RESEARCH ARTICLE

Effect of Ethanolic Leaf Extract of *Azadirachta indica* A. Juss (Neem) on Testicular Morphometry of New Zealand White Rabbits

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Abstract

Azadirachta indica (neem) is an ascent, evergreen medicinal plant native to Indian subcontinent and most of the African countries. Neem causes deleterious effects on the fertility of mammals and might be used to investigate its contraceptional use in stray animal population. The aim of the study was to assess the effect of ethanolic leaf extract of *A. indica* on testicular morphometry (length, width, thickness and volume) and histology of male New Zealand White rabbits. Eighteen healthy, adult male rabbits, aged 12 months old, weighing between 1.83–1.90 kg were randomly divided into three groups, each group comprised of 6 animals. Group A was kept as control with distilled water only, while Group B and C were given ethanolic leaf extract of *A. indica* @ 100 mg/kg and 200 mg/kg body weight/day for 60 days, respectively, in drinking distilled water. Testicular length, width and thickness of both testicles were measured on day 0, 15, 30, 60 and 120 after initiation of treatments by Vernier calipers. Volume was also calculated by standard formula. The results revealed significant decrease in morphometry of the testicles at day 60 over day 0 in treated groups, and it showed variable reversal in next 60 days after discontinuation of treatment.

Keywords: *Azadirachta indica*, Rabbits, Testicular morphometry.

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Introduction

Azadirachta indica (neem) belongs to *Meliaceae* family, is an evergreen tree, native to Indian subcontinent, Africa, America and other tropical regions (Pingale, 2010). Neem is found to contain a vast array of biologically active compounds, which are useful such as antimalarials, spermicidals, antituberculosis, anti-viral, anti-allergic, anti-enzymic and antifungal agents (Sharma and Bhattacharyya, 2005). A large number of biologically active components are present in seed, leaf, flower, bark, and roots of neem, which have great versatility in their use. In recent decades, there has been growing area of interest about the effect of different chemicals on developing male reproductive system (Sharma and Garu, 2011).

Various researchers reported that neem causes sterility in males (Santra and Manna, 2009; Auta and Hassan, 2016; Lisanti *et al.,* 2018). Flavonoids, steroids, alkaloids, tannins, and saponins are present in the neem leaf and alter the hypo-thalamo--pituitary-testis axis (Seriana *et al.,* 2021). Disruption in gonadotropin of male rats following feeding of leaf extract of neem leaf extract has been reported by several workers (Aladakatti *et al.,* 2005; Santra and Manna, 2009; Auta and Hassan, 2016; Lisanti *et al.,* 2018). The present study was carried out to assess the effect of ethanolic leaf extract of *A. indica* on testicular morphometry (length, width, thickness and volume) and histology of New Zealand White male rabbits.

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Materials and Methods

Preparation of Ethanolic Leaf Extract

Fresh leaves of *A. indica* were collected from the University campus Kumarganj, Ayodhya (India). The leaves of *A. indica* (neem) were properly washed, dried at room temperature,

and grinded to fine powder. The extraction of dried leaf powder was carried out as per standard protocol (Anonymous, 1983) using ethanol as a solvent. After filtration, ethanol was evaporated from extract through vacuum evaporator under low pressure and temperature (30°C). Finally, the extract in paste form was stored in deep freezer at -20°C.

Experimental Design

The study was conducted at small animal house of the College of Veterinary Science, Kumarganj, Ayodhya. Eighteen healthy, adult male New Zealand White rabbits, weighing 1.83-1.90 kg were included in this study. The animals were randomly divided into three groups, a control group (group A) and two experimental groups (group B and C) with 6 animals in each group. Animals of group B and C were treated with ethanolic leaf extract of *A. indica* @ 100 mg/kg body weight/day and 200 mg/kg body weight/day for 60 days, respectively, in distilled water as drinking water. Animals of the control group (group A) received only distilled water. The experiment was extended upto 120 days after 60 days of treatment without administration of the neem leaf extract.

