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# Sperm viability assessment using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide reduction assay of Swiss albino mice treated with *Diplazium esculentum*

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## ABSTRACT

*Diplazium esculentum* is the most commonly consumed fern throughout Asia and Oceania. Systemic toxicity and pathological effects on its consumption have already been demonstrated. But the spermicidal properties of the boiled *Diplazium esculentum* (BDE) have not yet been investigated. Here an attempt was made to investigate the effect of boiled *Diplazium esculentum* (BDE) on the viability of spermatozoa of adult Swiss albino mice, if any.3-(4,5-dimethylthiazol-2-y1)-2,5-diphenyltetrazolium bromide (MTT) reduction assay was performed for the assessment of the viability of spermatozoa of adult Swiss albino mice. Dehydrogenase, present in the mitochondria of the midpiece region of spermatozoa converts the yellow colored insoluble tetrazolium salt to purple colored water soluble formazan. This was measured spectrophotometrically using a microplate reader in the present study. Rates of MTT reduction were recorded before and after the incubation at 37°C for 1 h. The MTT reduction rate (change in optical density) for each group was determined by calculating the difference between the first and second reading of the microplate reader. Dose dependent inhibition of the viability of sperm was observed in case of all the treated animals when compared with the controls. The inhibition was statistically significant ($p < 0.001$) and directly proportional to the dose of the BDE. After 135 days and 180 days of treatment, at 320mg/kg body weight, the percentage inhibition of sperm viability was 40.51% and 53.12%, respectively. These results suggest that *D. esculentum*, even after boiling, possess spermicidal properties which may cause male infertility. Therefore, the consumption of *D. esculentum* is alarming and may act as an antifertility agent.

## INTRODUCTION

Declination of the male fertility has been a great concern as male infertility accounts for about 30% of infertility cases worldwide. Male infertility is an important issue, a common problem occurring worldwide [1]. There has been a steady accumulation of information regarding the screening of plants having antifertility properties in males. But, very few studies have been conducted so far to assess the effect of wild edible plants on male fertility. Increase in the consumption of these plants is due to the progressive decrease in the stock of cultivated crops [2]. But information on the possible toxic effects of most of the wild edible plants is too little to make the people aware about the hazardous effects of the consumption of these plants.

*Diplazium esculentum* (Koenig ex Retz.) Sw. (Family Athyriaceae) is one of the most common varieties and the most commonly consumed fern throughout Asia and Oceania. In India, young fronds of *D. esculentum* are popularly known as lingra in Northern India [3], rukja and lochanch in North Eastern India [4] and dheki sak in West Bengal, India [5]. The newly emerging coiled fronds are consumed after cooking as a seasonal vegetable during monsoon which continues for almost five months.

Very few studies have been conducted so far to assess the toxicological impact of this fern on human health. Interestingly, this fern is rejected as food by animals including cattle and insets. Studies conducted on rabbits and guinea pigs demonstrated systemic toxicity and several pathological effects of this fern [6]. Young fronds of *D. esculentum* collected from the high-altitude area of HarsilGangotri of North India had been found to have moderate level of ptaquiloside (Pta), a nor-sesquiterpenoid glycoside which is clastogenic, mutagenic and carcinogenic that cause enzootic bovine hematuria (EBH) in hill cattle in India and elsewhere [7]. Pta is considered as the causative agent for the location of tumors in the urinary bladder of ruminants and the ileum of rats [8]. However, the antifertility activity of this plant has not yet been studied.

