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# *In vitro* Impact Assessment of Aqueous Extract of *Sida cordifolia* Linn. Upon Rat Spermatozoa Parameters

**Mradu Gupta[1*], Saikat Chowdhury[1] and Sukumar Manna[2]**

*[1]Institute of Post Graduate Ayurvedic Education and Research, 294/3/1, A. P. C. Road, Kolkata, 700009, India.*
*[2]West Bengal University of Animal and Fishery Sciences, 37, K.B. Sarani, Kolkata, 700037, India.*

***Authors' contributions***

*This work was carried out in collaboration between all authors. Author MG designed the study, wrote the protocol and interpreted the data. Author SM designed and implemented the animal experimentation and laboratory studies. Author SC provided support to authors MG and SM in laboratory studies and data analysis. All authors read and approved the final manuscript.*

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## ABSTRACT

*Sida cordifolia* Linn. finds frequent mention in Ayurvedic System of Medicine for many therapeutic properties including beneficial effect upon male reproductive processes. Aim of present *in vitro* study was to evaluate impact of aqueous extract of *Sida cordifolia* roots on rat spermatozoa in terms of count, motility & morphology and through HOS study following standard assessment methods to assess any likely reproductive toxicity, spermicidal action, lethality or abnormalities in animal experimentation. Sexually mature (18–22 weeks old) male Wister rats weighing 180 to 260 gm were used. Sperms were collected from cauda epididymis of rat testes and sperm suspension was prepared by mixing them with 2 ml of 5% Sucrose at 37℃, dividing into 4 samples – control (A) and 3 drug treated samples (B, C & D) containing 10, 20 or 30 mg/ml of aqueous extract of *Sida cordifolia*. The various sperm parameters were observed under Olympus CX41 microscope. Sperm concentration, Progressive and Non-Progressive motility were calculated using the Neubauer Chamber. The viability and morphology of sperm suspensions were assessed using Eosin Y and

**Corresponding author: E-mail: mradu_gupta@hotmail.com;*

Nigrosin staining method using 400 X magnification under Phase contrast microscope. Hypo-osmotic swelling (HOS) test was used to evaluate the functional integrity of plasma membrane. Treatment with aqueous extract of *Sida cordifolia* resulted in no significant adverse impact upon sperm count up to 30 mg/ml dose, suggesting no noticeable spermicidal effect. Progressive motility increased marginally due to intervention of *Sida cordifolia* in a concentration-dependent manner from 40.00 ±1.07 in Sample A to 43.50 ± 2.05 in Sample D. Similarly, percentage of normal sperms and their viability exhibited steady enhancements with increasing concentrations of research drug. The HOS test indicated no adverse structural changes in plasma membrane integrity of sperms up to 30 mg/ml level. Thus, *in vitro* assessment of sperm motility, morphology and viability due to treatment with *Sida cordifolia* aqueous extract indicated no spermicidal or toxic effect and indicated small but significant enhancement in various parameters which was concentration-dependent.

*Keywords: Sida cordifolia; in vitro; sperm count; motility; viability.*

## 1. INTRODUCTION

Plants have been globally used since ancient times as a valuable and safe natural source of medicines and as agents of therapeutic utility. Male reproduction is a multifaceted process that involves the testes, epididymis, accessory sex glands and associated hormones. Testes perform two highly organized and intricate events, called spermatogenesis and steroidogenesis, which are vital for the perpetuation of life. Spermatogenesis, a highly dynamic and synchronized process, takes place within the seminiferous tubules of the testis with the support of somatic Sertoli cells, leading to the formation of mature spermatozoa from undifferentiated stem cells. The male reproductive system is extremely sensitive to various environmental factors such as life style, drugs, radiation, pollution and toxicants, the result of which could be functional alterations in adults [1-7].

Several natural and synthetic products are reported to target the testis at the hormonal level or during spermatogenesis or both. Many plants and minerals have been prescribed in the form of single or combined form to enhance reproductive processes and for the treatment of diseases related to the male genital organs since ancient times. One such plant which finds frequent mention in various texts relating to Ayurvedic System of Medicine for these properties is *Sida cordifolia* but presently *in vitro* analysis of its therapeutic properties or its possible adverse effect upon the vital sperm parameters has not been done till date.

