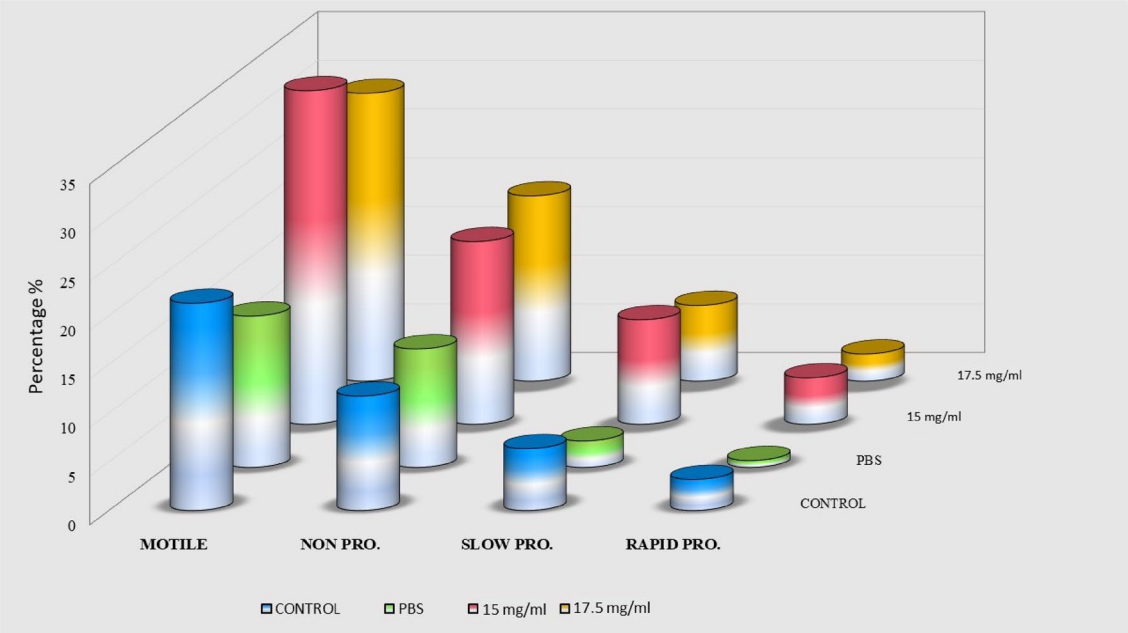

Fig. 1 Comparative evaluation of sperm motility profile in different conditions: total four groups that include control group, PBS (phosphate buffer saline) group, 15 mg/ml and 17.5 mg/ml concentration of *Putranjiva* seed extract treated group

Table 2 Mean (±SE) spermatozoa kinematics assessment with a comparison of treatment group spermatozoa and other controls

Seminal attribute	Normal value	Control	1X PBS	300P (15 mg/ml)	350P (17.5 mg/ml)
Curvilinear velocity (VCL, µm/s)	88.95 ± 1.72	89.22 ± 16.1	79.62 ± 15	69.45 ± 33.1	81.58 ± 13.9
Average path velocity (VAP, µm/s)	53.14 ± 1.24	41.71 ± 12.6	34.77 ± 5.1	33.44 ± 15.9	39.97 ± 17.4
Straight line velocity (VSL, µm/s)	49.39 ± 1.28	38.97 ± 13	32.02 ± 5.7	30.62 ± 14.9	37.77 ± 18.1
Linearity (LIN, %)	56.05 ± 1.21	38.54 ± 19	40.17 ± 3.7	38.31 ± 17.1	45.04 ± 13.7
Straightness (STR, %)	92.05 ± 0.39	92.14 ± 5.9	91 ± 4.7	77.9 ± 34.4	91.92 ± 3.8
Wobbling Index (WOB, %)	60.25 ± 1.08	46.85 ± 11.3	43.82 ± 3.5	41.84 ± 18.7	48.02 ± 12.7
Beat-cross frequency (BCF, hz)	16.81 ± 0.66	12.64 ± 3.9	11.15 ± 4	11.05 ± 5.2	15.14 ± 7.9
Lateral head displacement (ALH, µm)	2.35 ± 0.19	3.01 ± 0.7	2.85 ± 0.8	2.27 ± 1	3.37 ± 1.2
DNC ($\mu m^2/s$)	210.57 ± 16.67	240 ± 69.1	201.5 ± 84	161.8 ± 83.2	177.3 ± 65.4
DNM ($\mu m^2/s$)	4.48 ± 0.37	6.95 ± 3.3	6.4 ± 2.1	4.92 ± 2.4	6.6 ± 1.3

