CHAPTER – 5:

RESULTS

5. RESULTS

This chapter consists of two parts:

- The effect of boiled aqueous preparation of *D. esculentum* (BDE) on different *in vivo* and *ex vivo* parameters of Swiss albino mouse
- Comparative effect of crude- and boiled aqueous preparation of *D. esculentum* (CDE *vs.* BDE) on different *in vivo* and *ex vivo* parameters of Swiss albino mouse

5.1. THE EFFECT OF BOILED AQUEOUS PREPARATION OF *D. ESCULENTUM* (BDE) ON DIFFERENT *IN VIVO* AND *EX VIVO* PARAMETERS OF SWISS ALBINO MOUSE

5.1.1. Effect of BDE on the immune system of mouse

5.1.1.1. Assessment of the humoral immune responses (PFC and HA titre assay)

Significant decreases ($p < 0.001$) in number were observed in the formation of antibody secreting cells (plaques) in case of doses (80, 160, 320 mg/kg b.w) treated for longer durations (135 days and 180 days) (Table 2) when compared with the respective control groups. Table 1 and 2 showed dose- and time-dependent decrease in the HA titre value when they were compared with that of their respective controls. After 180 days of the treatment with different doses of BDE, 16 fold decreases in the titre value were observed when compared with the controls.

5.1.1.2. Measurement of body weight, relative spleen weight and counting of the splenocytes

Significant decreases in the body weights were observed in case of mice that were fed with BDE for longer durations (135 days and 180 days) ($p < 0.05$) when compared with their respective control groups (Group 1) (Table 2). Significant decreases ($p < 0.05$, $p < 0.01$ and $p < 0.001$) in the relative spleen weights were also observed in case of the treated groups when compared with the respective controls (Table 1 and 2). Results also showed that the number of splenocytes was also significantly decreased ($p < 0.01$ and $p < 0.001$) in case of BDE treated groups when compared with their respective controls (Table 1 and 2).

Table 1. Effect of BDE on the body weight, relative spleen weight, number of splenocytes, number of plaques formed, hemagglutination titer value and number of peritoneal macrophages of mice after 15 (S1) and 45 d (S2) of treatment.

	15 d (S1)				45 d (S2)			
Parameters observed (¶)	S1G1	S1G2	S1G3	S1G4	S2G1	S2G2	S2G3	S2G4
Body weight (g)	25.38 ± 0.49	25.36 ± 0.36	25.30 ± 0.65	25.25 ± 0.39	25.53 ± 0.48	25.35 ± 0.58	25.36 ± 0.53	25.33 ± 0.43
Relative spleen weight (g/100 g of body weight)	0.49 ± 0.008	0.48 ± 0.006	0.48 ± 0.003[a]	0.47 ± 0.005[b]	0.50 ± 0.005	0.48 ± 0.003[c]	0.47 ± 0.005[c]	0.45 ± 0.003[c]
Number of splenocytes (mean ± S.D.) $\times 10^6$/ml	31.84 ± 0.59	31.41 ± 0.47	30.77 ± 0.51[b]	30.50 ± 0.59[b]	31.89 ± 0.52	30.18 ± 0.48[c]	28.96 ± 0.44[c]	28.16 ± 0.45[c]
PFC/10^6 cells	120 ± 6.32	118.33 ± 4.08	110.83 ± 4.92[a]	107.5 ± 5.24[b]	120.83 ± 7.36	116.67 ± 2.58	110.83 ± 4.92[a]	106.67 ± 5.16[c]
HA titer value	1:160	1:160	1:160	1:80	1:160	1:80	1:40	1:40
Number of Macrophages (mean ± S.D.) $\times 10^6$	15.94 ± 1.05	15.57 ± 1.73	15.36 ± 1.78	15.14 ± 0.87	15.30 ± 1.05	14.56 ± 0.59	14.56 ± 1.00	14.18 ± 1.24

¶ All values (except HA titer) are mean ± SD of six observations.
[a] $p < 0.05$ when compared with Group 1 (control) (significantly different).
[b] $p < 0.01$ when compared with Group 1 (control) (significantly different).
[c] $p < 0.001$ when compared with Group 1 (control) (significantly different).

Table 2. Effect of BDE on the body weight, relative spleen weight, number of splenocytes, number of plaques formed, hemagglutination titer value and number of peritoneal macrophages of mice after 90 (S3), 135 (S4) and 180 d (S5) of treatment.

	90 d (S3)				135 d (S4)				180 d (S5)			
Parameters Observed (¶)	S3G1	S3G2	S3G3	S3G4	S4G1	S4G2	S4G3	S4G4	S5G1	S5G2	S5G3	S5G4
Body weight (g)	25.48 ± 0.41	25.28 ± 0.56	25.3 ± 0.42	24.86 ± 0.20	25.55 ± 0.35	25.38 ± 0.51	25.01 ± 0.29[a]	24.93 ± 0.22[a]	25.38 ± 0.51	25.03 ± 0.42	24.93 ± 0.32	24.7 ± 0.43[a]
Relative spleen weight (g/100 g of body weight)	0.49 ± 0.005	0.47 ± 0.006[c]	0.45 ± 0.002[c]	0.43 ± 0.005[c]	0.49 ± 0.010	0.46 ± 0.005[c]	0.43 ± 0.005[c]	0.42 ± 0.005[c]	0.49 ± 0.003	0.44 ± 0.005[c]	0.41 ± 0.005[c]	0.38 ± 0.007[c]
Number of splenocytes (mean ± S.D.) $\times 10^6$/ml	31.84 ± 0.59	29.44 ± 0.83[c]	28.10 ± 0.55[c]	26.77 ± 0.62[c]	32 ± 0.70	28.53 ± 0.55[c]	27.41 ± 0.48[c]	25.76 ± 0.59[c]	31.84 ± 0.87	27.84 ± 0.45[c]	25.97 ± 0.84[c]	23.73 ± 0.74[c]
PFC/10^6 cells	119.17 ± 5.85	105.83 ± 5.85[b]	98.33 ± 4.08[c]	89.17 ± 5.85[c]	112.50 ± 7.58	102.50 ± 8.80	92.50 ± 7.58[c]	69.17 ± 5.85[c]	115.47 ± 4.47	100 ± 7.07[c]	80 ± 7.07[c]	55 ± 4.47[c]
HA titer value	1:160	1:80	1:40	1:40	1:160	1:20	1:20	1:20	1:160	1:10	1:10	1:10
Number of Macrophages (mean ± S.D.) $\times 10^6$	16.05 ± 1.27	15.09 ± 1.39	14.72 ± 1.99	14.08 ± 1.23	16.26 ± 1.42	15.04 ± 1.10	13.22 ± 0.82[c]	14.34 ± 1.39[c]	17.06 ± 0.94	14.45 ± 1.23[b]	13.65 ± 1.48[c]	11.36 ± 0.77[c]

¶ All values (except HA titer) are mean ± SD of six observations.
[a] $p < 0.05$ when compared with Group 1 (control) (significantly different).
[b] $p < 0.01$ when compared with Group 1 (control) (significantly different).
[c] $p < 0.001$ when compared with Group 1 (control) (significantly different).

5.1.1.3. Effect of BDE on the number of peritoneal macrophages

As indicated in the Table 1 and 2, the number of the peritoneal macrophages decreased significantly in both dose- and time-dependent manner. After 15 day dose duration period, no significant decrease was observed in the mice that were treated with BDE at a dose of 320 mg/kg b.w., whereas after 180 days of treatment, significant decreases ($p < 0.01$ and $p < 0.001$) were observed in case of all the doses when compared with the respective control groups.

5.1.1.4. Effect of BDE on splenocyte proliferation

After 24 h, 48 h and 72 h of incubation, at 0 µg/ml (control), the number of splenocytes was $(11.57 \pm 0.93) \times 10^6$ cells/ml, $(16.16 \pm 0.59) \times 10^6$ cells/ml and $(12.96 \pm 0.59) \times 10^6$ cells/ml, respectively, whereas, at 200 µg/ml (highest dose), the number of splenocytes decreased remarkably to $(2.93 \pm 0.37) \times 10^6$ cells/ml, $(4.10 \pm 0.47) \times 10^6$ cells/ml and $(3.2 \pm 0.49) \times 10^6$ cells/ml, respectively. Therefore, the results clearly indicated that the number of splenocytes was significantly ($p < 0.001$) decreased in a dose-dependent manner in each case when they were compared with their respective controls (Figure 5 and 6). As shown in Figure 7, a significant ($p < 0.001$) dose-dependent increase in the percentage inhibition of the splenocyte proliferation has been observed in case of the BDE treated splenocytes when compared with the control (0 µg/ml). At 200 µg/ml, the percentage of inhibition was 35.04%. The IC_{50} value of BDE was 412.96 ± 12.13 µg/ml.

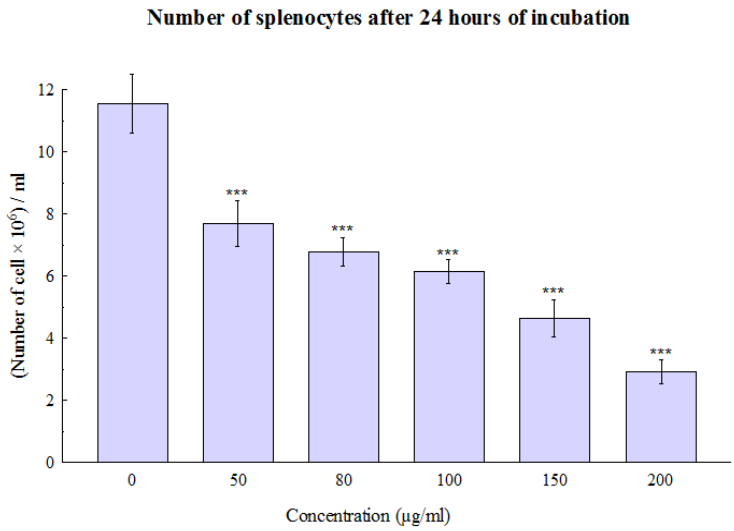


Figure 5. Effect of BDE on the primary cultured splenocytes after 24 h of incubation. Data represent the dose-dependent decrease in the number of splenocytes. The results are mean ± S.D. of six parallel observations. ***$p < 0.001$ vs. 0 µg/ml.

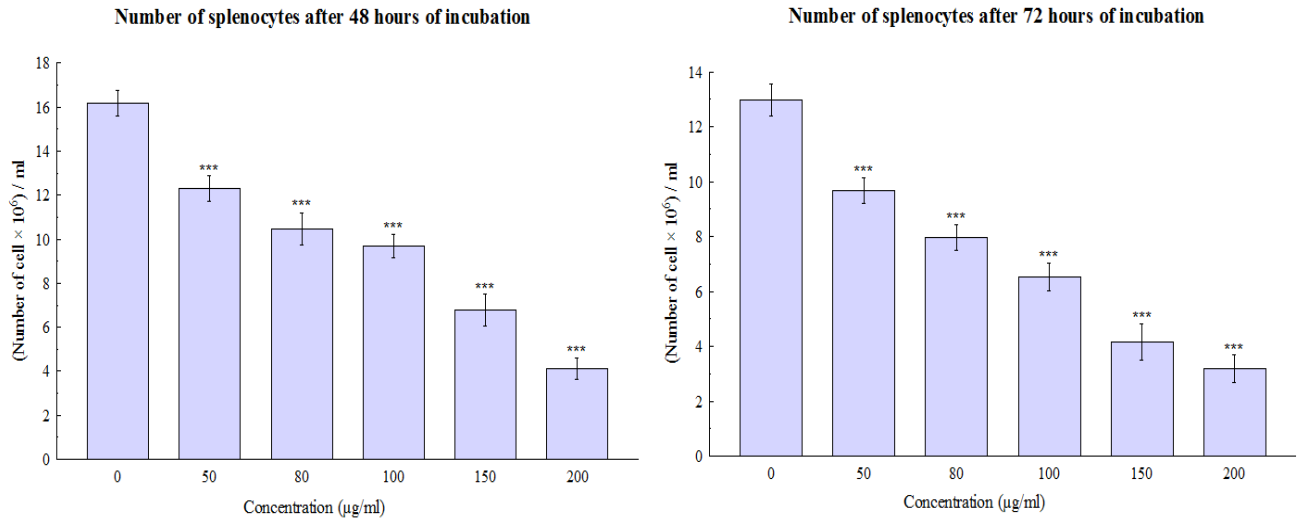


Figure 6. Effect of BDE on the primary cultured splenocytes after 48 h and 72 h of incubation. Data represent the dose-dependent decrease in the number of splenocytes. The results are mean ± S.D. of six parallel observations. ***p < 0.001 vs. 0 µg/ml.

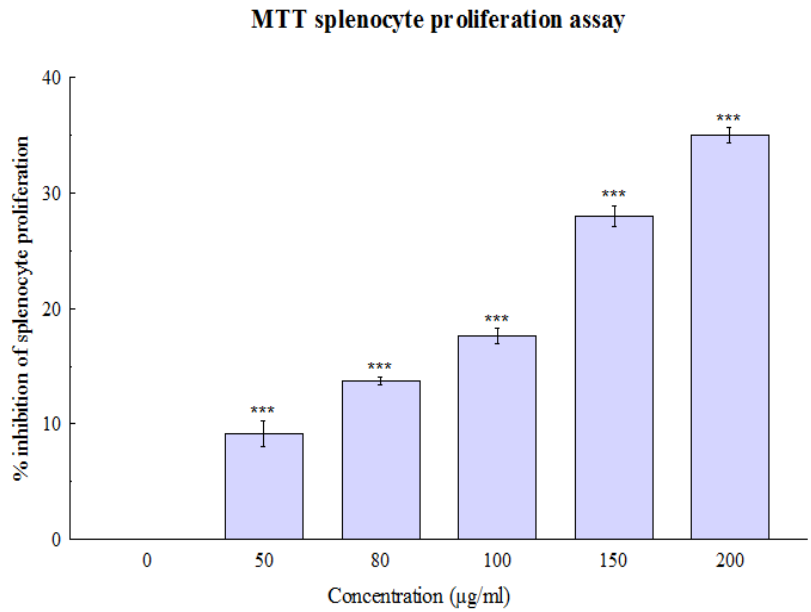


Figure 7. MTT splenocytes proliferation assay demonstrates dose-dependent increase in the percentage inhibition of splenocytes proliferation in the BDE treated splenocytes. At 200 µg/ml, the percentage of inhibition was 35.04%. The IC_{50} value of BDE was 412.96 ± 12.13 µg/ml. The results are mean ± S.D. of six parallel observations. ***p < 0.001 vs. 0 µg/ml.

5.1.1.5. Assessment of the effect of BDE on hemolysis

The hemolytic activity of BDE was increased significantly ($p < 0.001$) in a dose-dependent manner in case of mouse erythrocytes (Figure 8). Total hemolysis was obtained using 100 µl of Triton X-100 (0.1%) after 30 min of incubation (not shown in the figure). At 50 µg/ml, the percentages of hemolysis was 40.75%, whereas, the IC_{50} value was 61.78 ± 2.77 µg/ml.

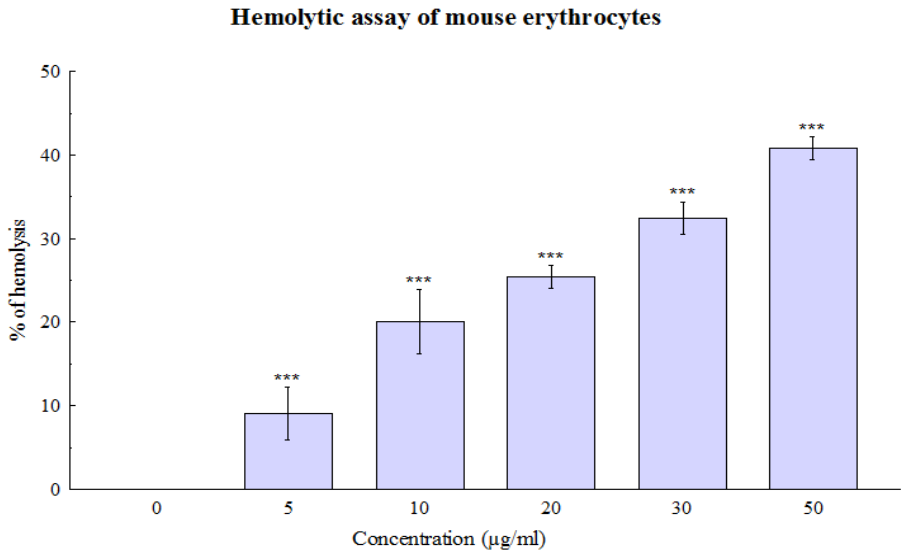


Figure 8. The hemolytic activity of BDE was increased significantly in a dose-dependent manner in case of mouse erythrocytes. At 50 µg/ml, the percentage of hemolysis was 40.75%. The IC_{50} value of BDE was 61.78 ± 2.77 µg/ml. The results are mean ± S.D. of six parallel observations. ***p < 0.001 vs. 0 µg/ml.