Testicular Morphometry

Testicular length, width and thickness of both testicles were measured on day 0, 15, 30, 60 and 120 after initiation of treatments by Vernier calipers. Testicular volume was calculated by the formula given by Paltiel *et al.* (2002). All the measurements were done on intact testicles.

$$\text{Testicular volume } (cm^3) = TL \times TW^2 \times 0.52$$

TL = Testicular Length, TW = Testicular Width

Testicular Histology

Inj. Xylazine hydrochloride @ 4 mg/kg body weight I/M was used to restrain and sedate the animals. Tissue samples were taken from the testicles of each group of animals using Bard Max Core biopsy instrument (16 G). Samples were collected on day 0 and 60 post-treatment and were stored in Bouin's solution. Tissue samples were processed in acetone-benzene sequence, embedded and blocked-in paraffin wax. Tissue sections of 5 µ thickness were obtained with the help of the microtome. The samples were mounted on a clean glass slide. The Hematoxylin and Eosin (H & E) staining was performed as per method described by Heehan and Hrapchak (1987).

Statistical Analysis

The data were analyzed by two-way ANOVA for testicular morphometry to compare the mean values between the days within the group and between different groups (Snedecor and Cochran, 1994).

Results and Discussion

Testicular Length (cm)

The mean right and left testicular length in different groups of rabbits before (0 day) and after treatment (day 15, 30, 60 and 120) are presented in the Table 1. After 60 days of treatment, significant ($p < 0.05$) reduction in mean right testicular length of group B (3.29 ± 0.10 vs 2.94 ± 0.06) and C (3.26 ± 0.04 vs 2.42 ± 0.08) was recorded in comparison to their pre-treatment values. Similarly, the mean left testicular length of group B (3.14 ± 0.047 vs 2.86 ± 0.05) and C (3.15 ± 0.02 vs 2.65 ± 0.06), reduced significantly ($p < 0.05$) in comparison to their pre-treatment values. The percent reduction in right and left testicular length was higher in group C (25.77 and 15.87%) as compared to group B (10.64 and 8.91%).

Testicular Width (cm)

The mean right and left testicular width in different groups of rabbits before (0 day) and after treatment (day 15, 30, 60 and 120) are shown in the Table 2. Perusal of the data given in table 2 revealed that after 60 days of treatment, a significant ($p < 0.05$) reduction in mean right testicular width of group B (1.06 ± 0.01cm *vs* 0.96 ± 0.01cm) and C (1.10 ± 0.03cm *vs* 0.89 ± 0.01cm) was observed in comparison to their pre-treatment values. Similarly, the mean left testicular width also reduced significantly ($p < 0.05$) in group B (1.01 ± 0.01 cm *vs* 0.88 ± 0.02 cm) and C (1.05 ± 0.02 cm *vs* 0.84 ± 0.01 cm). The percent reduction in right and left testicular width was highest in group C (19.09 and 20.00%) as compared to group B (15.43 and 12.87%).

Testicular Thickness (cm)

The mean right and left testicular thickness in different groups of rabbits before (0 day) and after treatment (day 15, 30, 60 and 120) are presented in the Table 3. The results revealed that after 60 days of treatment, significant ($p < 0.05$) reduction in mean right testicular thickness of group B (1.01 ± 0.02 cm *vs* 0.94 ± 0.01 cm) and C (1.06 ± 0.02 cm *vs* 0.84 ± 0.01 cm) was observed in comparison to their pretreatment values. Similarly significant ($p < 0.05$) reduction in mean left testicular thickness of group B (0.99 ± 0.01cm *vs* 0.90 ± 0.01cm) and C (1.00 ± 0.01cm *vs* 0.83 ± 0.01cm) was also observed. On day 60 post-treatment, the per cent reduction in right and left testicular thickness was highest in group C (20.25 and 17.00 %) followed by B (6.93 and 9.00 %). After 60 days of discontinuation of treatment (day 120), there was either significant or non-significant reversal in the testicular measures in both the extract treated groups (Tables 1-3).

Testicular Volume (cm^3)

The mean right and left testicular volume in different groups of rabbits before (0 day) and after treatment (day 15, 30, 60 and 120) are shown in the Table 4. On day 60 post-treatment, the mean right testicular volume (cm^3) declined significantly ($p < 0.05$) in group B (1.92 ± 0.03 cm^3 *vs* 1.43 ± 0.06 cm^3) and C (2.05 ± 0.05 cm^3 *vs* 1.00 ± 0.03 cm^3) in comparison to their pre-treatment values. Similarly, the mean left testicular volume reduced significantly ($p < 0.05$) in group B (1.65 ± 0.03 cm^3 *vs* 1.15 ± 0.06 cm^3) and C (1.78 ± 0.05 cm^3 *vs* 0.95 ± 0.03 cm^3). The per cent reduction in the mean right and left