parameter (LIN, STR, WOB, BCF, ALH, DNC and DNM) were described in Table 2. After evaluation of seed extract-treated spermatozoa by microscope as well as CASA there were three different combinations (control (S-TALP), 15 mg/ml extract treated, 1 × PBS) selected for further evaluation.

Quantification of X and Y spermatozoa

A total of five, *Putranjiva* seed extract-treated samples were analyzed, including control motile and control non-motile, 15 mg/ml extract treated motile and non-motile, 1X PBS motile. The thresholding tools were used for the designation of the droplet populations as double negative (gray), FAM positive/F9 Target (blue), VIC positive (green) and double-positive (brown) (Fig. 2). 2D fluorescence plot containing two amplicons, where differences in amplitude were observed due to differences in optimal annealing temperature. The number of targets and its percentage from each sample shows in Table 3. Results for F9 and SRY did not show any significant ($p > 0.05$) percentage difference for motile and non-motile sperm (Table 3).

Transcriptome profiling of seed extract-treated spermatozoa

The treatment group (15 mg/ml) was selected to evaluate the effects of seed extract on spermatozoa's transcriptome profile compared to the control group. More than 2 million reads

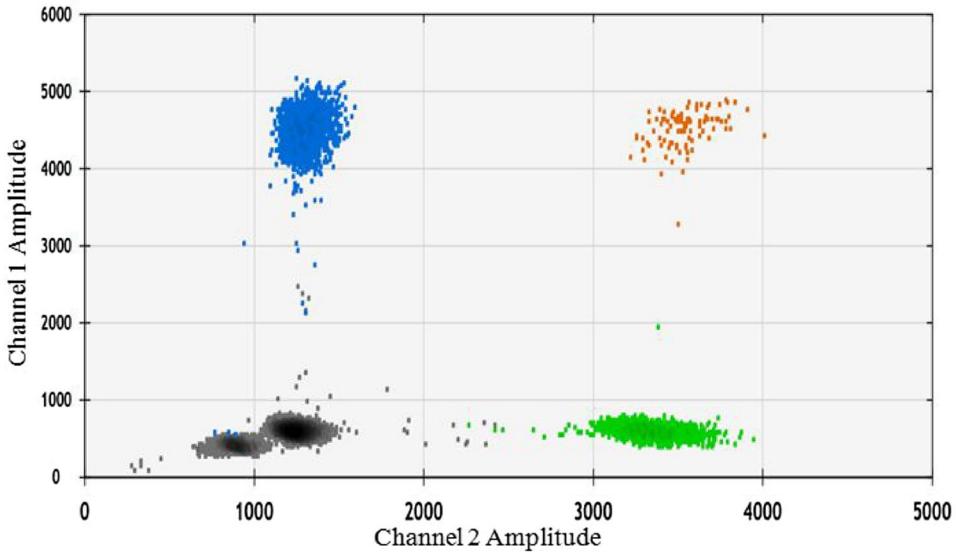

Fig. 2 In ddPCR 2D fluorescence plot containing two amplicons: gray colour in figure shows (double negative), blue colour (FAM positive/F9 target), green colour (VIC positive), brown colour (double-positive)

Table 3 F9 and SRY count from each *Putranjiva* seed extract treated and control sample