5.1.1.6. Effect of BDE on serum concentration levels of Th1 and Th2 cytokines

Significant decreases ($p < 0.05$, $p < 0.01$ and $p < 0.001$) were observed in both Th1 and Th2 cytokine concentrations in mice that were treated with different doses of BDE for 90, 135 and 180 days, when compared with their respective control groups (Table 4). After 15 and 45 days of treatment with different doses of BDE, the concentrations of IL-2, IL-4 and IL-10 did not decrease significantly, though after 45 days of treatment with BDE at 160 and 320 mg/kg bw, the concentrations of IFN-γ has been shown to decrease significantly ($p <0.05$) when compared with their respective controls (Table 3). After 180 days of treatment at with 80, 160 and 320 mg/kg bw of BDE, the concentration of all the cytokines as well as serum IgM decreased significantly when compared to their respective controls ($p < 0.001$) (Table 4 & Figure 40).

Table 3. Serum concentrations of different Th1 (IL-2 and IFN-γ) and Th2 (IL-4 and IL-10) cytokines of mice that are treated with different doses of BDE for 15 (S1) and 45 (S2) days.

Concentration of different cytokines[a]	15 days (S1)				45 days (S2)			
	S1G1	S1G2	S1G3	S1G4	S2G1	S2G2	S2G3	S2G4
IL-2 (pg/ml)	35.59 ± 1.50	35.44 ± 1.45	34.92 ± 0.17	34.74 ± 0.13	35.21 ± 1.18	35.34 ± 1.02	33.92 ± 0.24	35.30 ± 0.21
IFN-γ (pg/ml)	1147.61 ± 8.16	1144.48 ± 8.75	1144.42 ± 8.39	1143.97 ± 11.07	1149.27 ± 1.38	1139.81 ± 7.52	1134.42 ± 8.39*	1130.64 ± 4.68*
IL-4 (pg/ml)	136.81 ± 2.23	136.55 ± 0.76	135.89 ± 1.47	135.81 ± 1.68	136.14 ± 1.81	134.87 ± 2.79	134.55 ± 0.88	133.14 ± 1.14
IL-10 (pg/ml)	3477.09 ± 3.09	3472.06 ± 7.58	3461.05 ± 6.44	3460.42 ± 12.87	3466.42 ± 11.74	3463.73 ± 12.77	3458.05 ± 2.12	3457.42 ± 8.69

[a]All values are mean ± SD of three observations.
*$p < 0.05$ when compared with Group 1 (Control) (Significantly different).

Table 4. Serum concentrations of different Th1 (IL-2 and IFN-γ) and Th2 (IL-4 and IL-10) cytokines of mice that are treated with different doses of BDE for 90 (S3), 135 (S4) and 180 (S5) days.

Concentration of different cytokines[a]	90 days (S3)				135 days (S4)				180 days (S5)			
	S3G1	S3G2	S3G3	S3G4	S4G1	S4G2	S4G3	S4G4	S5G1	S5G2	S5G3	S5G4
IL-2 (pg/ml)	35.96 ± 1.51	34.56 ± 1.51	32.51 ± 0.61*	32.69 ± 0.22*	35.99 ± 1.55	32.04 ± 0.34**	30.76 ± 1.21***	29.09 ± 0.68***	35.99 ± 0.66	31.70 ± 0.52***	29.09 ± 1.06***	23.61 ± 1.09***
IFN-γ (pg/ml)	1152.27 ± 5.58	1136.48 ± 1.56**	1126.75 ± 5.14***	1119.97 ± 5.09***	1148.61 ± 4.91	1113.15 ± 6.77***	1098.75 ± 5.64***	1078.13 ± 5.34***	1150.94 ± 9.04	1013.15 ± 6.77***	984.73 ± 11.42***	902.99 ± 12.79***
IL-4 (pg/ml)	135.47 ± 1.31	131.22 ± 0.43**	130.22 ± 0.67**	129.81 ± 1.93**	136.81 ± 0.47	129.55 ± 0.76***	128.22 ± 0.46***	124.81 ± 1.93***	136.37 ± 0.87	126.55 ± 1.37***	125.97 ± 3.00***	119.81 ± 1.24***
IL-10 (pg/ml)	3467.09 ± 10.80	3457.06 ± 7.62	3441.38 ± 7.81	3440.76 ± 15.86*	3463.75 ± 4.52	3443.73 ± 3.02**	3434.71 ± 3.83***	3420.76 ± 6.64***	3470.42 ± 6.14	3407.06 ± 18.71***	3328.05 ± 9.52***	3227.42 ± 8.48***

[a]All values are mean ± SD of three observations.
*$p < 0.05$ when compared with Group 1 (Control) (Significantly different).
**$p < 0.01$ when compared with Group 1 (Control) (Significantly different).
***$p < 0.001$ when compared with Group 1 (Control) (Significantly different).

5.1.1.7. Effect of BDE on Th1 and Th2 cytokine production from primary cultured splenocytes

Figure 9 indicated significant concentration-dependent IL-2 decrease ($p < 0.001$) in con A induced splenocytes. At 0 µg/ml, the concentration of IL- 2 in splenocyte culture supernatant was 28.33 ± 0.58 pg/ml, whereas, at 200 µg/ml, the concentration of IL-2 decreased to 19.17±0.38 pg/ml. The amount of IFN-γ, IL-4 and IL-10 were also decreased significantly ($p < 0.01$ and $p < 0.001$) in concentration-dependent manner when compared with their respective controls. At 0 µg/ml of BDE, the concentrations of IFN-γ, IL-4 and IL-10 were 913.33 ± 5.77 pg/ml, 127.76 ± 2.21 pg/ml and 2208.33 ± 14.43 pg/ml, respectively, whereas, at 200 µg/ml, the concentrations of IFN-γ, IL-4 and IL-10 has been shown to decrease to 216.67 ± 5.77 pg/ml, 104.79 ± 2.45 pg/ml and 1325 ± 25 pg/ml, respectively (Figure 9).

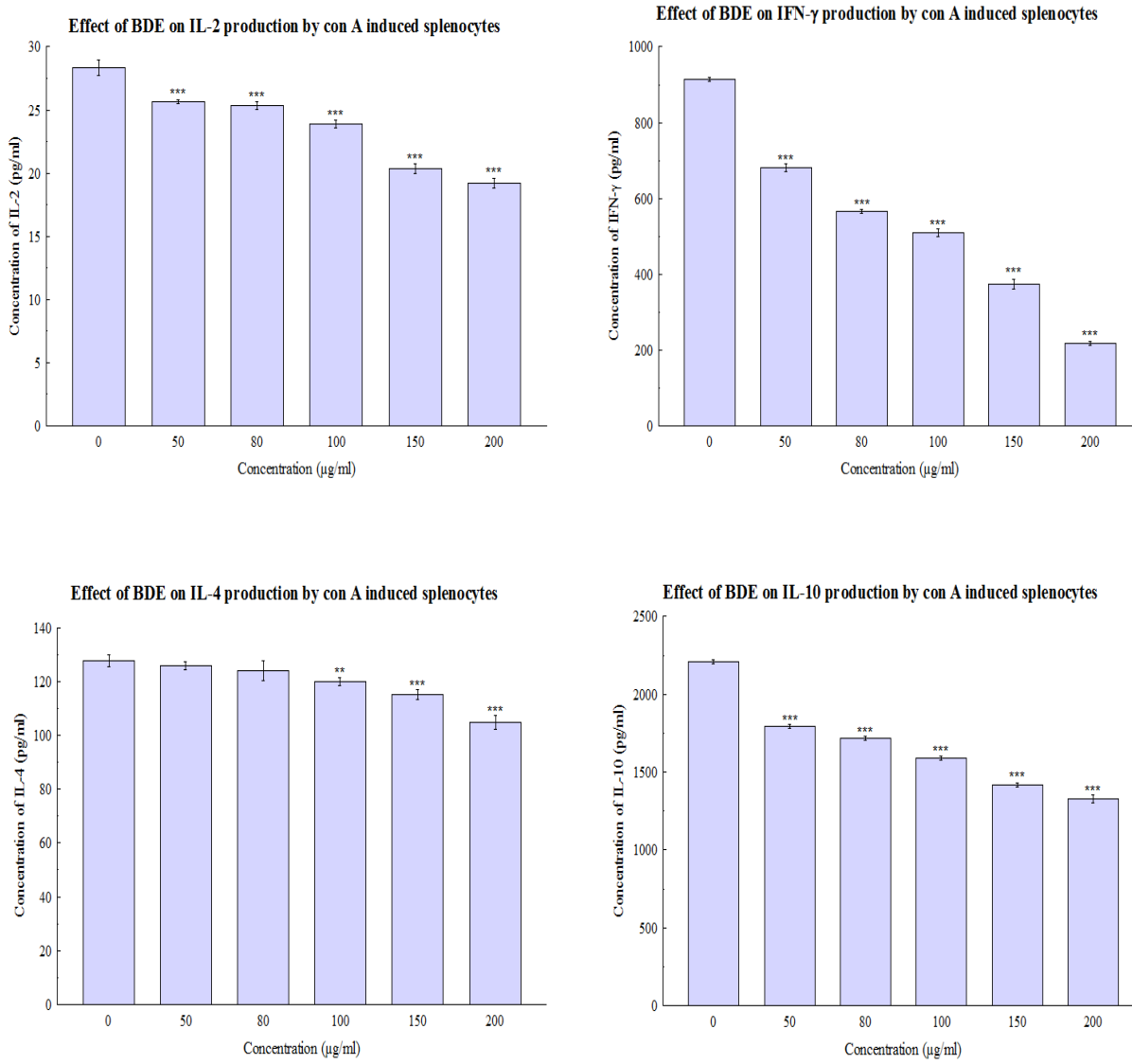
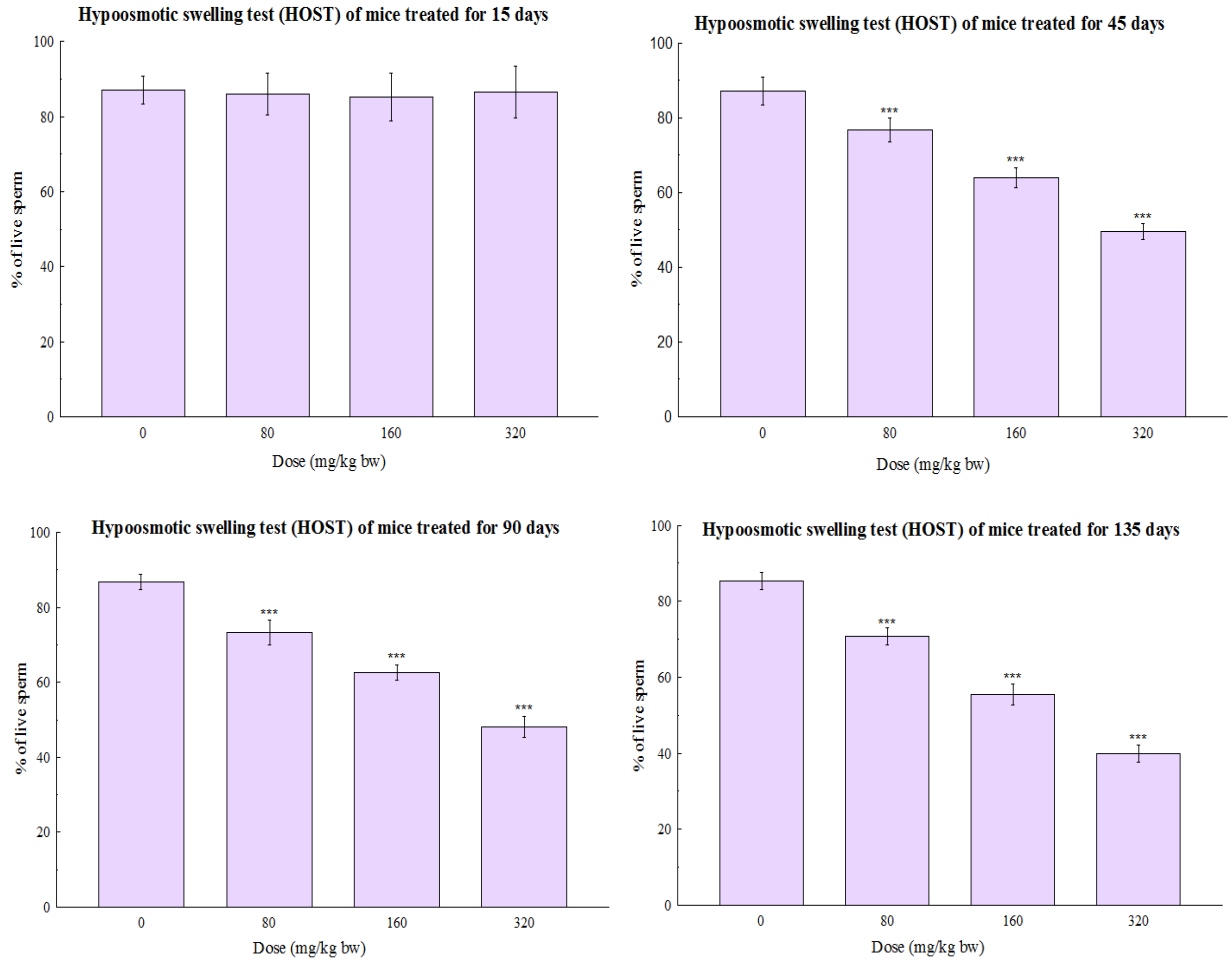


Figure 9: Effect of different concentrations (0-200 µg/ml) of BDE on different Th1 (IL-2 and IFN-γ) and Th2 (IL-4 and IL-10) cytokine production by con A induced splenocytes. Data represents significant concentration-dependent decrease in cytokine production. The results are mean ± S.D. of three parallel observations. ** p and ***$p < 0.001$ vs. 0 µg/ml.

5.1.2. Effect of BDE on the reproductive functions of mouse

5.1.2.1. Hypoosmotic swelling test (HOST)

Figure 10 indicated that no significant alterations were observed in sperm viability after 15 days of treatment with BDE. But, at 320 mg/kg bw of treatment with BDE for 45 days, significant decrease ($p < 0.001$) in the number of live sperm was observed when compared with the respective control group. After sub-chronic and chronic treatments (90, 135 and 180 days) with BDE at all the doses (80, 160 and 320 mg/kg bw), sperm viability reduces significantly ($p < 0.001$) when compared with the respective control groups.