*Sida cordifolia* Linn. belongs to the Malvaceae family and is one of the most useful medicinal plants in Ayurvedic literature. Also known as *Bala*, it is a small, erect, annual downy shrub and grows well through the plains of India, especially, in damp climates. The shrub grows up to 0.75 – 1.5 meters in height. The root and the stem are stout and strong. The tap root of the plant is odourless with slightly bitter taste and grayish yellow colour which constitutes a cluster 5-15 cm long with few lateral roots of smaller size. The leaves are 2.5-7 cm long and 2.5-5 cm broad, with 7-9 veins. They are chordate-oblong shaped, serrate and truncate. The flowers are small, yellow or white in colour, solitary and axillaries. The fruits are round in shape and very small in sized, 6-8 mm in diameter. The seeds are grayish black in colour and smooth. The plant flowers from August to December and fruiting occurs from October to January. It has been used as a cooling, astringent, aromatic, diuretic and tonic in Ayurvedic system of medicine for curing diseases like asthma, cough, fever, wound, skin diseases, heart diseases, facial paralysis, muscle and joint pain, swelling, inflammation, urinary infection, skin diseases, lack of sexual desire and unwanted weight loss. Its roots and seeds contain alkaloid ephedrine, vasicinol, vasicinone, β-sitosterol and stigmasterol and N-methyl tryptophan while the leaves of *Sida cordifolia* Linn. contain small amounts of both ephedrine and pseudoephedrine. Its pharmacological actions include hypoglycaemic, wound healing, anti-microbial, antioxidant, anti-inflammatory, analgesic, adaptogenic and hepato-protective activities [8,9].

The aim of the present *in vitro* study was to evaluate the impact of aqueous extract of *Sida cordifolia* roots on rat spermatozoa in terms of sperm count, sperm motility, sperm morphology and through HOS study following standard assessment methods. The objective was to assess any likely cytotoxic damage, lethality or abnormalities that could cause spermicidal action

or other reproductive toxicity in animal experimentation. These assessments could provide valuable information for the determination of potential therapeutic action of the research drug which can be prescribed for future in vivo spermatogenesis evaluations.

## 2. MATERIALS AND METHODS

### 2.1 Preparation of Plant Extract

The roots of *Sida cordifolia* Linn. were purchased from crude drug supplier of Katwa Chowrasta, Burdwan district and the plant samples were authenticated by the Research Officer, Botanical Survey of India, Howrah, India vide Ref. No. BSI/CNH/SF/Tech./2016. The crude drugs were washed, sun dried and crushed to particle size of 80 meshes. The powder of the dried roots of *Sida cordifolia* Linn was extracted by non-polar to polar solvents, namely Petroleum ether (60°-80°), Ethyl acetate, Chloroform, Acetone, alcohol and water using Soxhlet Apparatus by continuous hot extraction. The extracts obtained were filtered, concentrated by water-bath and finally dried and stored for further analysis. The aqueous extract of the research drug was used in this study.

### 2.2 Chemicals

Tri-Sodium citrate dehydrate (B. No. MK8M583268) was obtained from Merck Specialties Pvt. Ltd., Mumbai, India. Fructose (B. No. 503414), Sucrose (B. No. 503593), Eosin yellow stain solution-2% w/v (B. No. 409153) and Nigrosine solution-10% w/v (B. No. 407278) were obtained from NICE Chemicals Pvt. Ltd., Kerala, India.

### 2.3 Animals

Sexually mature (18–22 weeks old) male Wister rat weighing 180 to 260 gm were used for this study. All animals were procured from the Government of West Bengal approved breeder, M/s Satyacharan Ghosh, Kolkata and housed under standard environmental conditions with fixed 12 hr light/dark cycles and at a temperature of approximately 25℃ in the animal house of IPGAE&R, Kolkata. The animals were kept in standard polypropylene cages and provided with food (standard pellet diet) and water. These animals were acclimatized for a period of 14 days prior to performing any experiments. All experimental protocols were approved by the Institutional Animal Ethics Committee (IAEC).

### 2.4 Preparation of Sperm Suspension

Sperm suspension was prepared following the WHO manual 5$^{th}$ ed. [10]. The epididymis is a complex organ, where the immotile and infertile spermatozoa leaving the testis acquire both their motility and fertility. Sperm maturation mainly takes place within the proximal regions (caput and corpus) of the epididymis, whereas the cauda epididymidis plays a major role in the storage of mature sperm [5]. In this study, the sperms were collected from the cauda epididymis of a healthy male rat who was first acclimatized for 15 days in the animal house before dissection. The procedure was repeated for a total of three randomly selected healthy male rats and the average values obtained were used throughout the study. The sperm suspension was prepared by mixing the collected sperms with 2 ml of 5% Sucrose at 37℃ [11].