Sr. no	Sample ID	F9	Percentage	SRY	Percentage
1	300P (motile sperm)	25	51.22%	23.8	48.78%
2	300P (non-motile sperm)	31.1	47.77%	34	52.23%
3	Control S-TALP (motile sperm)	70	50%	70	50%
4	Control S-TALP (non-motile sperm)	33.6	49.77%	33.9	50.23%
5	1 × PBS (motile sperm)	43	51.86%	39.9	48.14%
6	NTC (no template control)	No call	0%	No call	0%

were generated for each sample with a mean read length of 400. When the data were further analysed, it has shown 65.80%, 82.05%, 64.34% and 92.52% of uniquely mapped reads and 34.20%, 17.95%, 35.66% and 7.48% of multiple mapped reads for control motile, control non-motile, treated motile and treated non-motile, respectively (Table S4). RNA sequence analysis identified 4143 genes were expressed with FPKM > 0, while 2111 genes were expressed at FPKM > 20 in the control motile spermatozoa sample. In the control non-motile spermatozoa sample, total of 3123 genes were expressed with FPKM > 0 and 2336 genes were expressed at FPKM > 20. Similarly, in the case of *Putranjiva*-treated motile and non-motile spermatozoa samples, 4658 and 2492 genes with FPKM > 0 and 2394 and 2404 genes identified with FPKM > 20, respectively. Top highly abundant identified genes with a description based on the highest FPKM value are depicted in table S5a-d.

Differential gene expression of control and treatment group spermatozoa

The spermatozoa treated with *Putranjiva* seed extract showed a significant change in the expression of 50 genes compared to the control sample. Out of 50 genes, 30 genes were up-regulated, while 20 genes were down-regulated in the motile spermatozoa. Seed-treated non-motile spermatozoa showed significant differential expression of 43 genes compared to the control. A total of 24 genes were found up-regulated, while 19 genes were found down-regulated (Tables 4, 5).

Functions of motile and non-motile spermatozoal transcript

The commonly expressed genes from control motile spermatozoa as well as treated motile spermatozoa were used for functional annotation. The genes associated with cell differentiation, cell division, in-utero embryonic developments, transcription and protein ubiquitination were found enriched among biological processes of the spermatozoa. Genes related to cellular components such as integral components of plasma, cell surface, cell junction, myelin sheath, cul3-RING ubiquitin ligase complex, cell body and others were significantly enriched. The genes associated with molecular functions were related to transcriptional activator activity, RNA polymerase II core promoter proximal region sequence-specific binding, nucleic acid binding and Poly(A)

Table 4 Differential gene (top ten) expression between control and *Putranjiva* seed extract-treated motile spermatozoa