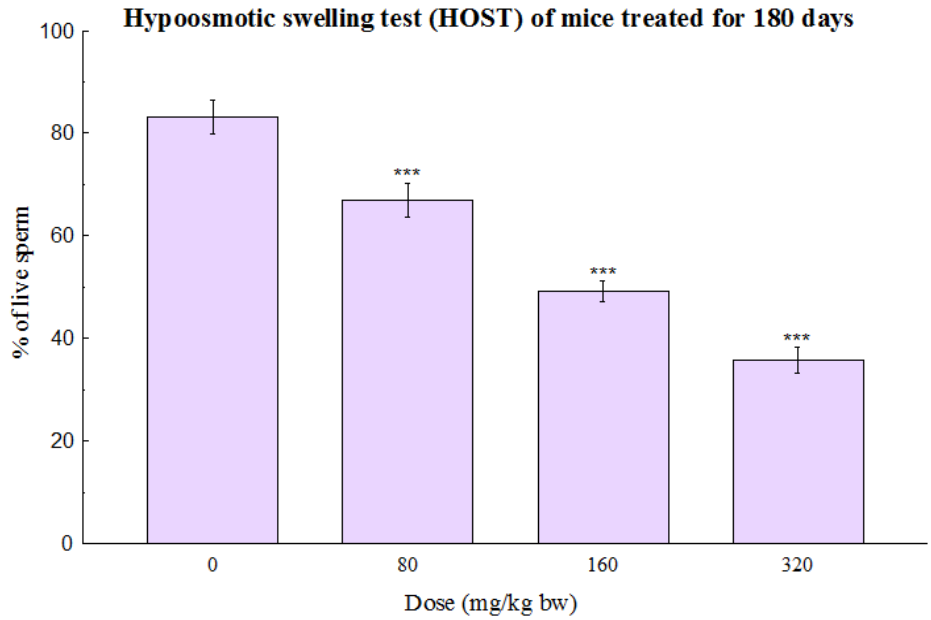
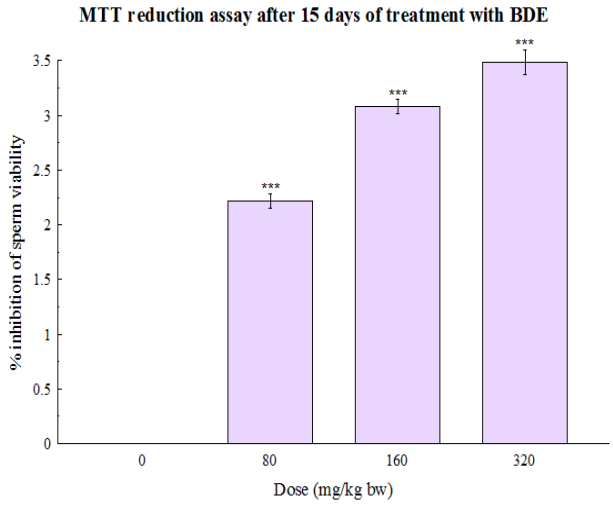


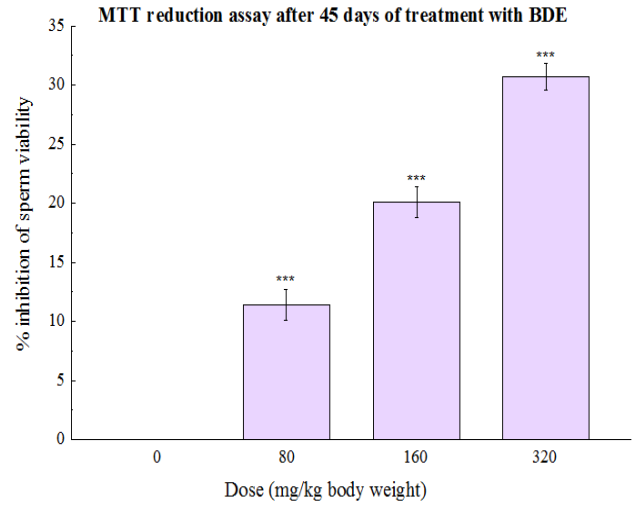
Figure 10: Hypo-osmotic swelling test (HOST) of spermatocytes demonstrates dose-dependent decrease in the percentage of live sperm in the mice treated with BDE for 15, 45, 90, 135 and 180 days. The results are mean ± S.D. of six parallel observations. ***$p < 0.001$ vs. control.

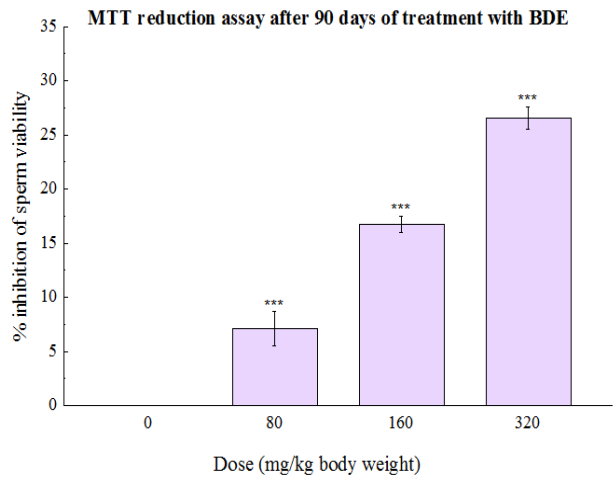
5.1.2.2. MTT reduction assay of live spermatozoa

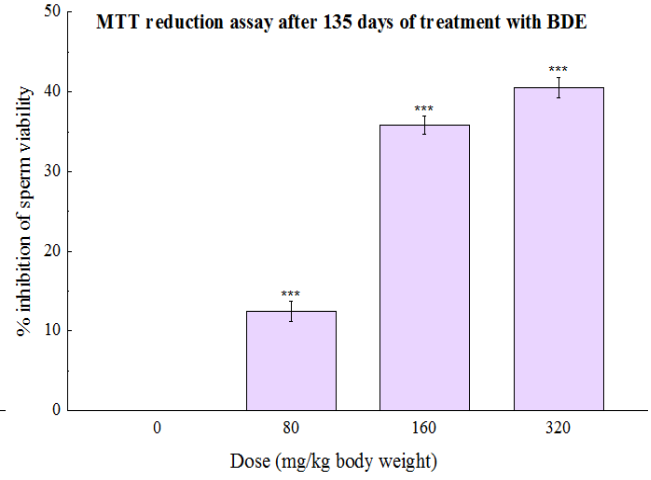
Results of the MTT reduction assay showed significant dose-dependent increased percentage of the inhibition of sperm viability in all of the cases. After 45 days of treatment, significant gradual dose-dependent increments ($p < 0.001$) in the percentage inhibition of sperm viability were observed in all of the treated doses, i.e., at 80 mg/kg bw (11.38%), 160 mg/kg bw (20.07%) and 320 mg/kg bw (30.69%), when compared with the control group (Figure 11). After 90 days of treatment, at 80 mg/kg bw, the percentage inhibition of sperm viability was 7.10%, whereas, at 160 mg/kg bw and 320 mg/kg bw, 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 11). This was also observed significantly after 135 days and 180 days of treatment with BDE. After 135 days of treatment, at 320 mg/kg bw, the percentage inhibition of sperm viability was 40.51% (Figure 11), whereas, after 180 days

of treatment with BDE, at 320 mg/kg bw, the percentage inhibition of sperm viability was increased remarkably up to 53.12% (Figure 11).









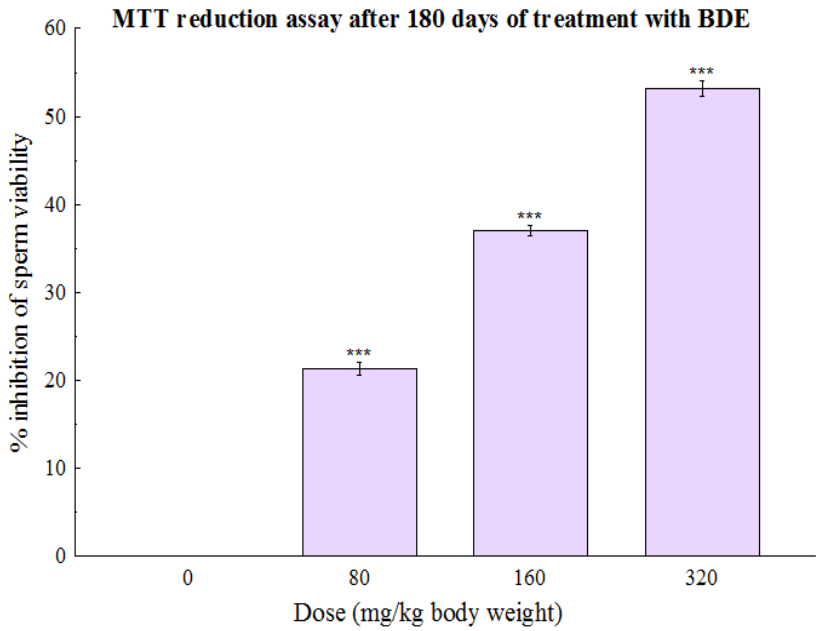


Figure 11: MTT reduction assay of spermatocytes demonstrates dose-dependent increase in the percentage inhibition of sperm viability in the mice treated with BDE for 15, 45, 90, 135, and 180 days. The results are mean ± S.D. of six parallel observations. ***p < 0.001 vs. control.

5.1.2.3. Body weight and relative weight of organs

No significant alterations in the body weight and relative organ weight have been observed in mice that were treated with all the doses for subacute and subchronic durations (Table 5). There were a significant decreases ($p < 0.05$, $p < 0.01$ and $p < 0.001$) in body weight of BDE treated mice (160 and 320 mg/kg bw) after 135 and 180 days of treatment when compared to the control group (Table 6). The relative weights of epididymis, seminal vesicle and prostate of animals treated with the BDE at the dose of 160 and 320 mg/kg bw have been decreased significantly ($p < 0.05$, $p < 0.01$ and $p < 0.001$) after 135 and 180 days of treatment.

Table 5: Effect of BDE on body weight and relative weight of testis and other sexual organs after subacute and subchronic doses

Parameters	Sub-acute dose I (15 days) (S1)				Sub-acute dose II (45 days) (S2)				Sub-chronic dose (90 days) (S3)			
	S1G1	S1G2	S1G3	S1G4	S2G1	S2G2	S2G3	S2G4	S3G1	S3G2	S3G3	S3G4
Body weight (g)	25.13 ± 0.62	25.03 ± 0.91	25.05 ± 0.82	25.27 ± 1.03	26.23 ± 0.52	26.03 ± 1.91	26.05 ± 0.82	25.27 ± 1.03	25.83 ± 0.52	25.03 ± 2.93	25.25 ± 0.87	25.27 ± 2.13
Relative testis weight (g/100 g of body weight)	0.98 ± 0.05	0.98 ± 0.07	0.98 ± 0.07	0.97 ± 0.06	0.96 ± 0.08	0.96 ± 0.07	0.95 ± 0.07	0.95 ± 0.06	0.94 ± 0.08	0.94 ± 0.07	0.94 ± 0.07	0.94 ± 0.06
Relative epididymis weight (g/100 g of body weight)	0.33 ± 0.03	0.34 ± 0.05	0.33 ± 0.02	0.34 ± 0.07	0.35 ± 0.03	0.34 ± 0.05	0.34 ± 0.02	0.33 ± 0.07	0.34 ± 0.03	0.33 ± 0.05	0.33 ± 0.02	0.32 ± 0.07
Relative seminal vesicle weight (g/100 g of body weight)	0.49 ± 0.06	0.48 ± 0.03	0.47 ± 0.05	0.47 ± 0.03	0.48 ± 0.06	0.47 ± 0.03	0.47 ± 0.05	0.46 ± 0.03	0.47 ± 0.06	0.47 ± 0.03	0.46 ± 0.05	0.45 ± 0.03
Relative prostate gland weight (g/100 g of body weight)	0.25 ± 0.01	0.26 ± 0.02	0.25 ± 0.05	0.24 ± 0.05	0.26 ± 0.01	0.26 ± 0.02	0.25 ± 0.05	0.25 ± 0.05	0.24 ± 0.01	0.24 ± 0.02	0.24 ± 0.05	0.23 ± 0.05

¶ Data shows no significant differences of the treated groups from the respective controls

Table 6: Effect of BDE on body weight and relative weight of testis and other sexual organs after chronic doses

Parameters	Chronic dose – I (135 days) (S4)				Chronic dose – II (180 days) (S5)			
	S4G1	S4G2	S4G3	S4G4	S5G1	S5G2	S5G3	S5G4
Body weight (g)	25.13 ± 0.18	24.86 ± 0.24	24.53 ± 0.15	24.12 ± 0.11	24.83 ± 0.32	23.03 ± 0.93	22.85 ± 0.77	22.27 ± 1.13
Relative testis weight (g/100 g of body weight)	0.95 ± 0.08	0.94 ± 0.07	0.94 ± 0.07	0.93 ± 0.06*	0.95 ± 0.01	0.93 ± 0.01	0.93 ± 0.01	0.91 ± 0.01**
Relative epididymis weight (g/100 g of body weight)	0.33 ± 0.01	0.31 ± 0.02	0.27 ± 0.01**	0.27 ± 0.01**	0.33 ± 0.02	0.33 ± 0.01	0.27 ± 0.01**	0.24 ± 0.01***
Relative seminal vesicle weight (g/100 g of body weight)	0.48 ± 0.01	0.46 ± 0.01	0.45 ± 0.01*	0.44 ± 0.01**	0.48 ± 0.01	0.43 ± 0.02*	0.42 ± 0.02**	0.37 ± 0.02***
Relative prostate gland weight (g/100 g of body weight)	0.24 ± 0.01	0.24 ± 0.01	0.23 ± 0.01	0.22 ± 0.01**	0.24 ± 0.01	0.22 ± 0.01*	0.21 ± 0.01**	0.19 ± 0.01***

*$p < 0.05$ when compared with Control (G1).

**$p < 0.01$ when compared with Control (G1).

***$p < 0.001$ when compared with Control (G1).

5.1.2.4. Effect of BDE on different biochemical parameters of sexual organs

Changes of biochemical parameters after different doses of treatment are outlined in Table 7 & 8. Proteins levels in serum, testis and epididymis of BDE treated mice (320 mg/kg bw) decreased significantly ($p < 0.05$) after 90 days of treatment. After 135 and 180 days of treatment at 320 mg/kg bw of BDE, protein contents in serum, testis and epididymis decreased significantly ($p < 0.001$) when compared with the respective controls. Cholesterol level in testis was decreased significantly ($p < 0.001$) in mice receiving BDE at the dose of 160 and 320 mg/kg bw after subchronic and chronic treatments. Fructose and α-glucosidase levels of BDE treated mice (at 160 and 320 mg/kg bw) significantly decreased ($p < 0.001$) after 135 and 180 days of treatment when compared with the control groups. The glycogen level in testis decreased significantly ($p < 0.01$ and $p < 0.001$) in the mice treated with BDE at 160 and 320 mg/kg bw for subchronic and chronic durations. Sialic acid concentration in epididymis decreased significantly ($p < 0.001$) in the mice treated with BDE at 160 and 320 mg/kg bw for 135 and 180 days of treatment when compared with the control groups. The concentration of prostatic citric acid decreased significantly ($p < 0.01$ and $p < 0.001$) in the mice treated with BDE at 160 and 320 mg/kg bw for subchronic and chronic durations. The testicular acid phosphatase concentration has also been decreased significantly ($p < 0.05$, $p < 0.01$ and $p < 0.001$) in a dose- and time dependent manner within a duration of 180 days of treatment of BDE at different doses, when compared to the respective control groups.

Table 7: Effect of BDE on different biochemical parameters of testis and other sexual organs after sub-acute (15 and 45 days) and sub-chronic doses (90 days)

Parameters	Sub-acute dose I (15 days) (S1)				Sub-acute dose II (45 days) (S2)				Sub-chronic dose (90 days) (S3)			
	S1G1	S1G2	S1G3	S1G4	S2G1	S2G2	S2G3	S2G4	S3G1	S3G2	S3G3	S3G4
Total Protein (serum) (mg/dl)	7.84 ± 0.75	7.74 ± 0.83	7.73 ± 0.59	7.82 ± 1.11	7.80 ± 0.81	7.56 ± 0.52	7.68 ± 0.27	7.60 ± 0.33	7.52 ± 0.53	7.21 ± 0.28	6.96 ± 0.32	6.91 ± 0.40*
Total Protein(testis) (mg/g)	0.50 ± 0.14	0.42 ± 0.15	0.43 ± 0.17	0.44 ± 0.20	0.52 ± 0.07	0.49 ± 0.07	0.47 ± 0.07	0.43 ± 0.04	0.56 ± 0.03	0.54 ± 0.03	0.52 ± 0.03	0.51 ± 0.01*
Total Protein (epididymis) (mg/g)	0.20 ± 0.07	0.19 ± 0.05	0.18 ± 0.07	0.19 ± 0.08	0.22 ± 0.06	0.18 ± 0.03	0.18 ± 0.03	0.19 ± 0.04	0.24 ± 0.05	0.20 ± 0.04	0.19 ± 0.05	0.17 ± 0.04*
Chelesterol (testis) (mg/g)	3.89 ± 0.18	3.79 ± 0.18	3.85 ± 0.16	3.85 ± 0.24	3.82 ± 0.14	3.84 ± 0.11	3.84 ± 0.09	3.75 ± 0.09	3.84 ± 0.11	3.69 ± 0.13	3.71 ± 0.18	3.48 ± 0.06*
α-glucosidase (epididymis) (mU/g)	4.81 ± 0.10	4.79 ± 0.10	4.75 ± 0.06	4.79 ± 0.15	4.82 ± 0.03	4.74 ± 0.10	4.72 ± 0.03	4.74 ± 0.11	4.80 ± 0.01	4.67 ± 0.05*	4.65 ± 0.06*	4.56 ± 0.06**
Fructose (seminal vesicles) (µM/g)	4.54 ± 0.16	4.50 ± 0.10	4.48 ± 0.02	4.49 ± 0.13	4.53 ± 0.08	4.47 ± 0.07	4.52 ± 0.06	4.48 ± 0.08	4.61 ± 0.10	4.54 ± 0.07	4.48 ± 0.08	4.53 ± 0.06
Glycogen (testis) (mg/g)	37.48 ± 2.35	37.66 ± 2.77	37.19 ± 4.30**	37.27 ± 4.58	39.78 ± 1.35	39.66 ± 2.25	39.19 ± 2.30	38.27 ± 3.53	38.52 ± 2.58	37.66 ± 3.25	37.19 ± 3.20	33.27 ± 0.53**
Sialic acid (epididymis) (µM/100 g tissue)	64.25 ± 2.21	64.35 ± 3.37	64.45 ± 2.39	64.25 ± 3.58	65.25 ± 2.21	65.11 ± 0.58	64.87 ± 2.02	64.80 ± 3.01	63.14 ± 0.69	63.02 ± 0.98	62.38 ± 0.36	61.78 ± 0.81*
Prostate citric acid (mg/g)	35.02 ± 2.52	34.75 ± 3.01	34.37 ± 1.36	33.33 ± 1.99	36.42 ± 2.52	36.25 ± 1.71	36.37 ± 1.56	35.33 ± 2.99	36.02 ± 1.52	35.75 ± 1.51	37.37 ± 0.56	31.33 ± 0.99*
Acid phosphatase (testis) (µM/min/g of tissue)	30.09 ± 5.20	28.60 ± 0.66	24.01 ± 1.50	23.83 ± 0.39	31.05 ± 2.79	31.98 ± 2.60	31.65 ± 2.32	22.46 ± 0.96**	30.86 ± 3.87	20.05 ± 2.97	21.65 ± 0.48**	18.50 ± 0.97***

*$p < 0.05$ when compared with Control (G1).