**Fig. 1. Testis along with the epididymis in rat**

### 2.5 Division of Prepared Semen Samples

The prepared sperm suspensions were divided into four equal parts and assigned to the following four samples:

Sample A: Control (untreated)
Sample B: *Sida cordifolia* Aqueous Extract (10 mg/ml)
Sample C: *Sida cordifolia* Aqueous Extract (20 mg/ml)
Sample D: *Sida cordifolia* Aqueous Extract (30 mg/ml)

The sperm count, motility, morphology and viability tests were done following the prescribed methods by adding the different concentrations (10, 20 & 30 mg/ml) of *Sida cordifolia* Aqueous Extract in physiological saline (pH 7.4) prepared by serial dilution, and 100 µL of each was mixed

with an aliquot of 20 µL of prepared sperm suspension. The sperm count, motility and other parameters of the each sample mixtures were observed within the next 30 minutes under microscope (Olympus CX41).

## 2.6 Epididymal Sperm Count

The epididymis functions as a storage repository for spermatozoa prior to ejaculation [10]. The sperm concentration for each epididymis was evaluated by using the Neubauer chamber [12]. Before using the Neubauer chamber, both sides of its walls were lightly wet and the cover slip was firmly placed onto the chambers. Approximately 10 µl of the prepared sperm suspension was transferred to the counting chambers of the Neubauer unit. The central square of the grid in an improved Neubauer chamber contains 25 large squares, each containing 16 smaller squares. More than 200 Spermatozoa were counted for each group and value was expressed as Mean ±SEM. The sperm concentration per epididymis was calculated by counting the number of spermatozoa in the 16 smaller squares and assessing it using the formula:

Sperm concentration in epididymis = $(\sum N_{1+2+....+16})/12$ million/ml

Where

$N_1$ = Sperm number in one smaller square
12 = factor of multiplication of dilution factor with thickness of cover slip of the Neubauer chamber


**Fig. 2. Neubauer chamber**

## 2.7 Determination of Sperm Motility

The effect upon sperm motility of the research drug was determined following a modified version of the procedure detailed by R.H. Aladakatti et al. [13]. The motility of each spermatozoon was graded as either 'Progressive motility (PR)', 'Non-Progressive motility (NP)', or 'Immotility' (IM) according to their rate of motility [10] during analysis in the Neubauer Chamber as described below.

a. Progressive motility (PR): Spermatozoa moving actively and swimming either mostly in a straight line or in a large circle, or sperm that zigzags but makes forward progression, with varying speed. (i.e., ≥ 25 µm/s at 37ºC and ≥ 20 µm/s at 20℃)
b. Non-Progressive motility (NP): All other patterns of motility with an absence of progression; the spermatozoa only vibrates in very tight circles, no forward progression is observed and the flagellar force hardly displaces the head; or when only a flagellar beat can be observed where speed < 5 µm/s
c. Immotility (IM): No movement

## 2.8 Assessment of Sperm Morphology and Viability

Sperm morphology and viability tests were done according to WHO manual 5th ed. 2010 [10]. All the study samples, namely the control and different concentrations of research drug treated sperm suspensions, were examined separately at 400 X under Phase contrast microscope using Eosin Y and Nigrosin staining method to assess viability and morphology of the sperm. The staining was done by mixing one drop of treated and control sperm suspensions with 2 drops of 2% Eosin Y on a clear microscope slide. After 20 seconds, 3 drops of 10% Nigrosin solution were added, the mixture was allowed to dry and observed under Phase contrast microscope at 400X magnification (Fig. 3). The proportion of live spermatozoa can be determined by using staining techniques that are based on the principle that dead cells with a damaged plasma membrane absorb certain stains, thus differentiating the live (unstained) spermatozoa from the dead (stained) cells. Sperm vitality assessments also provide a clear idea about the accuracy of the motility evaluation, since the percentage of dead cells should not exceed (within counting error) the percentage of immotile spermatozoa (as per Figs. 4 & 5). The presence of a large proportion of vital but immotile cells may be indicative of structural defects in the flagellum [14].