Therefore, it happened in our mind that this fern may have certain toxic substances which may be associated with antifertility especially with male sterility. Assessment of the metabolic status of spermatozoa can provide valuable information regarding the viability as well as characteristics of spermatozoa, which is directly correlated with male fertility. The reduction activity of spermatozoa depends on the ability of metabolically active spermatozoa to reduce specific stains. The ability of spermatozoa to reduce the resazurin redox dye [9-10] and the methylene blue dye [11] has been used to evaluate semen quality in boars and bulls, respectively. Yellow 3-(4,5-dimethylthiazol-2-y1)-2,5-diphenyltetrazolium bromide (MTT), a water-soluble tetrazolium salt dye, is converted to water-insoluble purple formazan by the succinate dehydrogenase system of an active mitochondria by the reductive cleavage of its tetrazolium ring [12]. Thus, the amount of formazan formed can be determined spectrophotometrically and can serve as an estimate of the number of active mitochondria, and hence the viable cells, in a sample [13-14]. The MTT assay has been evaluated successfully in different animal species [15-17], whereas literature pertaining to the use of this technique to evaluate the viability of the spermatozoa of *D. esculentum* fed adult Swiss albino mice is still lacking.Therefore, we have conducted this pilot study to investigate whether the boiled aqueous preparation of *D. esculentum* (BDE) affect the viability of the spermatozoa of adult Swiss albino mice, keeping in mind the fact that the local people consume this plant as food after cooking, not as raw material.

## MATERIALS AND METHODS

### Preparation of the plant material

Young *D. esculentum* plants were collected from different areas of North Bengal University campus, from the market where from the local people procure and also from the adjoining regions of Darjeeling, India. Plants were identified by Prof. A. P. Das, Plant Taxonomy Laboratory, Department of Botany, University of North Bengal and a voucher specimen (Accession No. 9602) was submitted to him.

Young frond of *D. esculentum* (100 g) was washed carefully by tap water, then cut into small pieces, and boiled with 1000 ml of distilled water for 30 min. The boiled plant material was then finely mixed by a mixer and dried in an incubator at $60^0$C until completely dried. This dried plant material (boiled *Diplazium esculentum*, BDE) was then kept at $4^0$C for future use.

### Animals and care

Male Swiss albino mice (25 ± 2 gm of body weight (b.wt.)) of 6-8 weeks of age were used for all the studies. They were housed in polypropylene cages, with dust-free paddy husk as bedding material. They were maintained in the animal house, Department of Zoology, University of North Bengal with food and water *ad libitum* under a constant 12 h dark/light cycle at an environmental temperature of 25 ± 2°C. All the experiments were performed after obtaining the approval from the Animal Ethical Committee (Registration No. 840/ac/04/CPCSEA).

### Dosage

Ninety six (96) male Swiss albino mice were divided in to four sets (S 1-4) and each set was sub-divided in to four groups (G 1-4). Therefore, each group contained six mice. Group 1 (G1) of all the sets were considered as control and 0.4 ml of distilled water was given orally. Group 2 (G2), Group 3 (G3) and Group 4 (G4) of all the sets were fed with 0.4 ml of BDE at the dose of 80 mg/kg b.wt., 160 mg/kg b.wt., and 320 mg/kg b.wt., respectively with the help of a syringe specially designed for this purpose. In this way, all groups of S1 (G1S1 to G4S1) were treated daily for 45 days, S2 (G1S2 to G4S2) daily for 90 days, S3 (G1S3 to G4S3) daily for 135 days and S4 (G1S4 to G4S4) daily for 180 days.

### Preparation of the sperm suspension and MTT reduction assay

Mice were sacrificed 24 h after the last dose by using proper anesthesia (chloroform and ether in a ration of 2:1). Sperm suspension was prepared according to the previously described method [18]. Caudal epididymis was separated and minced using a pair of small scissors to release the sperm into 10 ml warmed (37° C) physiological saline. The sperm suspension was placed in an incubator at 37° C for 10 minutes prior to perform the viability test. The MTT assay was performed according to the previously described method [19]. Briefly, the sperm suspension was diluted using phosphate buffered saline (PBS) and adjusted the number as $30x10^6$ spermatozoa/ml. To assess the sperm viability of S1 mice (G1S1, G2S1, G3S1, G4S1), twenty four wells of a 96-well microplate were used. One hundred microliters of sperm suspension from G1S1 mouse was placed in the first six wells of the first column of the microplate. Similarly, 100 µL of sperm suspension from G2S1, G3S1, and G4S1 mice was placed in the six wells of the second, third and fourth columns of the microplate, respectively. Therefore, a total of 24 wells of the microplate were occupied with sperm suspensions of all the groups of S1 mice. Then, 10 µL of MTT stock solution (5 mg/mL, dissolved in PBS; pH 7.0) was added to each of these 24 wells and mixed properly. The rates of MTT reduction (measured as optical density) were recorded immediately and after incubation at 37°C for 1 h using a microplate reader (Bio-Rad, USA). The MTT reduction rate (change in optical density) for each group was determined by calculating the difference between the first and second reading of the microplate reader. MTT reduction rates of the spermatozoa of S2, S3 and S4 mice were evaluated in the similar way as mentioned above.