Table 1: Right and left testicular length (Mean ± SE) in different groups of rabbits before and after treatment (cm)

		Treatment days				
Length	*Groups (n=6 each)*	*0*	*15*	*30*	*60*	*120*
Right testis	Group A (Control, Distilled water)	3.24 ± 0.11^{Aa}	3.22 ± 0.09^{Aa}	3.22 ± 0.11^{Aa}	3.25 ± 0.10^{Aa}	3.25 ± 0.10^{Aa}
	Group B (*A. indica* @ 100 g/kg b. wt.)	3.29 ± 0.10^{Aa}	3.22 ± 0.08^{Aab}	3.08 ± 0.07^{ABb}	2.94 ± 0.06^{Bc}	3.11 ± 0.09^{Aa}
	Group C (*A. indica* @ 200 g/kg b. wt.)	3.26 ± 0.04^{Aa}	3.09 ± 0.02^{Aa}	2.94 ± 0.04^{Bb}	2.42 ± 0.08^{Cc}	3.08 ± 0.04^{Aa}
Left testis	Group A (Control, Distilled water)	3.03 ± 0.06^{Aa}	3.05 ± 0.05^{Aa}	3.04 ± 0.07^{Aa}	3.06 ± 0.08^{Aa}	3.05 ± 0.08^{Aa}
	Group B (*A. indica* @ 100 g/kg b. wt.)	3.14 ± 0.07^{Aa}	3.05 ± 0.07^{Aab}	2.95 ± 0.06^{Ab}	2.86 ± 0.05^{Bb}	3.05 ± 0.06^{Aa}
	Group C (*A. indica* @ 200 g/kg b. wt.)	3.15 ± 0.02^{Aa}	3.04 ± 0.02^{Aa}	2.80 ± 0.08^{Bb}	2.65 ± 0.06^{Cb}	3.07 ± 0.04^{Aa}

Means with different superscripts within the row (a, b, c) and within the column (A, B, C) differ significantly for the right or left testes ($p < 0.05$).

Table 2: Right and left testicular width (Mean ± SE) in different groups of rabbits before and after treatment (cm)

		Treatment days				
Width	*Groups (n=6 each)*	*0*	*15*	*30*	*60*	*120*
Right testis	Group A (Control, Distilled water)	1.05 ± 0.01^{Aa}	1.05 ± 0.01^{Aa}	1.04 ± 0.01^{Aa}	1.04 ± 0.02^{Aa}	1.05 ± 0.01^{Aa}
	Group B (*A. indica* @ 100 g/kg b. wt.)	1.06 ± 0.01^{Aa}	1.03 ± 0.02^{Aab}	1.00 ± 0.01^{Ab}	0.96 ± 0.01^{Bb}	1.02 ± 0.01^{Aa}
	Group C (*A. indica* @ 200 g/kg b. wt.)	1.10 ± 0.03^{Aa}	1.03 ± 0.03^{Aa}	0.96 ± 0.02^{Bc}	0.89 ± 0.01^{Cd}	1.05 ± 0.03^{Aa}
Left testis	Group A (Control, Distilled water)	1.03 ± 0.01^{ABa}	1.02 ± 0.01^{Aa}	1.02 ± 0.01^{Aa}	1.04 ± 0.01^{Aa}	1.05 ± 0.01^{Aa}
	Group B (*A. indica* @ 100 g/kg b. wt.)	1.01 ± 0.01^{Ba}	0.98 ± 0.01^{Ba}	0.95 ± 0.01^{Bb}	0.88 ± 0.02^{Bc}	0.96 ± 0.01^{Cb}
	Group C (*A. indica* @ 200 g/kg b. wt.)	1.05 ± 0.02^{Aa}	0.99 ± 0.01^{ABb}	0.94 ± 0.01^{Bc}	0.84 ± 0.01^{Cd}	0.99 ± 0.02^{Bb}

Means with different superscripts within the row (a, b, c) and within the column (A, B, C) differ significantly for the right or left testes ($p < 0.05$).