Gene	Fold change	Gene description and function
Up-regulated genes		
ZAR1	27.23	Zygote arrest 1 it plays a role in the oocyte-to-embryo transition
CYP17A1	26.12	Cytochrome P450 family 17 subfamily A Member 1 involved in sexual development during fetal life and at puberty
HOXC11	26.73	Homeobox C11 has a role in RNA polymerase II proximal promoter sequence-specific DNA binding
IDH2	25.89	Isocitrate dehydrogenase (NADP (+)) 2, Mitochondrial plays a role in intermediary metabolism and energy production
APPL2	24.95	Adaptor protein, phosphotyrosine interacting with PH domain and leucine zipper 2. It is a signaling proteins to regulate cell proliferation, immune response, endosomal trafficking and cell metabolism
HOXB4	25.21	Homeobox B4. It is a sequence-specific transcription factor which is part of a developmental regulatory system
VAT1L	24.78	Vesicle amine transport 1 like.it involved in zinc ion binding
PRSS2	24.94	Serine Protease2 has serine-type endopeptidase activity and Ca binding
SRMS	25.54	Src-related kinase lacking C-terminal regulatory tyrosine and N-terminal Myristylation sites has protein tyrosine kinase activity
EPB42	24.89	Erythrocyte membrane protein band 4.2. It plays an important role in the regulation of erythrocyte shape and mechanical properties
Down-regulated genes		
IL16	− 25.91	Pro-interleukin-16 involved in cell cycle progression in T-cells
ABCF1	− 25.17	ATP binding cassette subfamily F member 1. It is required for efficient Cap- and IRES-mediated mRNA translation initiation
TOX2	− 26.33	TOX High Mobility Group Box Family Member 2 involved in the hypothalamo-pituitary–gonadal system
OTOP1	− 26.09	Otopetrin 1. It regulates purinergic control of intracellular calcium in vestibular supporting cells
CHMP4C	− 25.86	Charged multivesicular body protein 4C involved in multivesicular bodies formation and sorting of endosomal cargo proteins into MVBs
SP9	− 26.18	Sp9 transcription factor plays a key role in limb development
C13H20orf24	− 26.83	Protein-coding region of the C13H20orf24 cDNA ORF which is encoded by the open reading frame sequence
CDC34	− 26.02	Cell division cycle 34 has ubiquitin-protein transferase activity
FRMD3	− 26.92	FERM domain containing 3 involved in cytoskeletal protein binding
ADPRHL1	− 25.63	ADP-ribosylhydrolase like 1 has ADP-ribosylarginine hydrolase activity

Table 5 Differential gene expression between control and *Putranjiva* seed extract-treated non-motile spermatozoa

Gene	Fold change	Gene description and function
Up-regulated genes		
RPL31	26.73	Ribosomal protein L31 is a structural constituent of ribosome
MGC148328	26.51	uncharacterized LOC783058
RPS20	26.47	Ribosomal protein S20 is a structural constituent of ribosome
Down-regulated genes		
CDX1	− 26.57	Caudal type homeobox 1. It plays a role in the terminal differentiation of the intestine
DHRS3	− 26.67	Dehydrogenase/reductase 3. It has a role in catalyzes the reduction of all-trans-retinal to all-trans-retinol in the presence of NADPH
IFT122	− 30.12	Intraflagellar transport 122 is involved in cilia formation during neuronal patterning
LOC539818	− 31.87	Dolichyl-diphosphooligosaccharide-protein. Involved in glycosyltransferase subunit 1 pathway protein glycosylation

RNA binding. Genes related to KEGG pathways such as MAPK pathway, pathway in cancer, and others (Fig. 3).

For non-motile spermatozoa genes associated with biological processes such as positive regulation of transcription from RNA polymerase II promoter, and negative regulation