### Statistical analysis

Data have been presented as mean ± SD of six observations. Statistical analysis was performed using KyPlot version 2.0 beta 15 (32 bit) software. Differences in mean ± SD among different groups were statistically analyzed using one way ANOVA followed by Dunnett's test. A probability value of $p < 0.05$ was considered significant.

## RESULTS

Results of the MTT reduction assay showed significant dose-

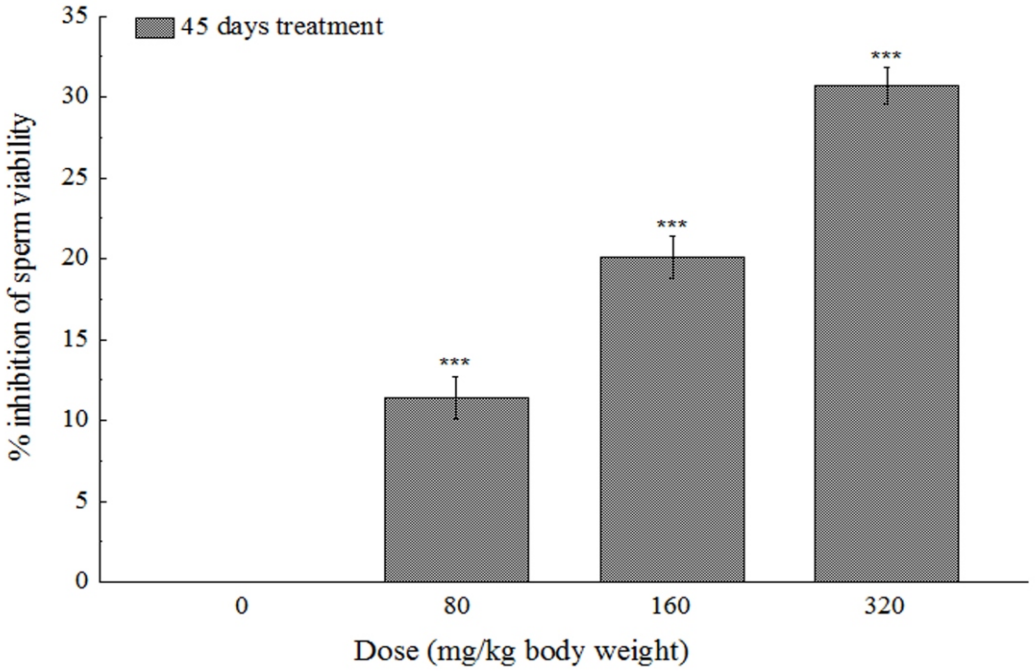


**Figure 1:** MTT reduction assay of spermatocytes demonstrates dose-dependent increase in the percentage inhibition of sperm viability in the mice treated with BDE for 45 days. At 320 mg/kg b. wt., the percentage inhibition of sperm viability was 30.69%. The results are mean ± S.D. of six parallel observations. ***$p < 0.001$ vs. Control.