**Fig. 3. Slide after staining with Eosin Y & Nigrosin**

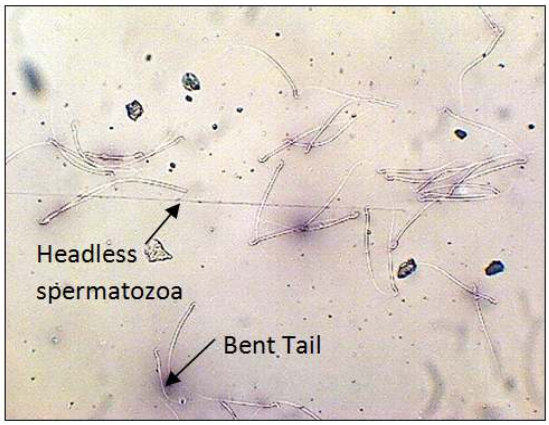



**Fig. 4. Viable sperm without staining showing headless and bent tail abnormality**

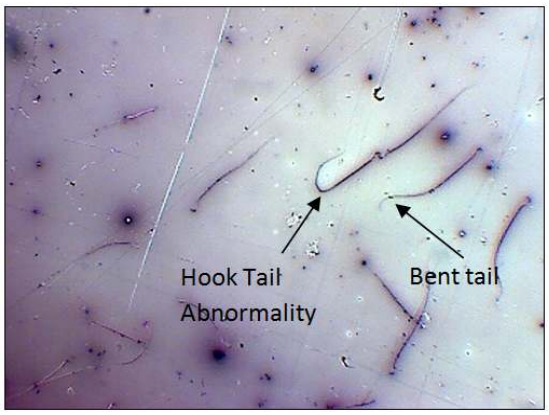



**Fig. 5. Dead sperm with stain showing hook tail and bent tail abnormality**

### 2.9 Assessment of Plasma Membrane Integrity

The Hypo-osmotic swelling (HOS) test is a relatively new assay used to evaluate the functional integrity of the sperm's plasma membrane [15]. The assay is based on the fact that fluid transport occurs across an intact cell membrane under hypo-osmotic conditions until equilibrium is reached. Due to the influx of fluid, the cell will expand and bulge, especially in the tail, and this change can be readily observed with a phase contrast microscope. The Control and different concentrations of research drug treated sperm suspensions were exposed to HOS solution (75 mM fructose and 20 mM sodium citrate) for at least 30 min at 37℃. The number of spermatozoa which exhibit characteristic swelling or tail coiling were counted under a phase contrast microscope (X 100).

## 3. RESULTS

### 3.1 Epididymal Sperm Count and Determination of Sperm Motility

The sperm count and motility values of the different samples are shown in Table 1. There was no appreciable change in the sperm count after introducing the research drug extract at different concentrations up to the level of 30 mg/ml level as elaborated in Figs. 6 and 7.

### 3.2 Sperm Morphology and Viability

Although normal seminal fluid contains a huge numbers of sperm, not all of these sperms will be viable where 'Viable' in its most basic sense means 'alive'. The morphological and viability studies of spermatozoa were done using Eosin Y and Nigrosin. No adverse morphological changes were found in the sperm head, mid-piece or tail of samples B, C and D which received the research drug in various concentrations when compared with untreated spermatozoa (Fig. 8). The percentage of sperms having normal morphology which was 54.50 ± 4.70 in Sample A increased to 56.00 ± 1.77 in case of Sample D. Similarly, the sperm viability also increased from 60.0 ±3.70% in Sample A to 61.50 ± 2.43% in case of Sample D (Fig. 9).

### 3.3 Hypo Osmotic Swelling Test (HOS Test)

No significant changes between the various treatment groups were observed during the HOS test. The number of spermatozoa which exhibited characteristic swelling or tail coiling in the control sample and various concentrations of research drug treated sperm suspensions exposed to HOS solution were assessed under the microscope, but no noticeable variations were observed under laboratory conditions.