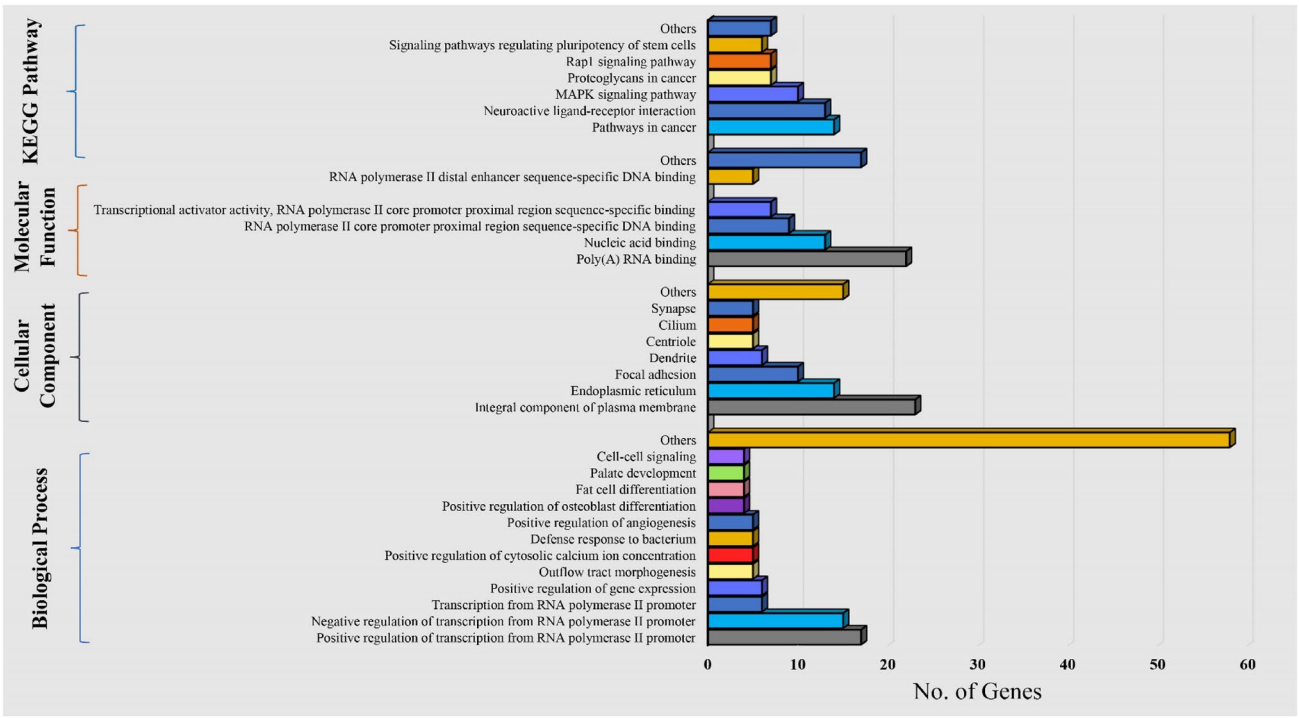

Fig. 3 Shows gene ontology graph from highly expressed common genes from control motile and *Putranjiva* seed extract treated motile spermatozoa. That genes classify into four major group (biological processes, cellular components, molecular function, KEGG pathway)

of transcription from RNA polymerase II promoter were significantly enriched in spermatozoa. An integral component of the plasma membrane, endoplasmic reticulum, focal adhesion and others were involved in cellular components. Genes associated with molecular functions such as heparin binding and others were significantly prominent in spermatozoa. Genes associated with KEGG pathways such as pathways in cancer and neuroactive ligand-receptor interaction were higher observed non-motile sperm (Fig. 4).

Discussion

In many cases of infertility, the problem encountered is low sperm motility. Advanced techniques for optimizing sperm function in this procedure are of evident value. Many herbal medicines have been reported to enhance sperm motility (Goswami et al. 2020). The present study demonstrated that dried ethanol extract of *Putranjiva* seed had considerable effects on the improvement of total motile sperm, non-progressive and slow progressive sperm cells. In the present study, CASA examination had shown a 10% increase in motility of *Putranjiva* seed-treated spermatozoa (31%) compared to the control (21%). In the present study, a sperm kinematics assessment such as VCL, VSL, LIN, VAP, ALH, DNC and DNM parameter does not show a significant difference compared to the control group. Results of sperm kinematics study on seed extract-treated spermatozoa are strongly in accordance with some previous reports (Das et al. 2015; Goswami et al. 2020).

The ddPCR did not show a significant difference in the percentage of X and Y spermatozoa in the control and treated group. Both treated and control has an average of 50% of X and Y sperm content. These findings indicate that there is no apparent impact of the treatment with *Putranjiva* extract on X or Y spermatozoa. So, it is nothing but might be a misconception related to its name, as providing any special powers to deliver only male offspring. However, it has historically been used to improve fertility and cure gynaecology and other illnesses.