**$p < 0.01$ when compared with Control (G1).

***$p < 0.001$ when compared with Control (G1).

Table 8: Effect of BDE on different biochemical parameters of testis and other sexual organs after chronic doses (135 and 180 days)

Parameters	Chronic dose – I (135 days) (S4)				Chronic dose – II (180 days) (S5)			
	S4G1	S4G2	S4G3	S4G4	S5G1	S5G2	S5G3	S5G4
Total Protein (serum) (mg/dl)	7.84 ± 0.49	7.12 ± 0.16**	6.68 ± 0.32***	6.35 ± 0.35***	7.85 ± 0.83	6.78 ± 0.41**	6.06 ± 0.30***	5.64 ± 0.55***
Total Protein (testis) (mg/g)	0.58 ± 0.04	0.53 ± 0.01*	0.51 ± 0.03***	0.48 ± 0.02***	0.56 ± 0.03	0.52 ± 0.03*	0.48 ± 0.02***	0.41 ± 0.01***
Total Protein (epididymis) (mg/g)	0.25 ± 0.04	0.20 ± 0.02*	0.18 ± 0.02***	0.14 ± 0.01***	0.28 ± 0.02	0.19 ± 0.01***	0.13 ± 0.01***	0.08 ± 0.01***
Chelesterol (testis) (mg/g)	3.85 ± 0.13	3.66 ± 0.08	3.53 ± 0.03**	3.42 ± 0.02***	3.82 ± 0.08	3.53 ± 0.12**	3.43 ± 0.07**	3.27 ± 0.06***
α-glucosidase (epididymis) (mU/g)	4.76 ± 0.06	4.63 ± 0.03*	4.61 ± 0.08*	4.52 ± 0.05**	4.77 ± 0.06	4.56 ± 0.06**	4.40 ± 0.05***	4.32 ± 0.06***
Fructose (seminal vesicles) (µM/g)	4.56 ± 0.11	4.46 ± 0.05	4.41 ± 0.07	4.36 ± 0.04*	4.52 ± 0.08	4.42 ± 0.04	4.35 ± 0.06*	4.30 ± 0.04**
Glycogen (testis) (mg/g)	39.58 ± 1.58	38.66 ± 2.25	33.19 ± 1.20**	30.27 ± 0.53***	40.58 ± 1.38	31.46 ± 2.85***	27.19 ± 3.28***	20.27 ± 3.53***
Sialic acid (epididymis) (µM/100 g tissue)	62.12 ± 3.24	56.71 ± 0.93*	50.03 ± 1.14***	41.06 ± 0.81***	63.59 ± 2.24	54.36 ± 3.36***	40.25 ± 3.35***	31.36 ± 2.54***
Prostate citric acid (mg/g)	37.02 ± 1.52	31.75 ± 1.51*	27.37 ± 2.56**	21.33 ± 0.99***	36.58 ± 2.27	25.48 ± 3.35***	21.36 ± 3.69***	18.47 ± 1.36***
Acid phosphatase (testis) (µM/min/g of tissue)	30.07 ± 5.12	23.94 ± 0.88**	20.34 ± 1.44***	16.29 ± 0.05***	33.72 ± 0.38	21.70 ± 0.60***	18.35 ± 1.02***	15.96 ± 1.57***

*p < 0.05 when compared with Control (G1).

**p < 0.01 when compared with Control (G1).

***p < 0.001 when compared with Control (G1).

5.1.2.5. Alterations in the architecture of testes

No significant morphological alterations in the architecture of testes were observed in subacute and subchronic doses and therefore, data was not presented. Morphological alterations of seminiferous tubules were observed only in the groups treated with 320 mg/kg bw of BDE for 135 and 180 days when compared to the control testis. Significant decreases in diameter, perimeter and area of the seminiferous tubules were observed in case of mice treated with 160 and 320 mg/kg bw of BDE for 180 days ($p < 0.01$ and $p < 0.001$) when compared with the control groups. The number of empty seminiferous tubules has been shown to increase with the increasing dose duration. Significant increases ($p < 0.05$ and $p < 0.001$) in the percentage of empty seminiferous tubules were also observed after treatment with 160 and 320 mg/kg bw of BDE for 135 and 180 days when compared with the control groups (Table 9). In the group treated with BDE at 320 mg/kg bw for 180 days, the epithelium was reduced to a single layer in few of the tubules [Figure 14 (A) & (B)]. Seminiferous tubules of mice treated with 320 mg/kg bw of BDE for 135 days [Figure 13 (A) & (B)] and 180 days [Figure 14 (A) & (B)] showed vacuolization in the tubules with reduced epithelial membranes layer, when compared to the control (Figure 12).

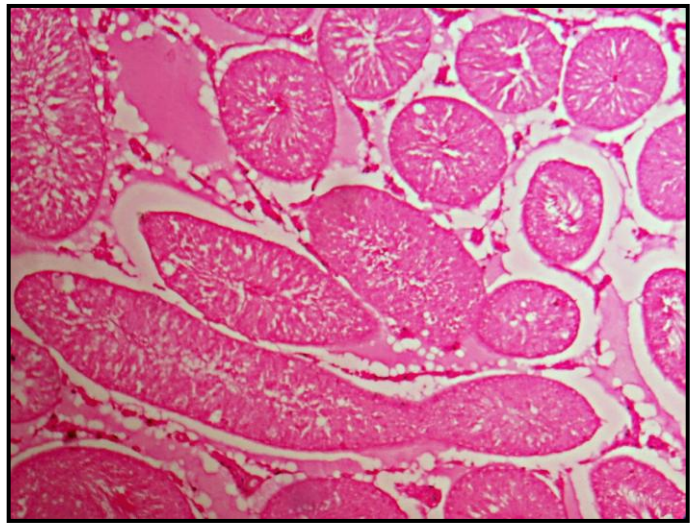


Figure 12: Histology of the testis of control mice (stained with haematoxylin–eosin) showing normal architecture in seminiferous tubules with intact epithelial membranes and no vacuolization.

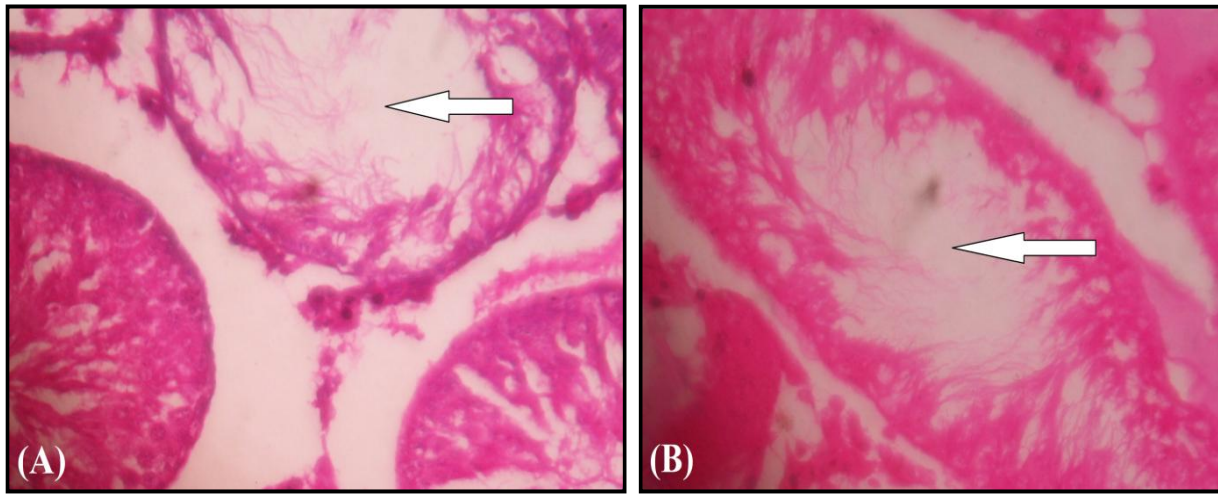


Figure 13 (A & B): Histology of the testis of mice treated with 320 mg/kg bw of BDE for 135 days (observed with a magnitude of ×400 under the microscope, stained with haematoxylin–eosin). Figure shows vacuolization in the seminiferous tubules (indicated by white arrow) with reduced epithelial membranes layer.

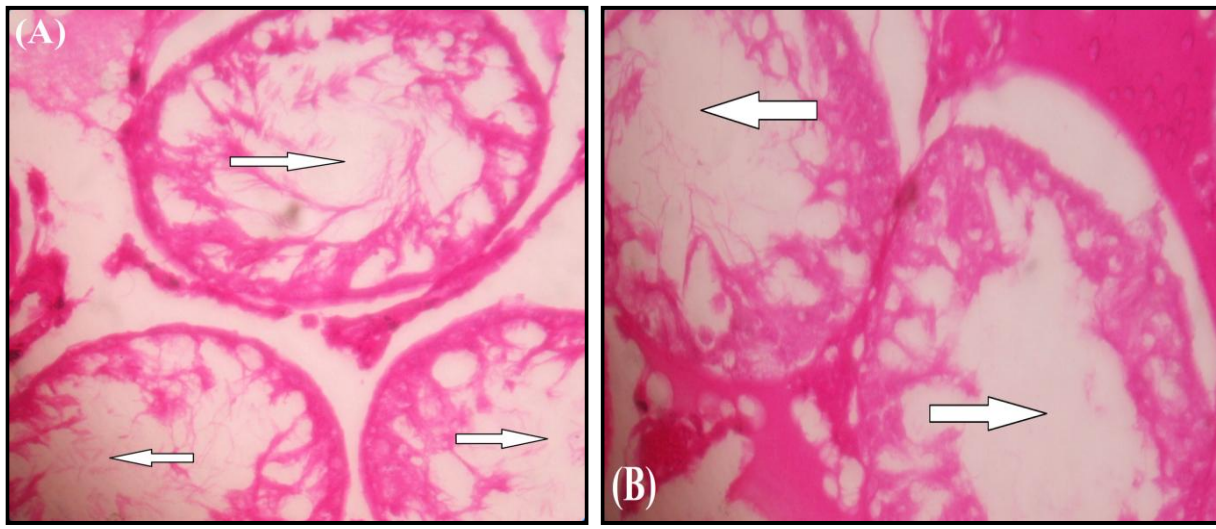


Figure 14 (A & B): Histology of the testis of mice treated with 320 mg/kg bw of BDE for 180 days (observed with a magnitude of ×400 under the microscope, stained with haematoxylin–eosin) showing increased vacuolization in the tubules (indicated by white arrow) with reduced epithelial membrane layers. The number of empty seminiferous tubules as well as inter-seminiferous tubular spaces has been shown to increase with the increased dose duration when compared to the control.

Table 9: Histomorphometric parameters of seminiferous tubules

Parameters	Chronic dose – I (135 days) (S4)				Chronic dose – II (180 days) (S5)			
	S4G1	S4G2	S4G3	S4G4	S5G1	S5G2	S5G3	S5G4
Diameter (in µm)	273.44 ± 9.25	256.91 ± 14.50	242.79 ± 14.87	240.76 ± 15.21*	271.92 ± 7.66	268.46 ± 9.73	253.79 ± 8.05	241.45 ± 7.17**
Perimeter (in µm)	964.35 ± 16.50	863.78 ± 12.55***	765.03 ± 15.34***	737.85 ± 12.83***	989.46 ± 30.27	564.31 ± 33.06***	365.11 ± 21.98***	239.64 ± 27.56***
Area (in sq. µm)	61122.62 ± 1927.22***	42978.20 ± 2631.77***	40954.62 ± 1574.45***	37948.97 ± 3270.21***	61583.03 ± 1644.35	50618.27 ± 1559.85***	40007.95 ± 1379.22***	32728.41 ± 3615.11***
Percentage of empty seminiferous tubules	11.60 ± 3.15	12.42 ± 3.13	15.21 ± 3.15	19.96 ± 1.99*	11.62 ± 2.78	16.52 ± 1.92	20.11 ± 2.55*	28.80 ± 3.36***

*p < 0.05 when compared with Control (G1).

**p < 0.01 when compared with Control (G1).

***p < 0.001 when compared with Control (G1).

5.1.2.6. Effect of D. esculentum on fertility and fecundity

As outlined in Table 10 and 11, the fertility as well as fecundity has been decreased in a dose- and time-dependent manner in BDE treated mice when compared to the respective control groups. The percentage of fertility (no. of viable pups) has decreased significantly ($p < 0.001$) with increasing dose of BDE, when compared with the respective control groups. After sub-chronic and chronic treatments at 320 mg/kg bw, no viable pups have been recorded. Similarly, percentage of fecundity has been decreased significantly ($p < 0.001$) with increasing dose of BDE, when compared with the respective controls. Hundred percent fertility losses observed at 320 mg/kg bw dose of BDE that were treated for subchronic and chronic durations. Similarly, 100% losses of fecundity observed in mice that were treated with 160 and 320 mg/kg bw of BDE for 180 days.

Table 10: Effects of the BDE on fertility of Swiss albino mouse after different days of treatment

Treatment	Ratio of males and females for fertility test	% of fertility (no. of viable pups) after different periods				
		15 days	45 days	90 days	135 days	180 days
0 mg/kg bw (Control)	½	100	100	100	100	100
80 mg/kg bw	½	100	100	100	100	80
160 mg/kg bw	½	100	80	80	50	50
320 mg/kg bw	½	50	50	0	0	0

Table 11: Effects of the BDE on fecundity of Swiss albino mouse after different days of treatment

Treatment	Ratio of males and females for fecundity test	% of fecundity after different periods				
		15 days	45 days	90 days	135 days	180 days
0 mg/kg bw (Control)	½	100	100	100	100	100
80 mg/kg bw	½	100	100	100	100	75
160 mg/kg bw	½	100	100	100	75	0
320 mg/kg bw	½	100	100	100	75	0

5.1.3. Effect of BDE on the cholinergic nervous system

5.1.3.1. In vivo experiment

5.1.3.1.1. Determination of acetylcholinesterase activity

The acetylcholinesterase activity of BDE treated Swiss albino mouse was determined by assessing the rate (R) of change in absorbance per min, by a previously described formula (Ellman et al., 1961). Results indicated that R, which was expressed in moles acetylthiocholine iodide (substrate) hydrolyzed per min per g of tissue, decreased significantly ($p < 0.01$ and $p < 0.001$) in a dose-dependent manner, indicating increased dose-dependent inhibition in the acetylcholinesterase activity in the BDE treated mice over a 30 d time period (Figure 15).