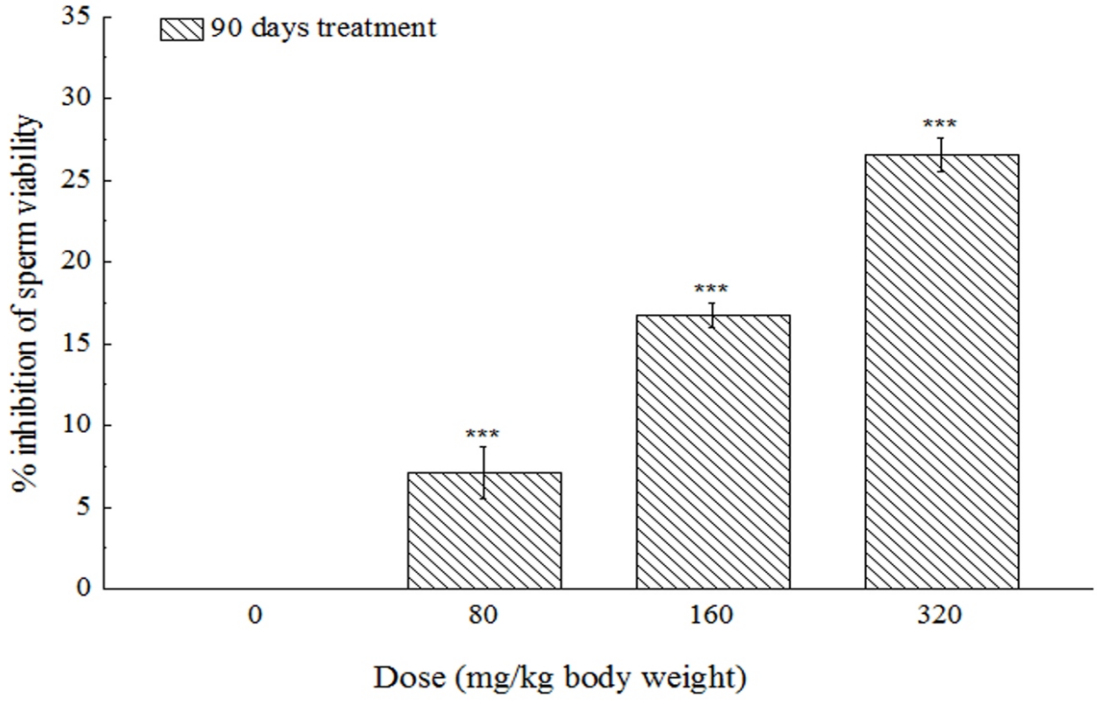


**Figure 2:** MTT reduction assay of spermatocytes demonstrates dose-dependent increase in the percentage inhibition of sperm viability in the mice treated with BDE for 90 days. At 320 mg/kg b. wt., the percentage of inhibition of sperm viability was 26.56%. The results are mean ± S.D. of six parallel observations. ***$p < 0.001$ vs. control.