**Table 1. Sperm parameter assessment**

| Sample | Sperm count (million/ml) | Sperm motility | | | Sperm morphology | Sperm viability (in %) |
|---|---|---|---|---|---|---|
| | | Progressive (in %) | Non-progressive (in %) | Immotile (in %) | Normal (in %) | |
| Sample A | 123.00 ±3.46 | 40.00±1.07 | 6.00±0.58 | 54.00±1.16 | 54.50±4.70 | 60.0±3.70 |
| Sample B | 121.00 ±6.00 | 41.50±1.38 | 5.50±0.89 | 53.00±1 .98 | 55.00±1.53 | 60.17±1.20 |
| Sample C | 121.00 ±7.04 | 43.00±1.07 | 5.00±0.73 | 52.00±1 .03 | 55.50±1.48 | 61.00±2.22 |
| Sample D | 122.00 ±8.30 | 43.50±2.05 | 4.90±0.68 | 51.60±1. 52 | 56.00±1.77 | 61.50±2.43 |

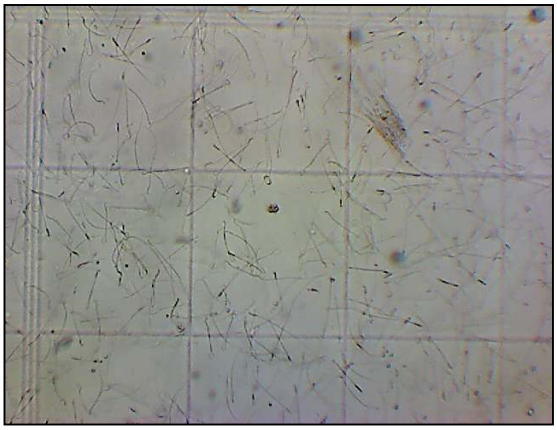


**Image of spermatozoa in control sample (Sample A) at 10X magnification**

**Image of spermatozoa in treated sample D (30 mg/ml) at 10X magnification**

**Fig. 6. Sperm count by using Neubauer chamber**

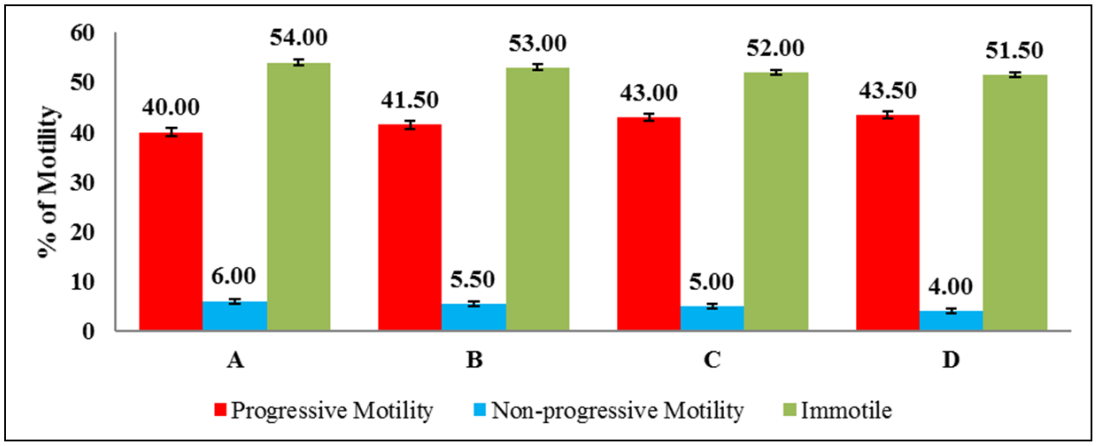
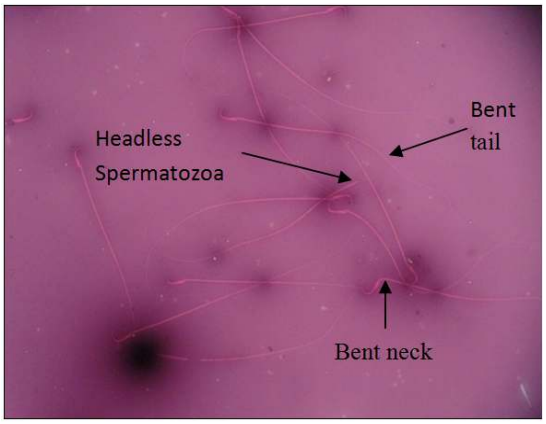