Table 3: Right and left testicular thickness (Mean ± SE) in different groups of rabbits before and after treatment (cm)

		Treatment days				
Thick-ness	*Groups (n=6 each)*	*0*	*15*	*30*	*60*	*120*
Right testis	Group A (Control, Distilled water)	0.99 ± 0.01^{Ca}	1.00 ± 0.01^{Aa}	1.00 ± 0.01^{Aa}	0.99 ± 0.01^{Aa}	1.00 ± 0.01^{Aa}
	Group B (*A. indica* @ 100 g/kg b. wt.)	1.01 ± 0.02^{BCa}	0.99 ± 0.01^{Aa}	0.97 ± 0.01^{Ab}	0.94 ± 0.01^{Bc}	0.97 ± 0.01^{Ab}
	Group C (*A. indica* @ 200 g/kg b. wt.)	1.06 ± 0.02^{Aa}	0.99 ± 0.01^{Ab}	0.94 ± 0.01^{Bc}	0.84 ± 0.01^{Cd}	0.98 ± 0.01^{Ab}
Left testis	Group A (Control, Distilled water)	0.98 ± 0.01^{Aa}	0.98 ± 0.01^{Aa}	0.98 ± 0.02^{Aa}	0.97 ± 0.01^{Aa}	0.97 ± 0.01^{Aa}
	Group B (*A. indica* @ 100 g/kg b. wt.)	0.99 ± 0.01^{Aa}	0.98 ± 0.01^{Aa}	0.93 ± 0.01^{Bc}	0.90 ± 0.01^{Bd}	0.95 ± 0.01^{Ab}
	Group C (*A. indica* @ 200 g/kg b. wt.)	1.00 ± 0.01^{Aa}	0.97 ± 0.01^{Ab}	0.92 ± 0.01^{Bc}	0.83 ± 0.01^{Cd}	0.96 ± 0.01^{Ab}

Means with different superscripts within the row (a, b, c) and within the column (A, B, C) differ significantly for the right or left testes ($p < 0.05$).

testicular volume was higher in group C (31.21 and 46.63%) as compared to group B (25.52 and 30.30%). Testicular volume also revealed either significant or non-significant reversal in both the extract treated groups after 60 days of discontinuation of treatment (day 120) (Table 4).

In all the treatment groups, the mean testicular length, width, thickness and volume reduced significantly ($p < 0.05$) on day 60 post-treatment in comparison to their pre-treatment values. In the present study, the reduction in the testicular length, width, thickness and volume might be due to reduction in the concentration of testosterone and protein content following treatment with ethanolic leaf extract of *A. indica*. Flavonoid, steroid, alkaloid, tannins, terpenoids and saponins like active compound are present in neem, which might have reduced the steroidal hormones level in the rabbit by affecting on hypo-thalamo-pituitary-testis axis (Seriana *et al.*, 2021).

Perusal of literature revealed no information regarding effect of ethanolic leaf extract of *A. indica* on testicular

Table 4: Right and left testic<ular volume (Mean ± SE) in different groups of rabbits before and after treatment (cm^3)

		Treatment days				
Volume	*Groups (n=6 each)*	*0*	*15*	*30*	*60*	*120*
Right testis	Group A (Control, Distilled water)	1.87 ± 0.04^{Aa}	1.85 ± 0.04^{Aa}	1.82 ± 0.04^{Aa}	1.84 ± 0.06^{Aa}	1.87 ± 0.08^{Aa}
	Group B (*A. indica* @ 100 g/kg b. wt.)	1.92 ± 0.03^{Aa}	1.75 ± 0.04^{Aa}	1.60 ± 0.04^{Bbc}	1.43 ± 0.06^{Bc}	1.69 ± 0.05^{Ab}
	Group C (*A. indica* @ 200 g/kg b. wt.)	2.05 ± 0.05^{Aa}	1.71 ± 0.02^{Ab}	1.41 ± 0.05^{Cc}	1.00 ± 0.03^{Cd}	1.76 ± 0.05^{Ab}
Left testis	Group A (Control, Distilled water)	1.67 ± 0.04^{ABa}	1.63 ± 0.04^{Aa}	1.62 ± 0.04^{Aa}	1.73 ± 0.06^{Aa}	1.73 ± 0.08^{Aa}
	Group B (*A. indica* @ 100 g/kg b. wt.)	1.65 ± 0.03^{Ba}	1.52 ± 0.04^{Ab}	1.40 ± 0.04^{Bc}	1.15 ± 0.06^{Bd}	1.48 ± 0.05^{Cb}
	Group C (*A. indica* @ 200 g/kg b. wt.)	1.78 ± 0.05^{Aa}	1.55 ± 0.02^{Ab}	1.28 ± 0.05^{Cc}	0.95 ± 0.03^{Cd}	1.58 ± 0.05^{BCb}

Means with different superscripts within the row (a, b, c) and within the column (A, B, C) differ significantly for the right or left testes ($p < 0.05$).