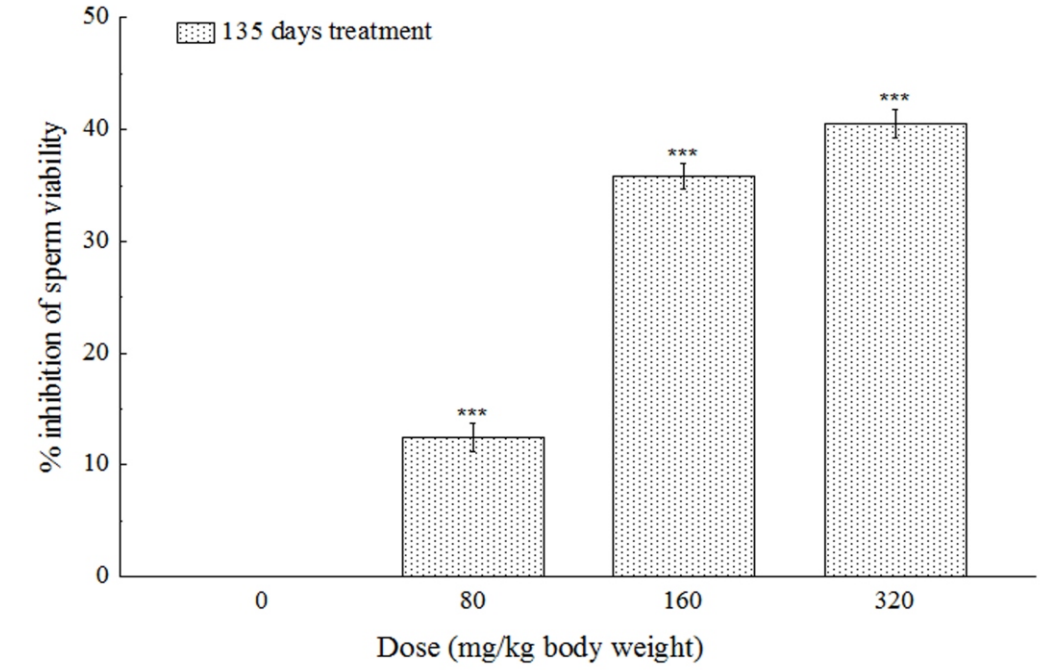


**Figure 3:** MTT reduction assay of spermatocytes demonstrates dose-dependent increase in the percentage inhibition of sperm viability in the mice treated with BDE for 135 days. At 320 mg/kg b. wt., the percentage of inhibition of sperm viability was 40.51%. The results are mean ± S.D. of six parallel observations. ***$p < 0.001$ vs. control.

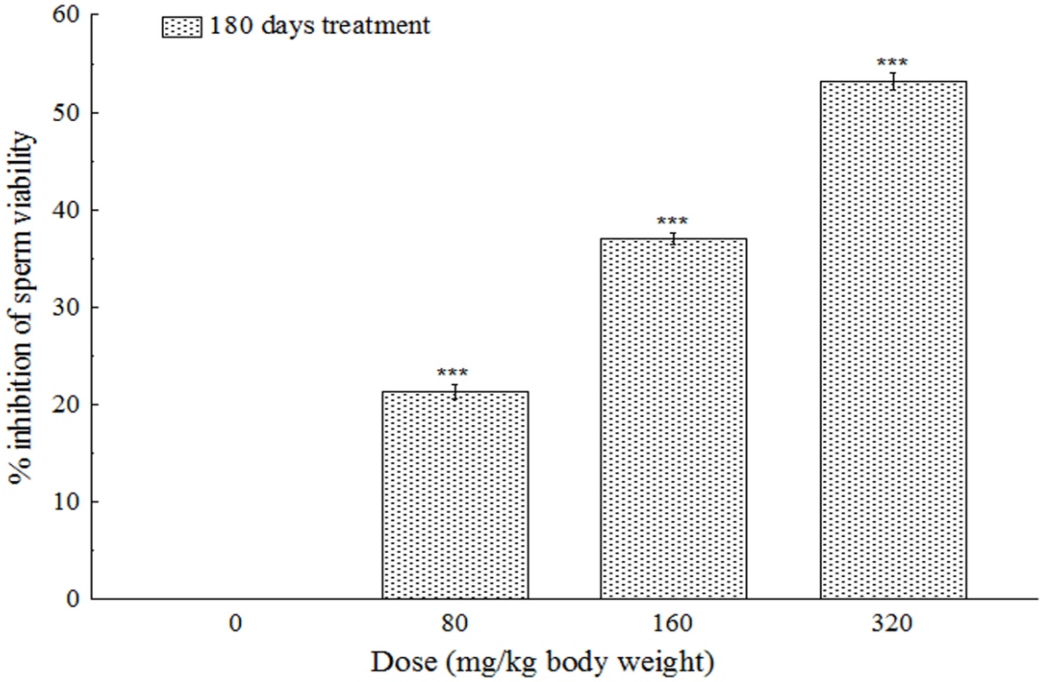


**Figure 4:** MTT reduction assay of spermatocytes demonstrates dose-dependent increase in the percentage inhibition of sperm viability in the mice treated with BDE for 180 days. At 320 mg/kg b. wt., the percentage of inhibition of sperm viability was 53.12%. The results are mean ± S.D. of six parallel observations. ***$p < 0.001$ vs. control.