**Fig. 7. Effect of different concentrations of *Sida cordifolia* aqueous extracts on sperm motility**

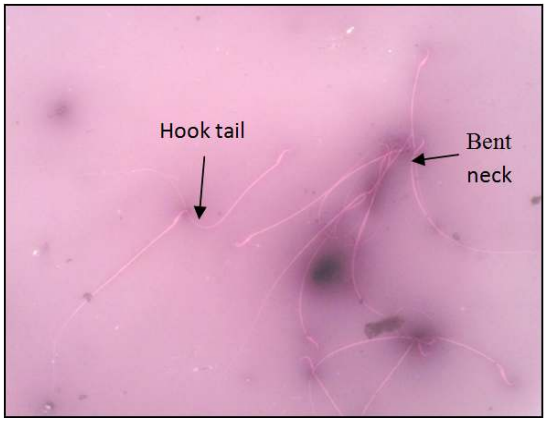
## 4. DISCUSSION

In India, *Sida cordifolia* or 'Bala' is considered to be one of the most valuable drugs in Ayurvedic medicine and has been widely used since ancient times. The roots, leaves, and stems are utilized as traditional medicines in chronic dysentery, general weakness, muscular weakness, gonorrhea, pain and inflammation of the joints and asthma. The roots of this plant are also indicated for piles, to promote aphrodisiac treatment for neurodegenerative diseases,

including Parkinson's disease, facial paralysis as well as in urinary and reproductive disorders. In this study, the aqueous extract of *Sida cordifolia* was used because this plant has been prescribed to the patients as a rejuvenator and tonic in the aqueous form of root powder for a long period in Ayurvedic traditional system of medicine. However, it was not evaluated in vivo till now for its possible impact on the spermatozoa in animal experiments. Therefore, the aqueous extract of this plant was evaluated and extraction was done in soxhlet apparatus following different solvent systems as per Ayurvedic pharmacopeia. The other extracts were not used since their extractive value was very low. During the study, treatment with the research drug, namely the aqueous extract of *Sida cordifolia* resulted in no significant adverse impact upon the sperm count up to 30 mg/ml dose.