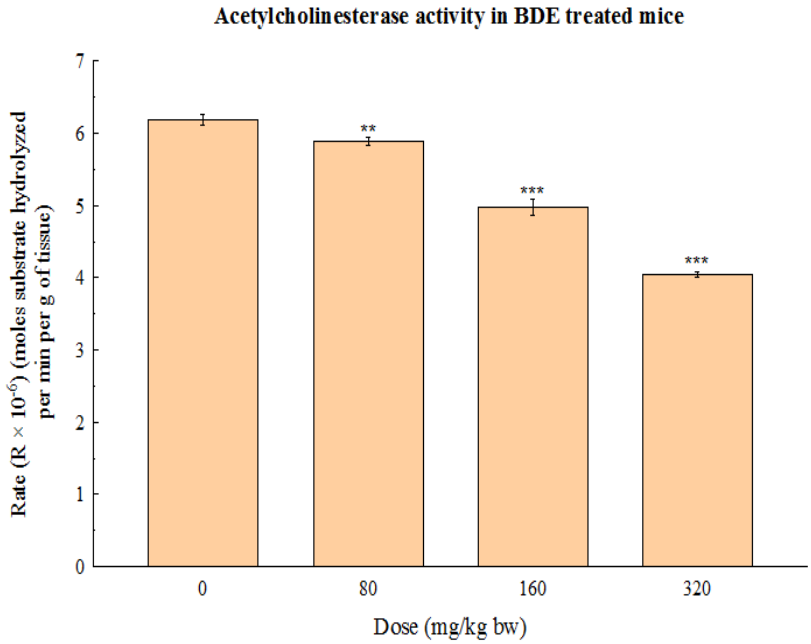


Figure 15: Acetylcholinesterase activity in BDE treated mice. Figure shows dose-dependent decrease in rate (R), which was expressed in moles acetylthiocholine iodide (substrate) hydrolyzed per min per g of tissue. The results are mean ± S.D. of three parallel observations. **p < 0.01 and ***p < 0.001 vs. control.

5.1.3.2. Ex vivo experiment

5.1.3.2.1. Assessment of acetylcholinesterase inhibitory activity

As indicated in Figure 16, a significant ($p < 0.001$) dose-dependent increase in the AChE inhibitory activity of *D. esculentum* has been observed. At 50 and 200 µg/ml, the percentage

inhibition of AChE was 8.63% and 54.39%, respectively. The IC_{50} value of MDE on AChE inhibitory activity has been found to be 272.97 ± 19.38 µg/ml.

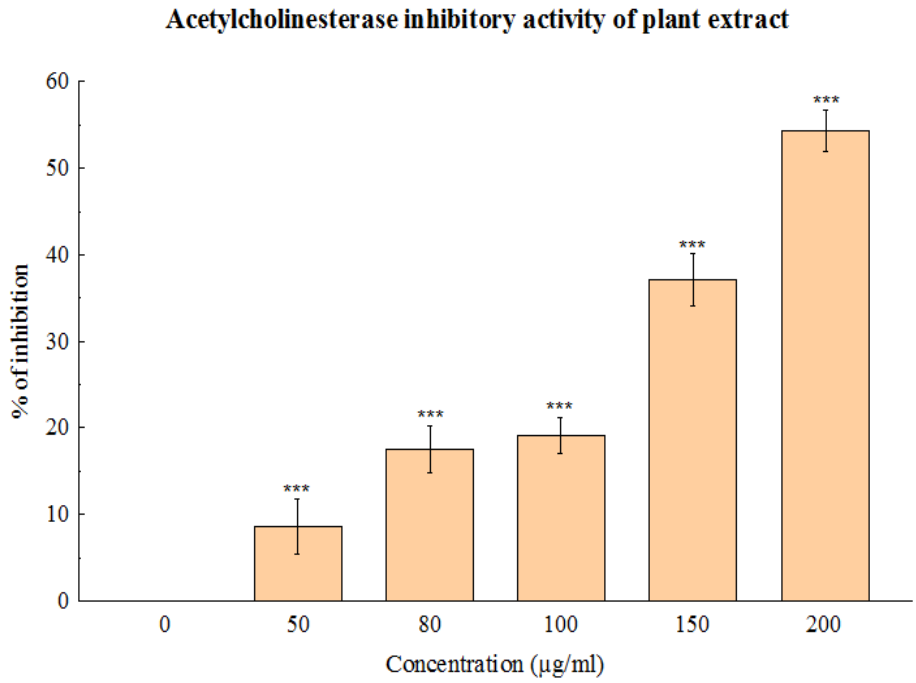


Figure 16: Acetylcholinesterase inhibitory activity of *D. esculentum* extract. The data represent the percentage inhibition of the enzyme acetylcholinesterase. The results are mean ± S.D. of six parallel measurements. ***$p < 0.001$ vs. 0 µg/ml. IC_{50} value of the plant extract was 272.97 ± 19.38 µg/ml.

5.1.3.2.2. Determination of NADH oxidase inhibitory activity

MDE inhibited NADH oxidase significantly ($p < 0.001$) in a dose-dependent manner (Figure 17). At 50 µg/ml, the percentage inhibition of NADH oxidase was 10.43%, whereas at 200 µg/ml, the percentage inhibition of NADH oxidase was increased to 43.99%. The IC_{50} value of MDE on NADH oxidase inhibition was 265.81 ± 21.20 µg/ml.

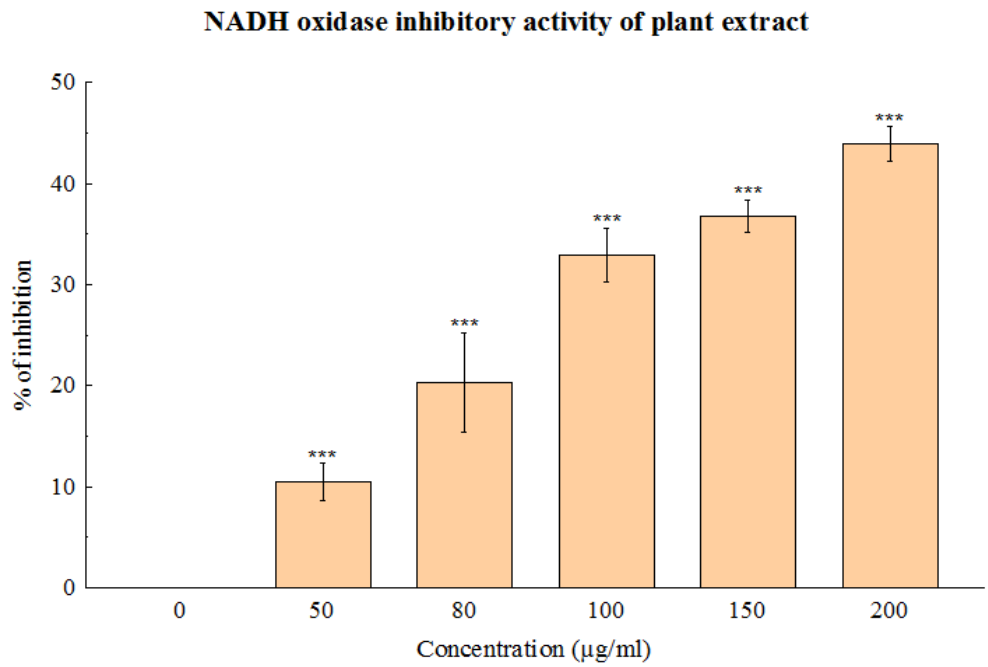


Figure 17: NADH oxidase inhibitory activity of *D. esculentum* extract. The data represent the percentage inhibition of the enzyme NADH oxidase. The results are mean ± S.D. of six parallel measurements. ***$p < 0.001$ vs. 0 µg/ml. IC_{50} value of the plant extract was 265.81 ± 21.20 µg/ml.

5.1.4. Antioxidant and free radical scavenging activities of *Diplazium esculentum*

5.1.4.1. Determination of total antioxidant activity by FTC and TBA method

As shown in Figure 18, the absorbance of the control at 500 nm increased to a maximal value of 0.84 on day 14, whereas vitamin E (α-tocopherol) and MDE increased to 0.57 and 0.61, respectively, on the same day. These differences were found statistically significant than the control ($p < 0.001$). The total antioxidant activity of vitamin E by FTC and TBA methods were 31.85% and 38.97%, respectively, whereas the total antioxidant activity of MDE by FTC and TBA methods were 27.41% and 33.22%, respectively.

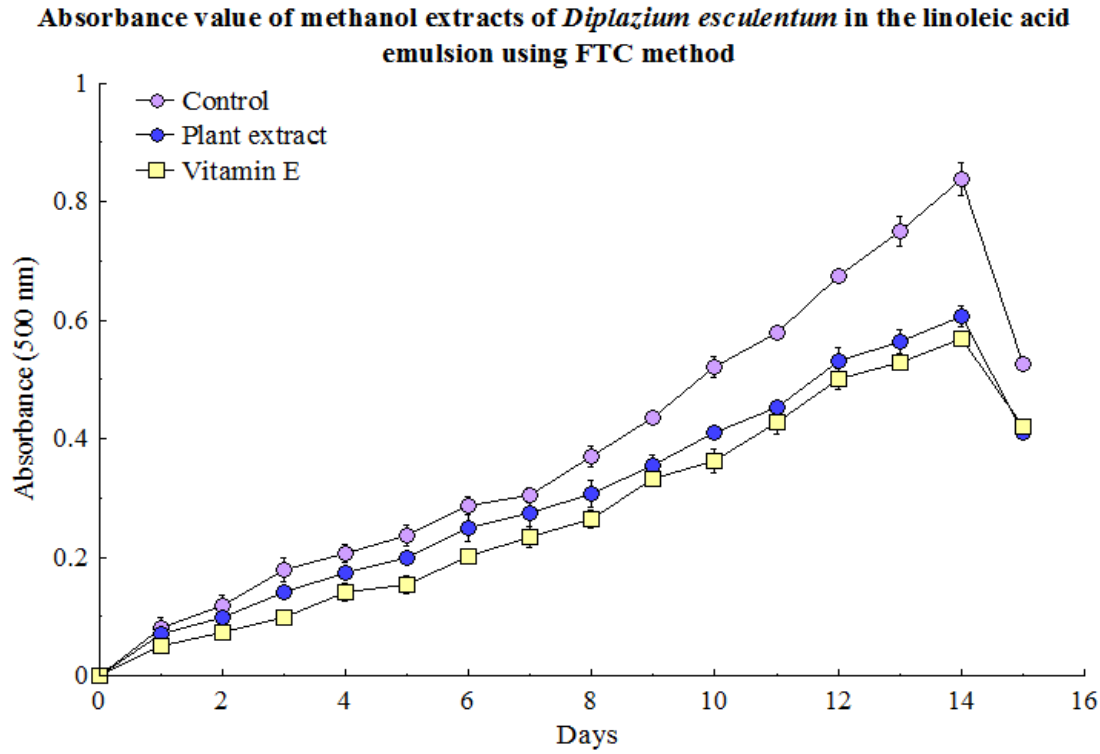


Figure 18: Absorbance value of the MDE in the linoleic acid emulsion using FTC method. The results are mean ± S.D. of six parallel measurements.

5.1.4.2. Total antioxidant activity by ABTS method

The total antioxidant activity of MDE was calculated from the decolorization of $ABTS^{\bullet+}$, which was measured spectrophotometrically at 734 nm. Interaction with the plant extract or standard trolox suppressed the absorbance of the $ABTS^{\bullet+}$ radical cation and the results, expressed as percentage inhibition of absorbance, are shown in Figure 19. The TEAC value of the extract was 0.21 ± 0.02.

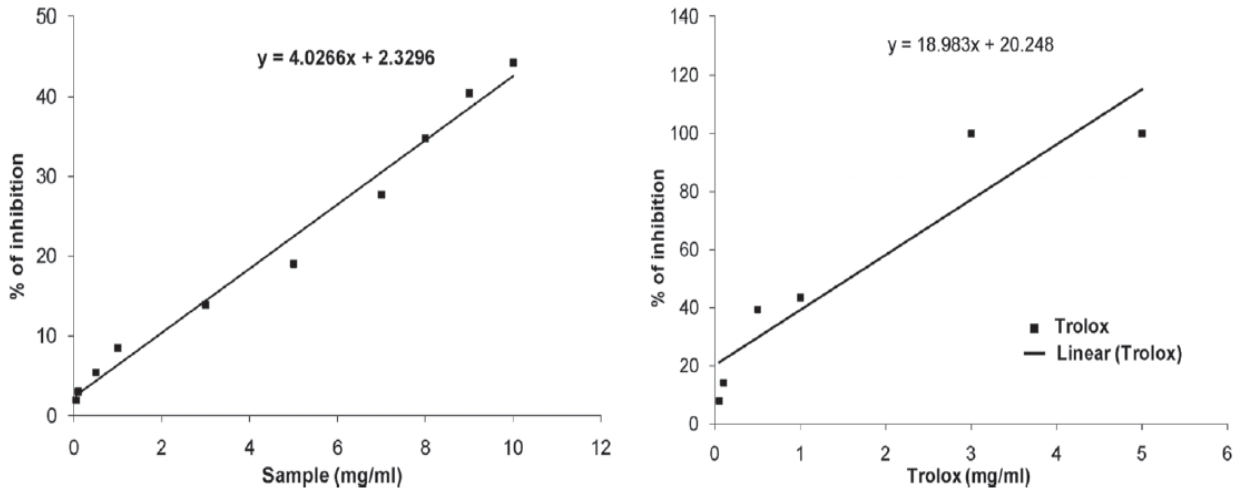


Figure 19: Total antioxidant activity of MDE and reference compound trolox on decolourization of ABTS radical cation. The percentage inhibition was plotted against the concentration of sample. All data are expressed as mean ± S.D. (n = 6).

5.1.4.3. DPPH radical scavenging activity

Figure 20 showed a significant ($p < 0.01$ and $p < 0.001$) dose-dependent increase in the percentage of DPPH radical scavenging by MDE, when compared with the standard α-tocopherol. At 50 µg/ml, the percentages of DPPH radical scavenging of the plant extract and standard were 8.75% and 11.07%, respectively, whereas, at 200 µg/ml, the radical scavenging was increased up to 38.12% and 42.69%, for MDE and the standard, respectively. The IC_{50} values of the plant extract and standard on DPPH radical scavenging were 402.88 ± 12.70 µg/ml and 324.86 ± 6.35 µg/ml, respectively.

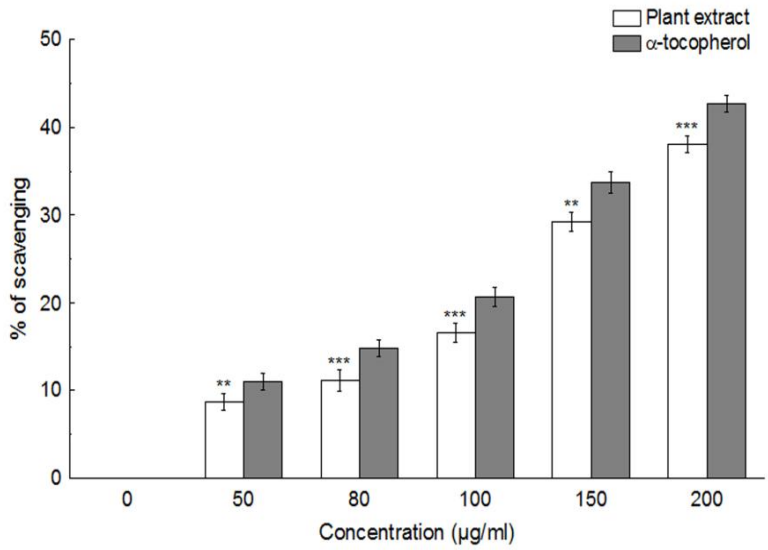


Figure 20: DPPH radical scavenging activity of *D. esculentum* extract. The data represent the effect of *D. esculentum* plant extract and α-tocopherol on the scavenging of DPPH radical. The results are mean ± S.D. of six parallel measurements. $^{**}p < 0.01$ and $^{***}p < 0.001$ vs. 0 µg/ml. IC_{50} value of the plant extract and the standard (α-tocopherol) were 402.88±12.70 µg/ml and 324.86±6.35 µg/ml, respectively.