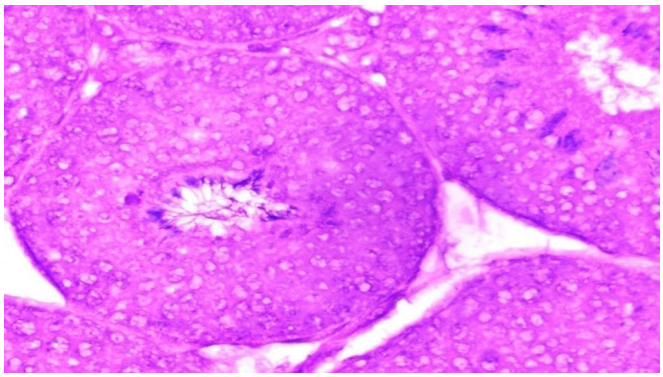

Fig. 1: Histology of testis (H&E staining; 400X) of rabbit in control group showing normal seminiferous tubules, basement membranes and spermatogenesis

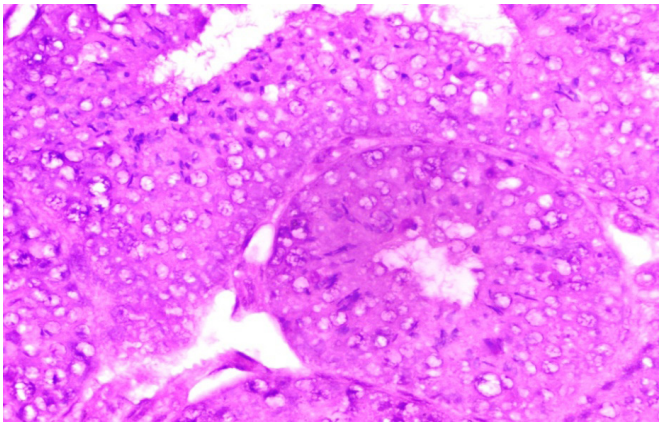

Fig. 2: Histology of testis (H&E staining; 400X) of Group B rabbit (*A. indica* @ 100 mg/kg b.wt.) showing homogenous fluid material in the interstitium with mild disorganization of spermatogonial cells

morphometry in male New Zealand White rabbits. However, our findings corroborate with the findings of Gois *et al.* (2019), who observed significant decline in the testicular length and width of male quails fed with ration containing 40% *A. indica* seeds. Singh (2020) also observed significant reduction in the testicular length, width, thickness and volume of male rabbits following treatment with methanolic stem extract of *Dendrophthoe falcata*.

Testicular Histology

The results of the histopathology of the testes (H&E staining) are shown in the Figs. 1 to 3. In control group, the testicular section showed normal histological pattern with normal seminiferous tubules, basement membranes and spermatogenesis. The seminiferous tubules appeared round or oval and surrounded by a basement membrane enclosing myoid cells. The seminiferous tubules were lined by stratified germinal epithelium, spermatogonia, sertoli cells, primary spermatocytes, secondary spermatocytes, round spermatids and spermatozoa into lumen (Fig. 1).

The histo-pathological sections of group B (ethanolic leaf extract of *A. indica* @ 100 mg/kg body weight/day for 60 days) rabbit testis (H&E Staining; 400X) showed abnormal histological pattern with homogenous fluid material in the interstitium with mild disorganization of spermatogonial cells (Fig. 2).

The histo-pathological sections of group C (ethanolic leaf extract of *A. indica* @ 200 mg/kg body weight/day for 60 days) rabbit testis (H&E Staining; 400X) showed abnormal histological pattern showing shrinking in the size of seminiferous tubules, small layers of germinative cells with vacuolation and clumping of spermatogonial cells (Fig. 3).