**Control: Image under microscope showing some abnormal spermatozoa (headless, bent tail and bent neck)**




**Sample D (30 mg/ml): Image under microscope showing some abnormal spermatozoa (headless, bent tail and bent neck)**

**Fig. 8. Slides showing assessment of sperm morphology and viability**

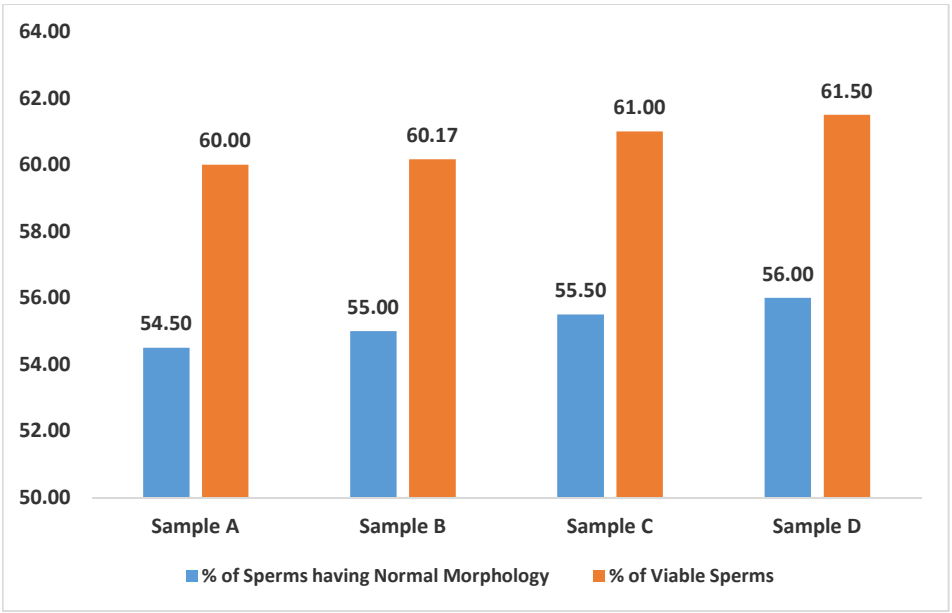



**Fig. 9. The effect of *Sida cordifolia* aqueous extract on sperm morphology and viability**

Sperm maturation mainly takes place within the proximal regions (caput and corpus) of the epididymis, whereas the cauda epididymidis plays a major role in the storage of mature sperm. Plant origin compound azorellanone significantly inhibited several functions that are essential for fertilization, such as sperm motility, sperm–zona binding, sperm acrosome reaction and $Ca^{2+}$ influx, and sperm protease activities [16]. Neem derivatives, viz., sodium nimbinate and sodium nimbidinate have been found to possess weak spermicidal action *in vitro* [17,18]. Praneem polyherbal cream has shown spermicidal effect at effective concentration to immobilization (100%) of sperm within 20 secs [19]. Kumbar et al. [20] and Aladakatti et al. [21] evaluated the effective concentration of nimbolide, an isoprenoid of neem leaf, to immobilize and kill 100% rat spermatozoa and suggested that isoprenoid of leaf is hydrophilic in nature and mixes immediately with water as well as body fluids and kills sperm within 20 secs. Azadirachtin - A, a tetranortritarpinoid of neem seed kernel, when tested on rat spermatozoa bestows the sperm-immobilizing effect either directly executing its effects by structural and functional modulation of the plasma membrane or by way of its synergism with blockage of some biochemical pathway likes energy utilization [22]. *A. indica* leaves also cause impairment of spermatogenesis and exhibit anti-androgenic effect in albino rats [23,24]. Therefore, the present study was done to evaluate its direct impact on the spermatozoa of rat animal following the standard spermicidal effects methods.

In this study, the sperms were collected from the cauda epididymis of a healthy male rat who was first acclimatized for 15 days in the animal house before dissection. The sperm count, motility, morphology and viability tests were done following the prescribed methods by adding the different concentrations (10, 20 & 30 mg/ml) of *Sida cordifolia* Aqueous Extract in physiological saline (pH 7.4) prepared by serial dilution, and 100 µL of each was mixed with an aliquot of 20 µL of prepared sperm suspension.The overall sperm count varied only between 121 to 123 million/ml during the study, suggesting no noticeable *in vitro* spermicidal effect of the research drug.

As far as sperm motility is concerned, there was no adverse impact upon motility due to intervention of *Sida cordifolia* Aqueous Extract at all doses. In fact, the progressive motility increased marginally from 40.00 ±1.07 in case of Sample A to 43.50 ±2.05 in case of Sample D. Accordingly, the percentage of immotile sperms decreased from 54.00 ±1.16 in case of Sample A to 51.50 ±1.52 in Sample D. Therefore, the *in vitro* treatment with aqueous extract of *Sida cordifolia* root powder had a little but consistent and significant positive impact upon the motility of sperms in this experiment. It was also observed that this positive impact on the sperm motility in case of semen samples was concentration-dependent in nature since the overall motility showed an increasing trend when the drug concentration was increased in various samples in the range of 10 mg/ml to 30 mg/ml may be due to its antioxidant activity of the plant having phenolic and flavonoid compounds. Plant sourced food antioxidants like vitamin C, vitamin E, carotenes, phenolic acids, phytate and phytoestrogens have been recognized as having the potential to reduce disease risk that can scavenge free radical. Researchers suggest that two-thirds of the world's plant species have medicinal value and many of them have great antioxidant potential. In fact, many plant species have similar antioxidant potential as that of synthetic antioxidants [25].

These findings about the positive *in vitro* effect of the research drug upon vital sperm parameters are also corroborated by the results obtained in respect of the morphology and viability of sperms. Both the percentage of normal sperms and their viability exhibited a trend of slow but steady enhancements with increasing concentration levels of research drug across the treatment groups.

Studies of the plasma membrane integrity of sperms in the HOS test indicated no significant adverse changes at the structural level in the research drug treated semen samples. No negative impact in the sperm plasma membrane, which could affect its efficacy, could be noticed up to the level of 30 mg/ml *Sida cordifolia* aqueous extract.

This study validates the effect of the research drug in enhancing the quality of semen as described in the Ayurvedic texts which may be due to the presence of phenolic compounds and its antioxidant properties.

## 5. CONCLUSION

*In vitro* assessment of the sperm quality in terms of its motility, morphological abnormalities and

viability due to treatment with aqueous extract of *Sida cordifolia* indicated no spermicidal or toxic effect. On the other hand, there was a small but significant enhancement in the sperm parameters, reduction in abnormalities and increase in viability of sperms which was found to be concentration-dependent.

## CONSENT

It is not applicable.

## ETHICAL APPROVAL

All authors hereby declare that "Principles of laboratory animal care" (NIH publication No. 85-23, revised 1985) were followed, as well as specific national laws where applicable. All experiments have been examined and approved by the appropriate ethics committee.

## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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