5.1.4.4. Hydroxyl radical scavenging activity

This assay shows the abilities of MDE and standard mannitol to inhibit hydroxyl radical-mediated deoxyribose degradation in a Fe^{3+}-EDTA-ascorbic acid and H_2O_2 reaction mixture. The results are shown in Figure 21. The IC_{50} values (Table 12) of MDE and the standard in this assay were 811.00 ± 23.73 µg/ml and 571.45 ± 20.12 µg/ml, respectively. Though the IC_{50} value of MDE was greater than that of the standard, at 200 µg/ml, the percentages of inhibition were 23.4% and 21.9% for *D. esculentum* and mannitol, respectively.

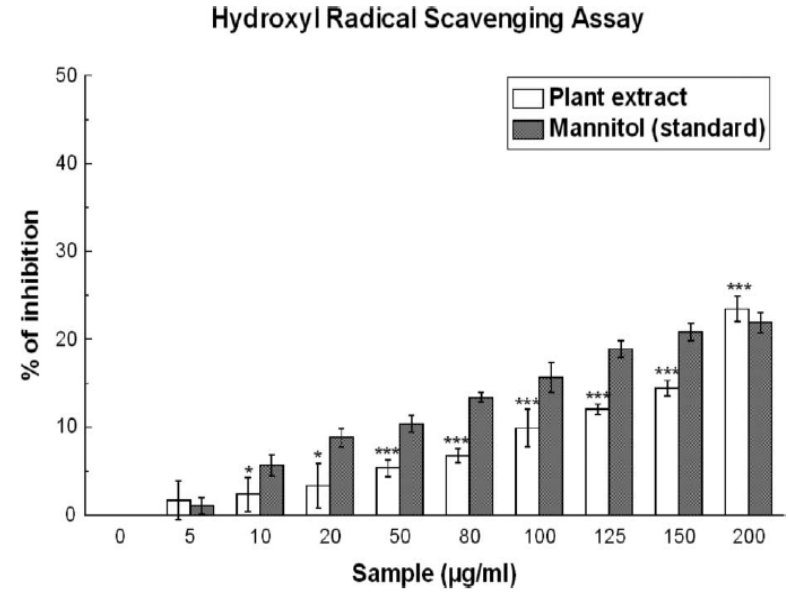


Figure 21: Hydroxyl radical scavenging activities of MDE and the reference compound mannitol. The data represent the percentage inhibition of deoxyribose degradation. The results are mean ± S. D. of six parallel measurements. $^{*}p < 0.05$ and $^{***}p < 0.001$ vs 0 µg/ml. IC_{50} values of MDE and standard are 811 ± 23.73 µg/ml and 571.45 ± 20.12 µg/ml, respectively.

5.1.4.5. Superoxide radical scavenging activity

Superoxide radicals, generated from the PMS-NADH coupling, can be measured by their ability to reduce NBT. The decrease in absorbance at 560 nm with MDE and the reference compound quercetin indicates their abilities to quench superoxide radicals in the reaction mixture (Figure 22), The IC_{50} values (Table 12) of MDE and quercetin on superoxide scavenging activity were 90.39 ± 2.22 µg/ml and 42.06 ± 1.35 µg/ml, respectively. At 20 µg/ml, the percentage of inhibition of the plant extract was 17.8%, whereas that of quercetin was 29.1%.

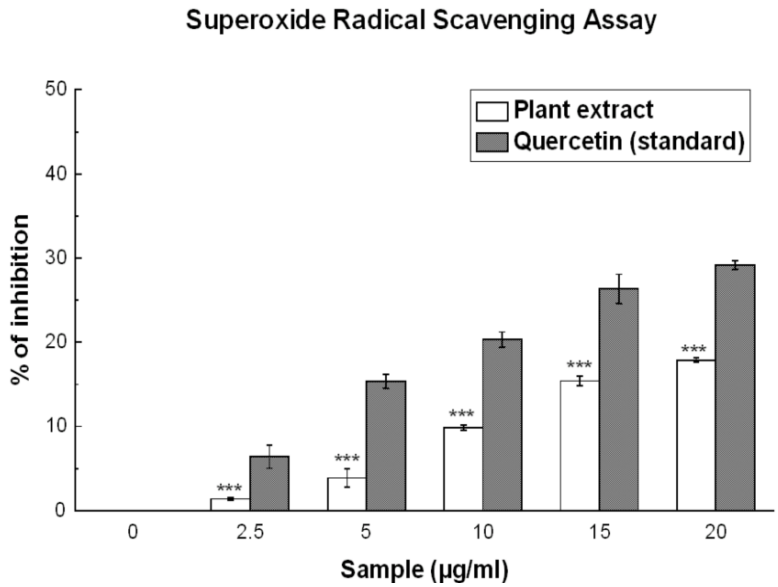


Figure 22: Scavenging effect of MDE and the standard quercetin on superoxide radical. All data are expressed as mean ± S.D. (n = 6). ***$p < 0.001$ vs 0 µg/ml. IC_{50} values of MDE and standard are 90.39 ± 2.22 µg/ml and 42.06 ± 1.35 µg/ml, respectively.

5.1.4.6. Nitric oxide radical scavenging activity

As shown in Figure 23, MDE also caused a moderate dose-dependent inhibition of nitric oxide with an IC_{50} value (Table 12) of 204.28 ± 18.31 µg/ml. Curcumin was used as a reference compound and 90.82 ± 4.75 µg/ml curcumin was needed for 50% inhibition. At 70 µg/ml, the percentage of inhibition of MDE was 23.2% whereas that of curcumin was 43.9%.

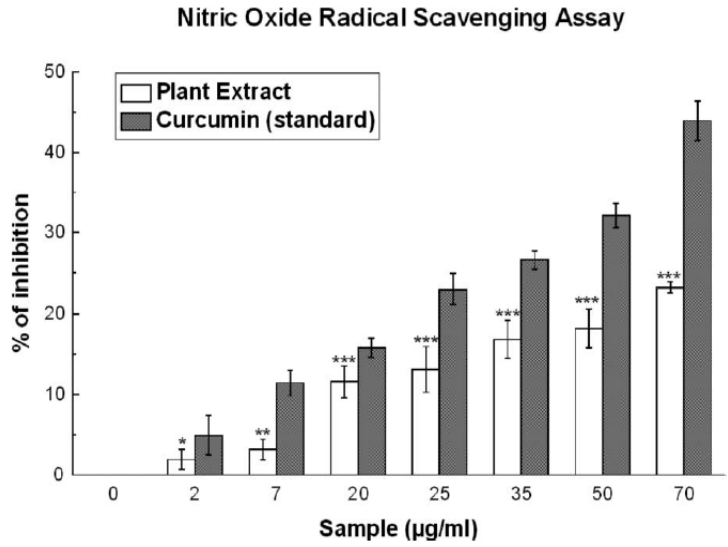


Figure 23: The data represent the percentage nitric oxide inhibition. Each value represents mean ± S.D. (n = 6). *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ vs 0 µg/ml. IC_{50} values of MDE and standard are 204.28 ± 18.31 µg/ml and 90.82 ± 4.75 µg/ml, respectively.

5.1.4.7. Hydrogen peroxide scavenging activity

Hydrogen peroxide scavenging activity was assayed by the FOX reagent method. Figure 24 shows that MDE has good H_2O_2 scavenging activity (IC_{50} = 4.17 ± 0.86 mg/ml) when compared with the standard sodium pyruvate (IC_{50} = 3.24 ± 0.30 mg/ml) (Table 12). At 2 mg/ml, the percentage of scavenging was 38.4% and 57.5% for *D. esculentum* and sodium pyruvate, respectively. At lower doses viz. 0.5 mg/ml, 0.8 mg/ml, and 1 mg/ml, the percentage of scavenging of the plant extract were 10.5%, 15.2% and 17.0%, respectively which were much higher than that of the sodium pyruvate (1.5%, 5.2% and 11.7% for 0.5 mg/ml, 0.8 mg/ml and 1 mg/ml, respectively).

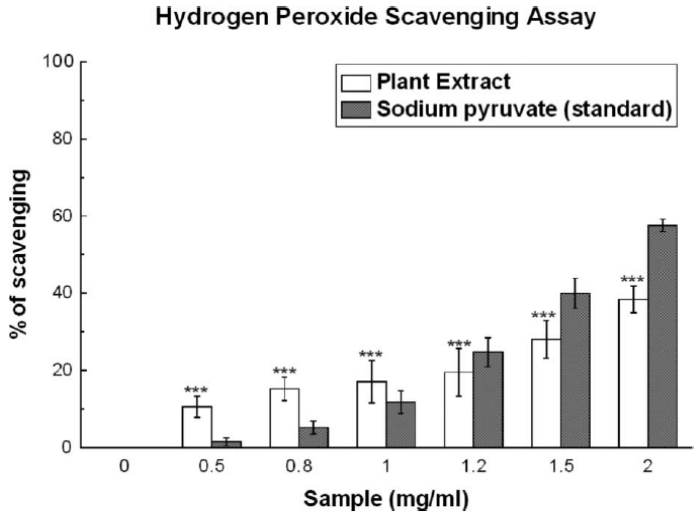


Figure 24: Effect of MDE and sodium pyruvate on the scavenging of H_2O_2. The data represent the percentage H_2O_2 scavenging. All data are expressed as mean ± S.D. (n = 6). ***$p < 0.001$ vs 0 mg/ml. IC_{50} values of the plant extract and standard are 4.17 ± 0.86 mg/ml and 3.24 ± 0.30 mg/ml, respectively.

5.1.4.8. Peroxynitrite scavenging activity

Figure 25 shows that the peroxynitrite scavenging activity of MDE is concentration dependent. The calculated IC_{50} value was 3.35 ± 0.33 mg/ml which was much greater than that of the reference compound gallic acid (IC_{50} = 0.87 ± 0.05 mg/ml) (Table 12), indicating that MDE is not a good peroxynitrite scavenger when compared to gallic acid. At 200 µg/ml, the scavenging percentages were 7.5% and 15.4% for *D. esculentum* and gallic acid, respectively.

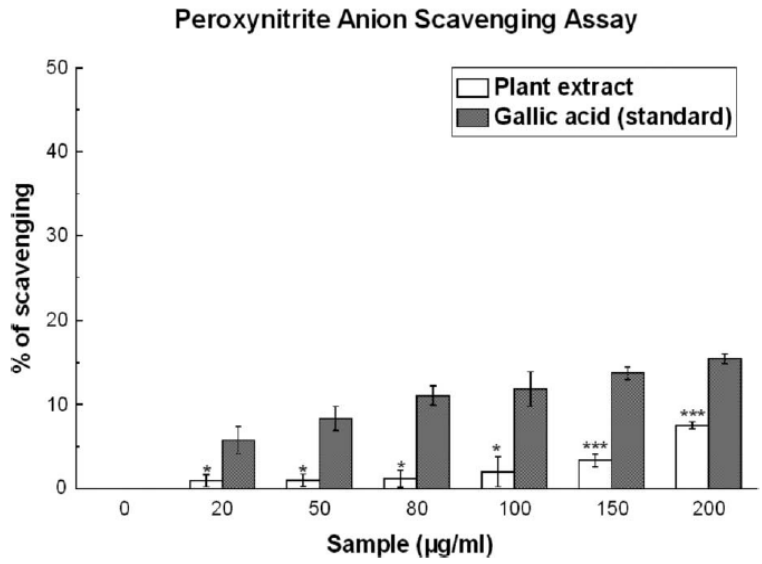


Figure 25: The peroxynitrite anion scavenging activity of MDE and the standard gallic acid. Each value represents mean ± S.D. (n = 6). *$p < 0.05$ and ***$p < 0.001$ vs 0 µg/ml. IC_{50} values of MDE and standard are 3.35 ± 3.33 mg/ml and 0.87 ± 0.05 mg/ml, respectively.

5.1.4.9. Singlet oxygen scavenging activity

MDE has moderate singlet oxygen scavenging activity when compared to that of lipoic acid (Figure 26). The IC_{50} value (Table 12) of the test sample was 278.88 ± 6.02 µg/ml whereas that of lipoic acid was 46.15 ± 1.16 µg/ml. At 200 µg/ml, the percentage scavenging of MDE was 42.2% whereas that of lipoic acid was 75.3%.

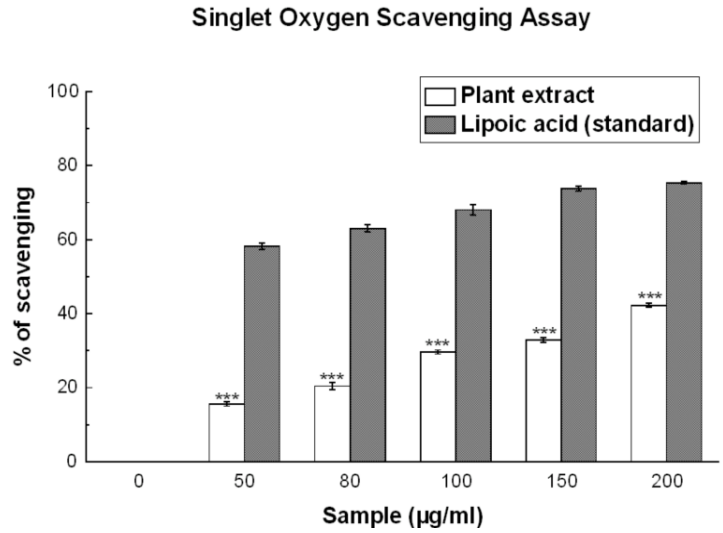


Figure 26: Effects of MDE and the standard lipoic acid on the scavenging of singlet oxygen. The results are mean ± S.D. of six parallel measurements. ***$p < 0.001$ vs µg/ml. IC_{50} values of MDE and standard are 278.88 ± 6.02 µg/ml and 46.15 ± 1.16 µg/ml, respectively.

5.1.4.10. Hypochlorous acid scavenging activity

Figure 27 shows that *D. esculentum* possesses an efficient hypochlorous acid scavenging activity (IC_{50} = 338.96 ± 11.60 µg/ml) when compared to that of ascorbic acid (IC_{50} = 235.95 ± 5.75 µg/ml) (Table 12). At 100 µg/ml, the percentage scavenging of MDE was 22.9% whereas that of ascorbic acid was 35.4%. At lower doses viz. 10 µg/ml and 20 µg/ml, the percentage scavenging of MDE were 3.9% and 5.6%, respectively which were higher than that of ascorbic acid (3.4% and 5.0% for 10 µg/ml and 20 µg/ml, respectively).

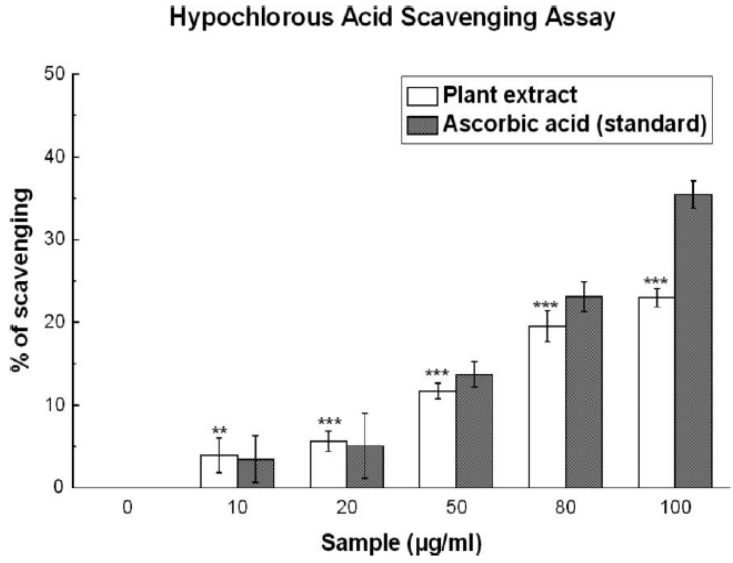


Figure 27: Hypochlorous acid scavenging activities of MDE and the standard ascorbic acid. All data are expressed as mean ± S.D. (n = 6). **$p < 0.01$ and ***$p < 0.001$ vs 0 µg/ml. IC_{50} values of MDE and standard are 338.96 ± 11.60 µg/ml and 235.95 ± 5.75 µg/ml, respectively.

5.1.4.11. Fe^{2+} chelation

Ferrozine produces a violet complex with Fe^{2+}. In the presence of a chelating agent, complex formation is interrupted and as a result the violet colour of the complex is decreased. The results [Figure 28 (a) and Figure 28 (b)] demonstrated that the formation of the ferrozine-Fe^{2+} complex was inhibited in the presence of the test and reference compounds. The IC_{50} values (Table 12) of MDE and EDTA were 1.33 ± 1.13 mg/ml and 0.001 ± 0.000 mg/ml, respectively. At 120 µg/ml, the percentage inhibition of MDE was 14.72% whereas at 20 µg/ml that of EDTA was 99.34%.