The transcriptome profiling of the control as well as treated group had showed approximate profile of 14 K genes. The majority of the abundant spermatozoa transcripts were intact, and these transcripts might play a role in sperm function, fertilization events and early zygote development upon their transfer to the oocytes (Sendler et al. 2013). In the present study, we observed that treated motile spermatozoa showed significant differential expression of ZAR1, CYP17A1, APPL2, HOXB4 and SP9 genes involved in the embryo development process. The transcripts (MAP7, PTK2, PLK1S1, MYH9 and PRKCZ) involved in centrosome organization were observed in control, and treated spermatozoa, implying that the presence of these transcripts

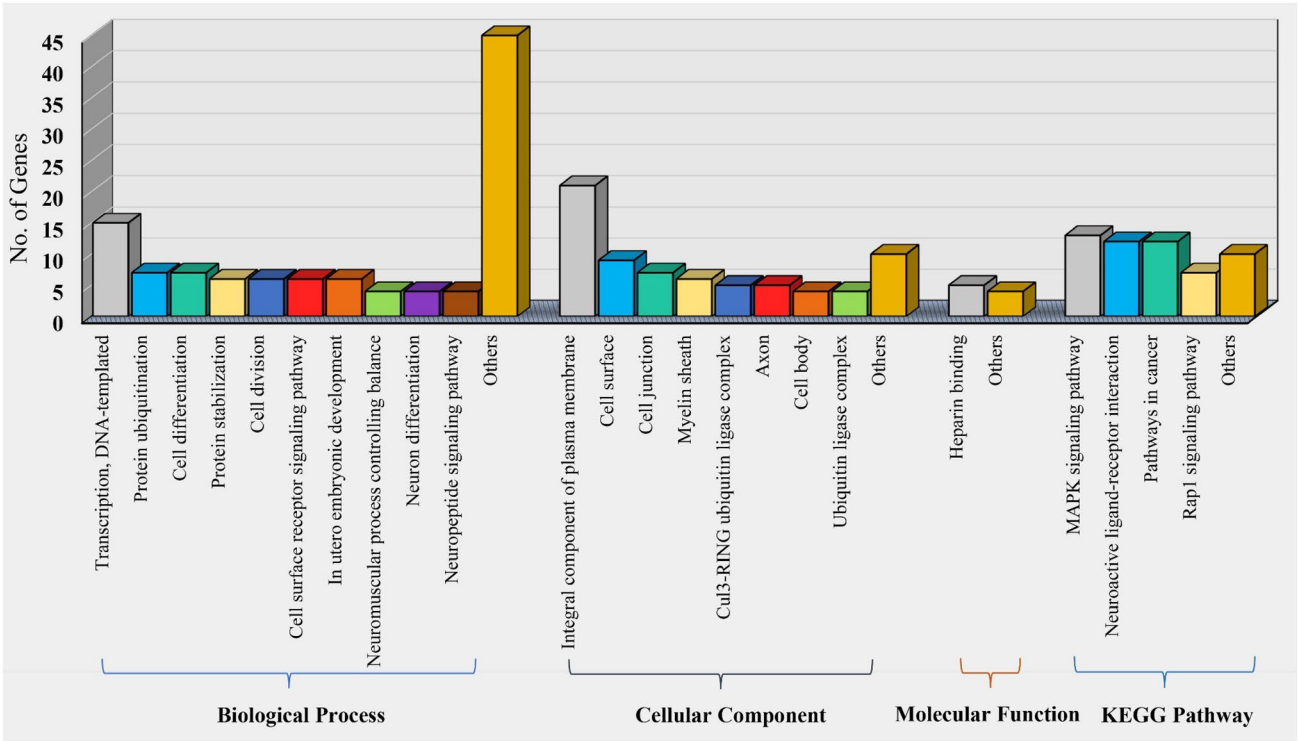

Fig. 4 Shows gene ontology graph from highly expressed common genes from control non-motile and *Putranjiva* seed extract treated non-motile spermatozoa. That genes classify into four major group (biological processes, cellular components, molecular function, KEGG pathway)

might reflect events associated with spermatogenesis and sperm function (Comizzoli et al. 2006).