The seminiferous tubules showed disruption of vacuolation, clumping of spermatogonial cells and lumen contains few spermatozoa with large amount of tissue debris.

Sperm production cannot proceed optimally to completion without a continuous supply of androgen (Seetharam *et al.,* 2003). Leydig cells influence the seminiferous tubules by maintaining a high concentration of testosterone in the peritubular compartments of the testis (Mali *et al.,* 2001; Gupta *et al.,* 2006). In the present study, due to degeneration of Leydig cells the testosterone concentration was reduced significantly ($p < 0.05$) after treatment, which might be the cause of spermatogenesis arrest (Bhasin *et al.,* 1988; Sarkar *et al.,* 1997). Spermatogenesis is controlled by action of testosterone hormone. It is necessary for completion of meiotic division,

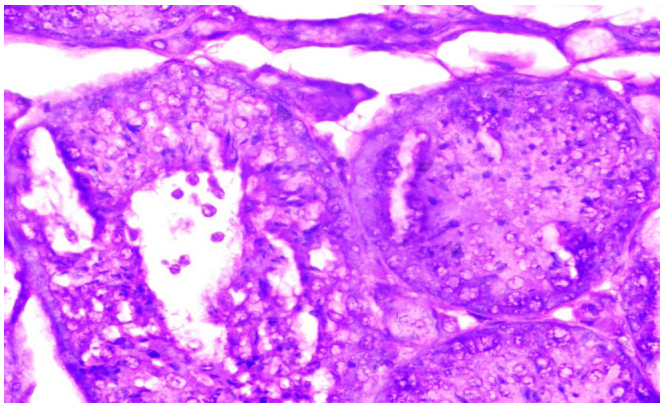

Fig. 3: Histology of testis (H&E stanning; 400X) of Group C rabbits *(A. indica* @ 200 mg/kg b.wt.) showing shrinkage in the size of seminiferous tubules, absence of spermatogenesis, with vacuolation and clumping of the spermatogonial cells

differentiation of spermatid cells (Poccia, 2011) and for starting the process of meiosis in spermatocyte cells. Testosterone plays crucial role in the first meiotic phase of the diakinesis stage.

Perusal of literature revealed no information regarding effect of ethanolic leaf extract of *A. indica* on histological structure of testis in rabbits. So, our results could not be compared. However, our findings agree with the findings of Gowda *et al.* (1996), who observed alteration in testicular structure and arrest of spermatogenesis in rabbits following feeding of processed neem (*A. indica*) kernel meal incorporated diets. Similarly, Mishra and Singh (2005), Morovati *et al.* (2009), Auta and Hassan (2016), El-Beltagy *et al.* (2020) also recorded significant alteration in histological structure of testis and arrest of spermatogenesis in male rats following treatment with aqueous leaf extract of *A. indica*, commercial neem extract Neem Azal-T/S, aqueous wood extract of *A. indica*, and ethanolic leaf extract of *A. indica*, respectively.

Conclusion

Ethanolic leaf extract of *A. indica* (neem) showed significant alteration in morphometry and histology of the testicles on day 60 over day 0. In the present study, it can be concluded that ethanolic leaf extract of *A. indica* (neem) has contraceptive like effect in male rabbits, as it showed variable reversal in next 60 day after discontinuation of treatment.

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ANNOUNCEMENT: SVSBT-NS-2022

IX Annual Convention and National Seminar of SVSBT

The ***IX Annual Convention*** and ***National Seminar*** of The Society for Veterinary Science & Biotechnology ***(SVSBT) on "Recent Biotechnological Advances in Health and Management to Augment Productivity of Livestock and Poultry"*** will be **organized at Ramayanpatti, Tirunelveli - 627 358, Tamil Nadu, during September 22-24, 2022** (Thursday, Friday & Saturday) by Veterinary College & Research Institute, Tirunelveli - 627 358, TANUVAS, (TN). The detailed Brochure cum Invitation showing Theme Areas/ Sessions, Registration Fee, Bank Details for online payment and deadlines, etc. has been floated on the Whats Apps and e-mails. Accordingly, the organizing committee of ***SVSBT NS-2022 invites abstracts*** of original and quality research work on theme areas of seminar limited to 250 words by e-mail on svsbttnns2022@gmail.com or mopandian69@gmail.com latest by 30th August, 2022 for inclusion in the Souvenir cum Compendium to be published on the occasion.

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