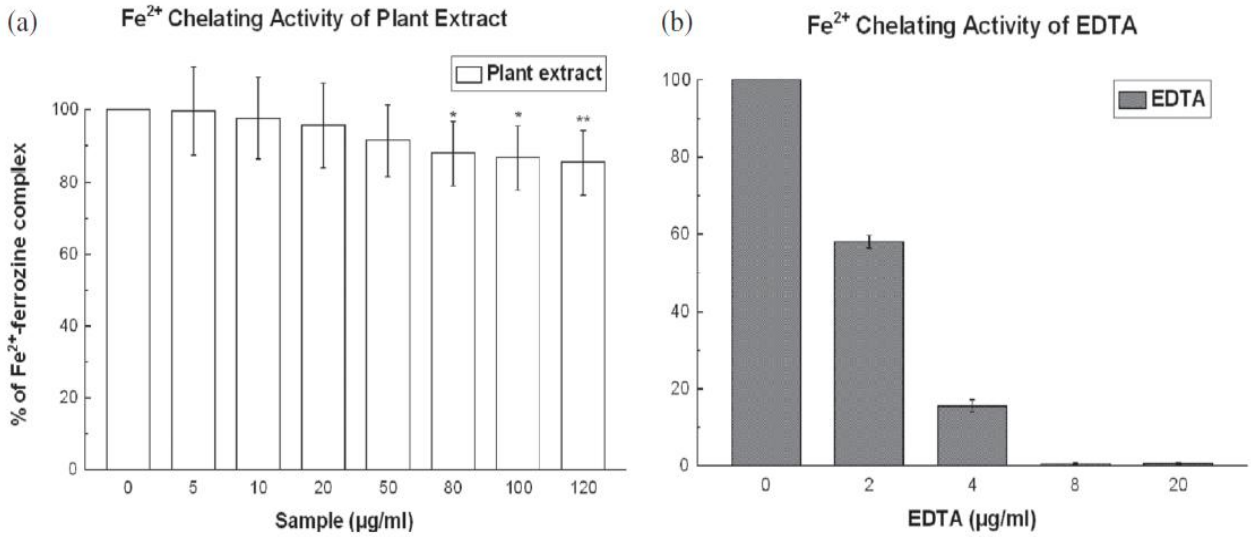


Figure 28: Effects of *D. esculentum* plant extract and EDTA on Fe^{2+}-ferrozine complex formation is shown. The data are expressed as percentage inhibition of chromogen formation. The results are mean ± S.D. of six parallel measurements. *$p < 0.05$ and **$p < 0.01$ vs 0 µg/ml. IC_{50} value of the plant extract and the standard were 1.33 ± 1.13 mg/ml and 0.001±0.00005 mg/ml, respectively.

5.1.4.12. Reducing power

As illustrated in Figure 29, Fe^{3+} was transformed to Fe^{2+} in the presence of MDE and the reference compound ascorbic acid to measure the reductive capability. Although the activity of ascorbic acid was better than MDE with absorbance values of 0.05 and 0.47 at 1 mg/ml for MDE and reference compound, respectively, still MDE showed moderate reducing capability.

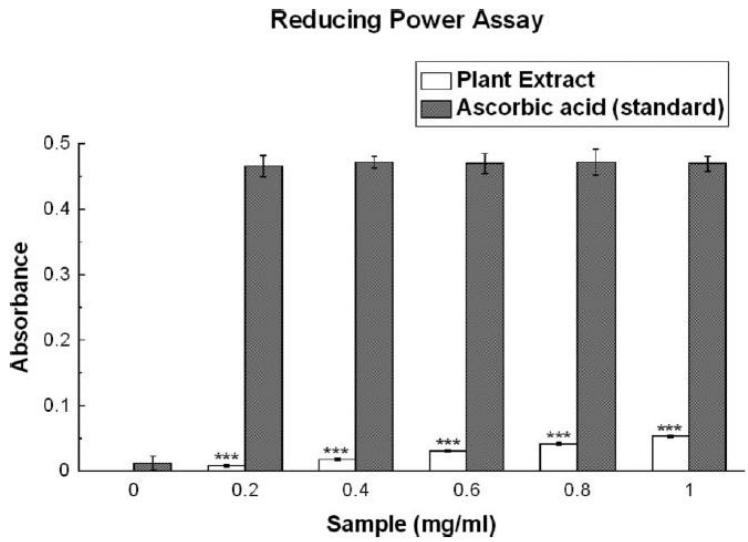


Figure 29: Reducing Power Assay. The reductive abilities of MDE and the standard ascorbic acid. The absorbance (A_{700}) was plotted against concentration of sample. Each value represents mean ± S.D. (n = 6). ***$p < 0.001$ vs 0 mg/ml.

5.1.4.13. Lipid peroxidation inhibition assay

The IC_{50} values (Table 12) of MDE (141.67 ± 4.19 µg/ml) and the standard (6.76 ± 0.17 µg/ml) supported the fact that the inhibitory efficiency of MDE was poor compared to standard trolox. As shown in Figure 30, the increase in lipid peroxidation inhibition with increasing concentration of MDE reflects its antioxidant property.

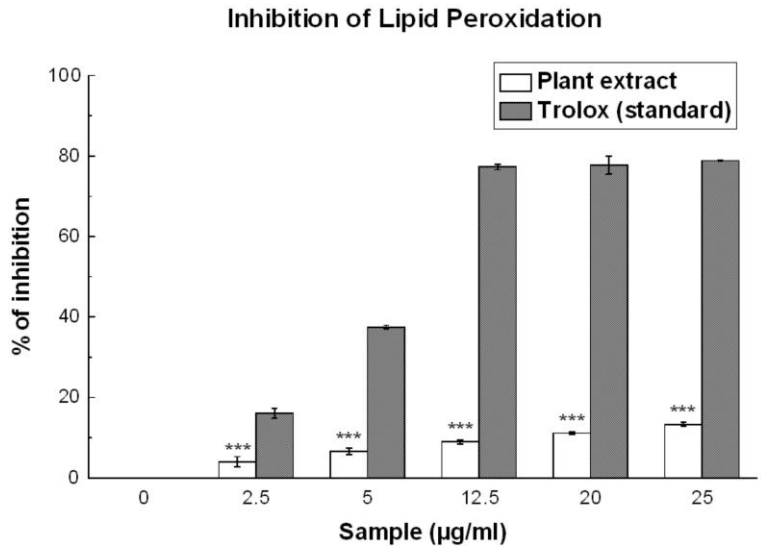


Figure 30: The data is expressed as the percentage of lipid peroxidation inhibition of brain homogenate, induced by Fe^{2+}/ascorbic acid. Each value represents mean ± S.D. (n=6). ***$p < 0.001$ vs 0 µg/ml. IC_{50} values of MDE and standard are 141.67 µg/ml and 6.76 µg/ml, respectively.

5.1.4.14. Determination of total phenolic content

MDE showed 126.67 ± 8.16 mg gallic acid equivalent phenolic content in 1 g dried plant extract.

5.1.4.15. Determination of total flavonoid content

MDE showed 94.33 ± 6.12 mg quercetin equivalent flavonoid content in 1 g dried plant extract.

Table 12. Scavenging of reactive oxygen species, iron chelating and lipid peroxidation inhibition activity (IC_{50} values) of *Diplazium esculentum* (MDE) and reference compounds.

Activity	Extract/Reference	IC_{50}[#]
Hydroxyl radical (OH•) scavenging	*Diplazium esculentum*	811.00 ± 23.73
	Mannitol	571.45 ± 20.12 (6)***
Superoxide anion ($O_2^{\bullet -}$) scavenging	*Diplazium esculentum*	90.39 ± 2.22
	Quercetin	42.06 ± 1.35 (6)***
Nitric oxide radical (NO) scavenging	*Diplazium esculentum*	204.28 ± 18.31
	Curcumin	90.82 ± 4.75 (6)***
Hydrogen peroxide (H_2O_2) scavenging	*Diplazium esculentum*	4.17 ± 0.86
	Sodium pyruvate	3.24 ± 0.30 (6)[N.S.]
Peroxynitrite ($ONOO^-$) scavenging	*Diplazium esculentum*	3.35 ± 0.33
	Gallic acid	0.87 ± 0.05 (6)***
Singlet oxygen ($^1O_2$) scavenging	*Diplazium esculentum*	278.88 ± 6.02
	Lipoic acid	46.15 ± 1.16 (6)***
Hypochlorous acid (HOCl) scavenging	*Diplazium esculentum*	338.96 ± 11.60
	Ascorbic acid	235.95 ± 5.75 (6)***
Iron chelating	*Diplazium esculentum*	1.33 ± 1.13
	EDTA	0.001 ± 0.000 (6)*
Lipid peroxidation inhibition	*Diplazium esculentum*	141.67 ± 4.19
	Trolox	6.76 ± 0.17 (6)***

[#]Units of IC_{50} for all activities are μg/ml, except H_2O_2 scavenging, peroxynitrite scavenging, and iron chelating where the units are mg/ml. Data are expressed as mean ± S.D. Data in parenthesis indicate number of independent assays.

EDTA: ethylenediamine tetraacetic acid; N.S.: not significant.

*$p < 0.05$; ***$p < 0.001$ vs. *Diplazium esculentum*.

5.1.5. Qualitative analysis of phytochemicals

Qualitative phytochemical analysis revealed the presence of secondary metabolites like terpenoids, flavonoids, cardiac glycosides, tannins, etc. (Table 13).

Table 13. Qualitative analysis of the phytochemicals of *D. esculentum*

Phytochemicals analysed	Terpenoid	Gylcoside	Alkaloids	Steroid	Tannin	Phlobatannins	Saponin	Flavonoid	Phenols
Present in *D. esculentum*	+	+	+	+	+	–	+	+	+

5.1.6. Effect of *D. esculentum* on the some major organs of mouse (*viz.* liver and kidney)

5.1.6.1. General condition, symptoms and mortality

All animals appeared to tolerate well the acute BDE dose and no mortality occurred in any of the treatment groups. No abnormal behavior or cases of diarrhea and soft feces were observed except for the mice treated with 320 mg/kg bw BDE in the initial days of chronic toxicity study. In general, dosing of BDE in mice did not induce any clinical signs of toxicity in acute regimen.

5.1.6.2. Acute study

The single-dose study in mice revealed no untoward physiological events that may arise out of an acute exposure of a test material in target species. Both feed intake and body weight gain (data not shown) of treated groups were not significantly different from that of untreated control mice. Feed conversion efficiency of untreated control and BDE administered groups were 15.2%, 14.9%, 14.2% and 13.9%, respectively. Relative weight of organs (Table 14) in mice of control and treatment groups did not show significant difference. No mortality, no alteration in growth and normal state of vital organs of adult mice in this study showed that they can tolerate maximum recommended dose (Schilter et al., 2003) of BDE. Based on this data, we conducted

sub-acute (45 days) and subchronic (90 days) and chronic (135 and 180 days) dietary study in adult mouse.

5.1.6.3. Subacute, subchronic and chronic toxicity studies

The data showed significant loss of feed intake and body weight of treated group mice from that of untreated control mice in subacute and subchronic and chronic toxicity studies (Table 15). The feed conversion efficiency were 14.8%, 13.2% and 12.4% in case of mice that were treated daily for 180 days at 80 mg/kg bw, 160 mg/kg bw and 320 mg/kg bw, respectively, when compared to the control (15.5%), indicating significant ($p < 0.05$) dose-dependent decrease in feed conversion efficiency. The growth of mice was also significantly reduced in treatment groups when compared to that of controls. Gross examination of vital organs such as liver, kidney and testis of mice from treated and control groups, and microscopic examination of tissue sections prepared from these organs reveal alterations in their histological architecture that could be attributed to BDE intake at different doses.

Table 14: Acute toxicity study of BDE on body weight and relative organ weight of Swiss albino mouse

Dosage group (g BDE/kg bw)	Final body weight (g)	Relative organ weight (g/100 g bw)							
		Brain	Heart	Spleen	Liver	Kidney	Adrenal	Testis	Ovary
Group A: untreated control	22.13 ± 0.62	1.83 ± 0.04	0.71 ± 0.05	0.33 ± 0.05	5.31 ± 0.50	1.99 ± 0.07	0.024 ± 0.005	0.98 ± 0.05	0.18 ± 0.02
Group B: 1 g/kg bw	25.03 ± 0.91	1.85 ± 0.05	0.70 ± 0.06	0.31 ± 0.03	5.27 ± 0.43	2.02 ± 0.05	0.025 ± 0.006	0.98 ± 0.07	0.18 ± 0.02
Group C: 2 g/kg bw	25.05 ± 0.82	1.83 ± 0.06	0.69 ± 0.05	0.32 ± 0.04	5.37 ± 0.57	1.98 ± 0.08	0.020 ± 0.004	0.98 ± 0.07	0.17 ± 0.03
Group D: 4 g/kg bw	25.27 ± 1.03	1.81 ± 0.04	0.69 ± 0.03	0.31 ± 0.04	5.42 ± 0.40	1.99 ± 0.10	0.023 ± 0.005	0.97 ± 0.06	0.17 ± 0.03

Table 15: Chronic toxicity study (180 days) of BDE on body weight and relative organ weight of Swiss albino mouse

Dosage group (g BDE/kg bw)	Final body weight (g)	Relative organ weight (g/100 g bw)							
		Brain	Heart	Spleen	Liver	Kidney	Adrenal	Testis	Ovary
Group A: untreated control	22.13 ± 0.62	1.83 ± 0.04	0.71 ± 0.05	0.33 ± 0.05	5.31 ± 0.50	1.99 ± 0.07	0.024 ± 0.005	0.98 ± 0.05	0.18 ± 0.02
Group B: 80 mg/kg bw	22.03 ± 0.91	1.82 ± 0.05*	0.70 ± 0.06	0.31 ± 0.03	5.27 ± 0.43*	1.98 ± 0.05	0.022 ± 0.006	0.96 ± 0.07	0.17 ± 0.02
Group C: 160 mg/kg bw	21.05 ± 0.82**	1.81 ± 0.06**	0.69 ± 0.05	0.30 ± 0.04	5.22 ± 0.57*	1.95 ± 0.08*	0.020 ± 0.004	0.93 ± 0.07*	0.16 ± 0.03
Group D: 320 mg/kg bw	20.27 ± 1.03***	1.80 ± 0.04**	0.68± 0.03**	0.28 ± 0.04**	5.15 ± 0.40**	1.90 ± 0.10**	0.015 ± 0.005*	0.88 ± 0.06**	0.15 ± 0.03*

*p < 0.05 when compared with control (Significantly different).

**p < 0.01 when compared with control (Significantly different).

***p < 0.001 when compared with control (Significantly different).

Table 16 and Table 17 represent serum biochemistry of mice fed with BDE at different doses in the subacute, subchronic and chronic toxicity study. Activities of serum enzymes such as AST, ALT, LDH, ALP, ACP and GGT (which indicate liver function) were significantly increased after repeated oral dosing of BDE. Total bilirubin, which is involved in lipid metabolism, in mice across the treated groups was increased significantly when compared to the control. In untreated control and treated mice, clinical chemical endpoints of kidney function such as creatinine and urea levels were also compared. The concentrations of urea and creatinine have been found to increase significantly in subchronic and chronic doses when compared to the respective control groups.