The presence of transcripts associated with motility, such as CATSPER and SPAG, indicates male fertility and a knockout study has shown that SPAG genes are also important for spermatozoa structural integrity (Tamburrino et al. 2014). The SPAG transcript was expressed in the control and treated group, whereas CATSPER transcripts were highly expressed (FPKM: 2780.34) in the treatment group compared to the control group. In the treated group, we observed a high expression of *AKAP3* (*FPKM:2342.52*) transcript, which has a role in sperm motility and capacitation. Spermatozoa carry fertilin transcripts (ADAM1B, ADAM2 and ADAM32) along with the corresponding proteins. These proteins regulate sperm motility in the reproductive tract and sperm-zona pellucid binding and penetration (Civetta 2003; Gur and Breitbart 2008). Thus, an absence of these proteins might affect fertility (Chen et al. 1999). Spermatozoal motility is correlated with mitochondrial function, which regulates energy generation, apoptosis and calcium homeostasis. The study confirms the existence of embryogenesis-associated transcripts ZP3, which is highly expressed in the treatment group compared to the control group.

The studies conducted so far suggest that spermatozoal transcripts have a role in fertilization and early embryonic development. In the present study, placental development-associated transcripts such as PAG5, PAG7 and PAG10, were observed in both control and treatment groups. In mouse, cold shock protein with nucleic acids binding properties, YBX2 regulates male fertility (Yang et al. 2005), and YBX1 is essential for the survival of embryos (Lu et al. 2006), which are both expressed in the control and treatment group. PARP11 plays a role in nuclear envelope stability and nuclear remodelling during spermatogenesis, which was observed in control as well as treated spermatozoa. We observed the presence of transcripts such as *KAT8, CKB* and *IGF1R in control and treatment group*, which involved in sperm biology, including sperm development, motility and sperm-egg interaction.

Conclusion

The study elucidated the rich knowledge of the traditional use of the Putaranjiva plant for increasing fertility. The current study demonstrated the significant response of the *Putranjiva* seed ethanolic extract (@15–17.5 mg/ml) on Gir bull spermatozoa motility. Transcriptome profile shows higher expression of transcripts involved in sperm motility in treated spermatozoa than control groups (CATSPER and AKAP3). The treated motile spermatozoa showed

significant differential expression of genes (ZAR1, HOXB4, SP9) involved in the embryo development process. Thus, it concludes that the ethanolic seeds extract of putaranjiva induces sperm motility and does not show any biased effect on gender-specific chromosomes. Such study can be helpful by providing new avenues in future pharmacological evaluation and natural drug discovery.

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Author contributions JMI—data curation; methodology, original draft; MRP—data curation; formal analysis, software, visualization; AVG—data curation; formal analysis, software, supervision, validation; SSP—data curation; formal analysis, software, validation; BKT—formal analysis, review and editing; SJJ—conceptualization, investigation, resources, supervision, validation; CGJ—investigation, resources, supervision, validation; PGK—conceptualization, funding acquisition, investigation, project administration, resources, supervision, validation, review and editing.

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Data availability Data generated and analysed under this study is available with Accession Numbers: PRJNA545120; GEO: GSE131849.

Declarations

Conflict of interest The authors declare that they have no conflict of interest in the publication, though two of the authors are Editorial board members of this journal.

Compliance with ethical standards This article does not contain any studies involving animals.

References

Aravindakshan T, Nainar A, Nachimuthu K (1998) A simple and efficient method for isolating IIIGH molecular weight DNA from bovine sperm. Indian Vet J 75(4):314–317

Chen MS, Tung KS, Coonrod SA, Takahashi Y, Bigler D, Chang A, Yamashita Y, Kincade PW, Herr JC, White JM (1999) Role of the integrin-associated protein CD9 in binding between sperm ADAM 2 and the egg integrin α6β1: implications for murine fertilization. Proc Natl Acad Sci 96(21):11830–11835

Civetta A (2003) Positive selection within sperm-egg adhesion domains of fertilin: an ADAM gene with a potential role in fertilization. Mol Biol Evol 20(1):21–29