dependent increased percentage of the inhibition of sperm viability in all of the cases. After 45 days of treatment, significant gradual dose-dependent increments ($p < 0.001$) in the percentage inhibition of sperm viability were observed in all of the treated doses, i.e., at 80mg/kg b. wt. (11.38%), 160mg/kg b. wt. (20.07%) and 320mg/kg b. wt. (30.69%), when compared with the control group (Figure 1). After 90 days of treatment, at 80mg/kg b. wt., the percentage inhibition of sperm viability was 7.10%, whereas, at 160mg/kg b. wt. and 320mg/kg b. wt., the percentage inhibitions of sperm viability were 16.69% and 26.56%, respectively. Therefore, significant gradual dose-dependent increments ($p < 0.001$) in the percentage inhibition of sperm viability were observed in all of the treated doses, when compared with the respective control groups (Figure 2). This was also observed significantly after 135 days and 180 days of treatment with BDE. After 135 days of treatment, at 320mg/kg b.wt., the percentage inhibition of sperm viability was 40.51% (Figure 3), whereas, after 180 days of treatment with BDE, at 320mg/kg b.wt., the percentage inhibition of sperm viability was increased remarkably up to 53.12% (Figure 4).

## DISCUSSION

Studies on the effects of plant products on the male reproductive system and fertility are comparatively few and far fetched [20]. In the present study, the effect of boiled aqueous preparation of *D. esculentum* (BDE) on the metabolic activity of the spermatozoa of adult Swiss albino mice clearly establishes that BDE can affect male reproductive system and cause infertility through its spermicidal properties. Mosmann (1983) used MTT tetrazolium salt to assess the cellular viability, proliferation, and cytotoxicity of lymphocytes. Additionally, the MTT assay has been used in many studies to evaluate the viability of different cells [21-23]. The present study provides new information on the MTT assay for sperm viability assessment in *D. esculentum* fed adult Swiss albino mice. Formation of MTT formazan granules or spikes around the midpiece region of spermatozoa showed that mitochondria contain a succinate dehydrogenase system that converts MTT to formazan. The presence of formazan granules in the midpiece region identifies the viability of spermatozoa. Results indicated a strong correlation between the MTT reduction rate and the viability of spermatozoa. A strong correlation between MTT reduction and the viability of spermatozoa has also been found in bovines, stallions, boars, fowl, and humans [15-17] [24-25]. The MTT reduction rate was taken successfully after 1 h of incubation time. This is due to the fact that spermatozoa are very active cells and rich in mitochondria; therefore, the reduction of MTT by spermatozoa is faster than other cells. Other studies have already revealed that sperm viability is positively related to sperm quality parameters like acrosome integrity, mitochondrial activity and these parameters also correlate positively with fertility [26]. The male accessory sex organs, viz. epididymis and vas deferens are androgen dependent target organs that manifest differential sensibility to androgens for the maintenance of their structure and function. Any change in the circulating androgens would affect the internal microenvironment of epididymis and thereby lead to alter the sperm motility and metabolism [27]. Present study showed that the rate of MTT reduction decreased gradually with the increase in dose, in all the groups. After 135 days and 180 days of treatment, at the dose of 320mg/kg b. wt., the percentage inhibition of sperm viability was increased remarkably up to 40.51% and 53.12%, respectively. Treatment with the ethanolic extract of *Sarcostemma secamone* to adult male rat has been shown to reduce the number of female impregnation, number of implantation and also the number of viable fetuses, when mated with fertile females [20]. These could be due to the decrease in sperm density, viability and motility, which supports our findings of having reduced sperm viability due to the treatment of boiled aqueous preparation of *D. esculentum*, and therefore, indicated that *D. esculentum,* may possess antifertility activity, probably due to its spermicidal properties. The differences in the mean values among the treatment groups were greater than would be expected by chance; there were statistically significant differences ($p < 0.001$). To isolate the group or groups that differ from the others, we use a multiple comparison procedure. All pair-wise multiple comparison procedures (Dunnett's method) were also performed for the authentication of the results.

## CONCLUSION

*Diplazium esculentum*, the vegetable fern, is extensively used as a palatable food throughout Asia, Oceania and especially in the Northern part of West Bengal where we reside. Considering the findings of the present study, it can be concluded that *D. esculentum*, even boiled, possesses potent spermicidal properties. This is the first report on the assessment of the reproductive dysfunction due to the intake of the edible *D. esculentum*, and thereby to make people aware about the hazards of its consumption and it will advance the existing knowledge of this fern in relation to human health.

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