Comizzoli P, Wildt DE, Pukazhenthi BS (2006) Poor centrosomal function of cat testicular spermatozoa impairs embryo development in vitro after intracytoplasmic sperm injection. Biol Reprod 75(2):252–260

Das DC, Sinha NK, Patsa MK, Das M (2015) Investigation of herbals for the treatment of leucorrhoea from south West Bengal, India

Emasushan M, Britto J (2018) Preliminary phytochemical profiling and antifungal activity of the seeds and pericarp of *Putranjiva* roxburghii Wall. Pharma Innov J 7(4):107–110

Fénichel P, Gharib A, Emiliozzi C, Donzeau M, Ménézo Y (1996) Stimulation of human sperm during capacitation in vitro by an adenosine agonist with specificity for A2 receptors. Biol Reprod 54(6):1405–1411

Goswami P, Laskar MA, Basak M (2020) A review on medicinal plants of North Eastern Region with potential antifertility activity. Asian J Pharm Res Dev 8(3):162–165

Gupta M (2016) A review of pharmacological properties, pharmacognosy and therapeutic actions of *Putranjiva roxburghii* Wall. (Putranjiva). Int J Herb Med 4(6):104–108

Gur Y, Breitbart H (2008) Protein synthesis in sperm: dialog between mitochondria and cytoplasm. Mol Cell Endocrinol 282(1–2):45–55

Liu J, Liang P, Yin C, Wang T, Li H, Li Y, Ye Z (2004) Effects of several Chinese herbal aqueous extracts on human sperm motility in vitro. Andrologia 36(2):78–83

Lu ZH, Books JT, Ley TJ (2006) Cold shock domain family members YB-1 and MSY4 share essential functions during murine embryogenesis. Mol Cell Biol 26(22):8410–8417

Miller D, Ostermeier GC, Krawetz SA (2005) The controversy, potential and roles of spermatozoal RNA. Trends Mol Med 11(4):156–163

Parrish JJ (2014) Bovine in vitro fertilization: in vitro oocyte maturation and sperm capacitation with heparin. Theriogenology 81(1):67–73

Raval NP, Shah TM, George L-B, Joshi CG (2019) Insight into bovine (*Bos indicus*) spermatozoal whole transcriptome profile. Theriogenology 129:8–13

Salisbury GW, VanDemark NL (1961) Physiology of reproduction and artificial insemination of cattle

Selvaraju S, Parthipan S, Somashekar L, Kolte AP, Krishnan Binsila B, Arangasamy A, Ravindra JP (2017) Occurrence and functional significance of the transcriptome in bovine (*Bos taurus*) spermatozoa. Sci Rep 7(1):1–14

Sendler E, Johnson GD, Mao S, Goodrich RJ, Diamond MP, Hauser R, Krawetz SA (2013) Stability, delivery and functions of human sperm RNAs at fertilization. Nucleic Acids Res 41(7):4104–4117

Tamburrino L, Marchiani S, Minetti F, Forti G, Muratori M, Baldi E (2014) The CatSper calcium channel in human sperm: relation with motility and involvement in progesterone-induced acrosome reaction. Hum Reprod 29(3):418–428

Van der Ven H, Jeyendran R, Al-Hasani S, Tünnerhoff A, Hoebbel K, Diedrich K, Krebs D, Perez-Pelaez S (1988) Glass wool column filtration of human semen: relation to swim-up procedure and outcome of IVF. Hum Reprod 3(1):85–88

Vidhya U, Nishteswar K (2015) *Putranjiva*-a herb for pumsavana (male progeny facilitator). Int J Ayurveda Pharma Res 3:11–16

Yang J, Medvedev S, Reddi PP, Schultz RM, Hecht NB (2005) The DNA/RNA-binding protein MSY2 marks specific transcripts for cytoplasmic storage in mouse male germ cells. Proc Natl Acad Sci 102(5):1513–1518