Table 16: Biochemical measurements in serum of mice fed with BDE for 15, 45 and 90 days

Parameters	Sub-acute dose I (15 days)				Sub-acute dose II (45 days)				Sub-chronic dose (90 days)			
	0 mg/kg bw	80 mg/kg bw	160 mg/kg bw	320 mg/kg bw	0 mg/kg bw	80 mg/kg bw	160 mg/kg bw	320 mg/kg bw	0 mg/kg bw	80 mg/kg bw	160 mg/kg bw	320 mg/kg bw
AST (Units/ml)	20.68 ± 0.05	20.75 ± 0.08	20.73 ± 0.11	20.76 ± 0.09	20.47 ± 0.03	20.47 ± 0.06	20.60 ± 0.05***	20.63 ± 0.04***	20.50 ± 0.08	20.63 ± 0.04**	20.80 ± 0.05***	20.94 ± 0.03***
ALT (Units/ml)	19.73 ± 0.12	19.75 ± 0.10	19.80 ± 0.10	18.79 ± 0.08	19.65 ± 0.10	19.85 ± 0.12**	20.11 ± 0.05***	20.60 ± 0.08***	20.60 ± 0.08	20.89 ± 0.10***	21.98 ± 0.09***	22.77 ± 0.09***
LDH (Units/L)	126.36 ± 0.49	126.42 ± 0.68	126.58 ± 0.54	127.21 ± 0.68	123.90 ± 0.62	124.78 ± 0.47*	126.26 ± 0.52***	127.87 ± 0.46***	124.63 ± 0.87	126.97 ± 0.09***	128.76 ± 0.80***	131.66 ± 0.75***
ALP (K.A. Units)	10.24 ± 0.16	10.23 ± 0.19	10.31 ± 0.14	10.28 ± 0.09	10.24 ± 0.10	10.26 ± 0.13	10.64 ± 0.11***	10.83 ± 0.13***	10.19 ± 0.11	10.38 ± 0.15*	10.78 ± 0.09***	11.29 ± 0.09***
Total ACP (K.A. Units)	1.29 ± 0.06	1.28 ± 0.09	1.32 ± 0.12	1.25 ± 0.12	1.32 ± 0.11	1.31 ± 0.06	1.44 ± 0.10	1.70 ± 0.09***	1.31 ± 0.07	1.52 ± 0.08**	1.81 ± 0.10***	2.24 ± 0.11***
Prostatic ACP (K.A. Units)	0.24 ± 0.01	0.24 ± 0.02	0.23 ± 0.03	0.25 ± 0.05	0.23 ± 0.02	0.24 ± 0.02	0.24 ± 0.02	0.25 ± 0.02	0.23 ± 0.01	0.24 ± 0.02	0.24 ± 0.01	0.25 ± 0.01*
γ-glutamyl transferase (Units/L)	13.96 ± 0.58	13.99 ± 0.28	14.09 ± 1.02	14.25 ± 0.58	14.53 ± 1.28	15.65 ± 0.67	15.68 ± 0.86	16.27 ± 1.07*	13.42 ± 1.05	14.17 ± 1.28	16.25 ± 0.97***	17.10 ± 0.73***
Total bilirubin (mg/dl)	0.52 ± 0.03	0.53 ± 0.04	0.51 ± 0.06	0.52 ± 0.02	0.50 ± 0.02	0.52 ± 0.02	0.51 ± 0.02	0.54 ± 0.02**	0.51 ± 0.02	0.52 ± 0.01	0.52 ± 0.01	0.54 ± 0.02**
Urea (mg/dl)	17.62 ± 0.68	17.69 ± 0.64	17.85 ± 0.69	18.12 ± 0.37	17.56 ± 0.80	17.56 ± 0.48	18.45 ± 1.02	19.04 ± 0.81*	17.62 ± 0.65	17.92 ± 0.56	18.62 ± 0.91*	19.41 ± 0.57***
Creatinine (mg/dl)	1.22 ± 0.11	1.23 ± 0.09	1.24 ± 0.11	1.24 ± 0.13	1.20 ± 0.08	1.21 ± 0.12	1.23 ± 0.08	1.26 ± 0.14	1.23 ± 0.06	1.31 ± 0.08	1.38 ± 0.11*	1.41 ± 0.09**

*$p < 0.05$ when compared with control (Significantly different).

**$p < 0.01$ when compared with control (Significantly different).

***$p < 0.001$ when compared with control (Significantly different).

Table 17: Biochemical measurements in serum of mice fed with BDE for 135 and 180 days

Parameters	Chronic dose – I (135 days)				Chronic dose – II (180 days)			
	0 mg/kg bw	80 mg/kg bw	160 mg/kg bw	320 mg/kg bw	0 mg/kg bw	80 mg/kg bw	160 mg/kg bw	320 mg/kg bw
AST (Units/ml)	20.67 ± 0.04	20.80 ± 0.39	21.88 ± 0.06***	22.84 ± 0.09***	20.90 ± 0.04	21.92 ± 0.09***	23.49 ± 0.09***	25.70 ± 0.05***
ALT (Units/ml)	20.72 ± 0.10	21.87 ± 0.11***	22.93 ± 0.10***	23.62 ± 0.10***	20.71 ± 0.12	22.02 ± 0.09***	23.19 ± 0.08***	24.48 ± 0.09***
LDH (Units/L)	126.26 ± 0.59	128.60 ± 0.87***	132.73 ± 0.89***	137.81 ± 0.87***	124.87 ± 1.20	128.86 ± 1.30***	137.78 ± 1.04***	142.02 ± 1.06***
ALP (K.A. Units)	10.21 ± 0.11	11.18 ± 0.10***	11.31 ± 0.12***	11.84 ± 0.10***	10.21 ± 0.09	11.45 ± 0.14***	12.44 ± 0.09***	13.27 ± 0.05***
Total ACP (K.A. Units)	1.27 ± 0.10	1.99 ± 0.12***	2.68 ± 0.14***	3.49 ± 0.11***	1.27 ± 0.12	2.27 ± 0.12***	3.27 ± 0.10***	4.34 ± 0.14***
Prostatic ACP (K.A. Units)	0.22 ± 0.01	0.24 ± 0.01*	0.28 ± 0.01***	0.29 ± 0.01***	0.22 ± 0.01	0.26 ± 0.01***	0.30 ± 0.01***	0.33 ± 0.01***
γ-glutamyl transferase (Units/L)	13.33 ± 0.57	14.57 ± 1.20	15.68 ± 0.74***	16.87 ± 1.10***	13.80 ± 0.49	15.25 ± 1.09*	16.02 ± 0.69***	17.67 ± 0.85***
Total bilirubin (mg/dl)	0.51 ± 0.01	0.61 ± 0.02***	0.67 ± 0.02***	0.72 ± 0.02***	0.51 ± 0.01	0.81 ± 0.02***	0.92 ± 0.02***	1.62 ± 0.02***
Urea (mg/dl)	17.64 ± 0.51	21.16 ± 0.83***	21.38 ± 0.94***	23.41 ± 1.13***	18.38 ± 0.73	24.69 ± 1.58***	30.62 ± 1.46***	37.33 ± 1.64***
Creatinine (mg/dl)	1.24 ± 0.06	1.36 ± 0.07*	1.45 ± 0.06***	1.48 ± 0.08***	1.24 ± 0.04	1.57 ± 0.13***	1.77 ± 0.11***	2.07 ± 0.13***

*$p < 0.05$ when compared with control (Significantly different).

**$p < 0.01$ when compared with control (Significantly different).

***$p < 0.001$ when compared with control (Significantly different).

The histological study did not reveal any changes in the in liver and kidney tissue architecture except for the chronic dose, where mice were treated with BDE at 320 mg/kg bw for 180 days. Figure 31 (b) revealed disorganized portal area in the liver of BDE treated mice when compared to the controls [Figure 31 (a)]. Similarly, deformed distal tubules in the kidney have been observed in the BDE treated mice [Figure 32 (b)] when compared to the control group [Figure 32 (a)].

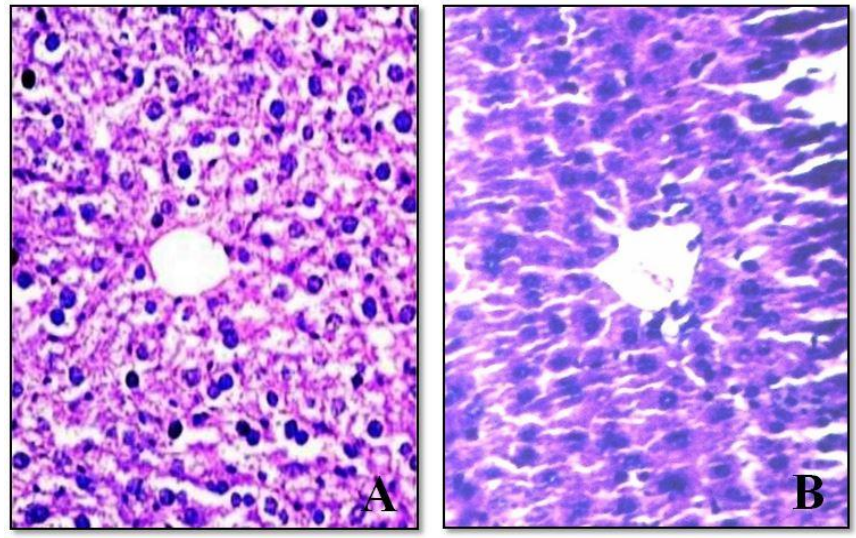


Figure 31: Histological architecture of liver in Control (A) and BDE treated mouse (B). Figure (B) shows liver of mice that received 320 mg/kg of bw of BDE (for 180 days). It displays liver with pleiomorphoinuclei, dilated blood vessels and lymphocytes surrounding ducts.

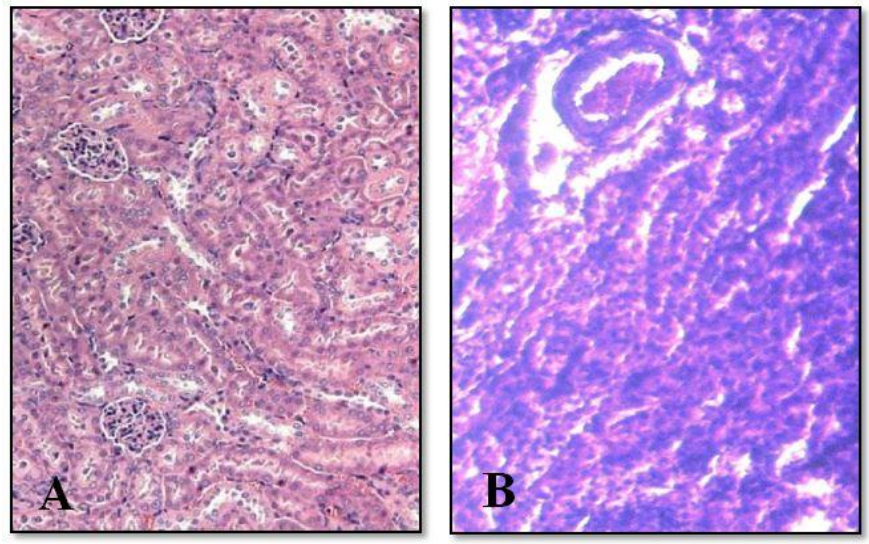


Figure 32: Histological architecture of kidney in Control (A) and BDE treated mouse (B). Figure (B) shows kidney of mice that received 320 mg/kg of bw of BDE (for 180 days). It displays kidney with tubular degeneration, dilation and inflammatory infiltrate.

5.2. COMPARATIVE ANALYSIS OF THE EFFECTS OF CDE AND BDE ON DIFFERENT *IN VIVO* AND *EX VIVO* PARAMETERS OF SWISS ALBINO MOUSE

Several parameters have been investigated to compare the effects of CDE and BDE *in vivo* and *in vitro*. The experimental procedures used to investigate these parameters have already been described in previous sections. The results observed are presented in this section.

5.2.1. Comparison of the effect of CDE and BDE on immunomodulation

5.2.1.1. Splenocyte count

Dose-dependent decreases in splenocyte number have been observed in both in CDE and BDE treated mice after 180 days of treatment, though significant differences ($p < 0.05$, $p < 0.01$, and $p < 0.001$) were there between the cell number of CDE and BDE treated mice at all the doses (Figure 33). At control group, the number of splenocytes for CDE and BDE treated mice were $(31.25 \pm 1.05) \times 10^6$/ml and $(31.84 \pm 0.87) \times 10^6$/ml, respectively, whereas, after 320 mg/kg bw of CDE and BDE, the cell numbers decreased to $(17.07 \pm 1.48) \times 10^6$/ml and $(23.73 \pm 0.74) \times 10^6$/ml, respectively.

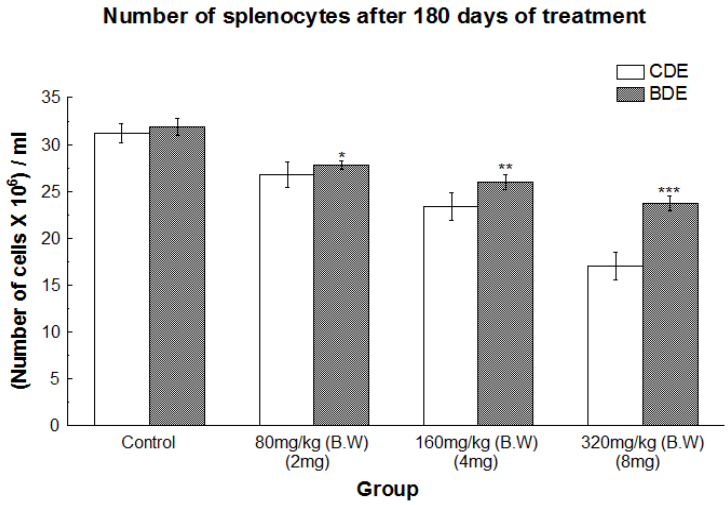


Figure 33: Comparison between the numbers of splenocytes of CDE vs BDE treated mice after 180 days of treatment. *p < 0.05 at 80 mg/kg bw, *p < 0.01 at 160 mg/kg bw, and *p < 0.001 at 320 mg/kg bw (CDE vs BDE).

5.2.1.2. PFC and HA titre assay

Figure 34 indicates the dose-dependent decrease in the number of plaque forming cells in spleen of mice treated with both CDE and BDE, though the decrease was 5% higher in case of CDE treated mice than that of BDE after 180 days of treatment. At 0 mg/kg bw, the numbers of PFC were 496.67 ± 8.16 and 495 ± 10.49, for CDE and BDE, respectively. At 320 mg/kg bw, the PFC numbers have decreased to 386.67 ± 8.16 and 406.67 ± 8.16, for CDE and BDE, respectively. Table 18 showed dose-dependent decrease in the HA titre value in both CDE and BDE treated mice when they were compared with that of their respective controls. After 180 days of the treatment with 360 mg/kg bw of CDE and BDE, the observed titre value of CDE and BDE treated mice were 1/10 and 1/20, respectively, 32- and 16 folds higher than that of their respective controls.

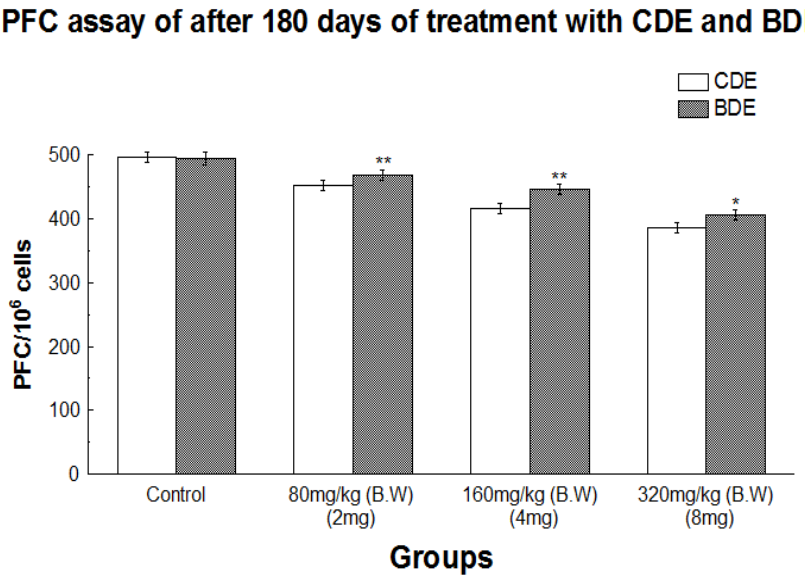


Figure 34: Comparison between the numbers of plaque forming cells in the spleen of CDE and BDE treated mice. **p < 0.01 at 80mg/kg bw and at 160mg/kg body weight (CDE vs BDE) and *p < 0.05 at 320mg/kg body weight (CDE vs BDE).

Table 18: Effect of CDE and BDE on the HA titre value in mice after 180 days of treatment

Group	Titre value	
	CDE	BDE
(I) Control	1 : 320	1 : 160
(II) 80mg/kg (B.W) (2mg)	1 : 160	1 : 10
(III) 160mg/kg (B.W) (4mg)	1 : 40	1 : 10
(IV) 320mg/kg (B.W) (8mg)	1 : 10	1 : 10

5.2.1.3. Comparison of the effect of CDE and BDE on the in vitro splenocyte viability and proliferation (MTT assay) as well as RBC hemolysis

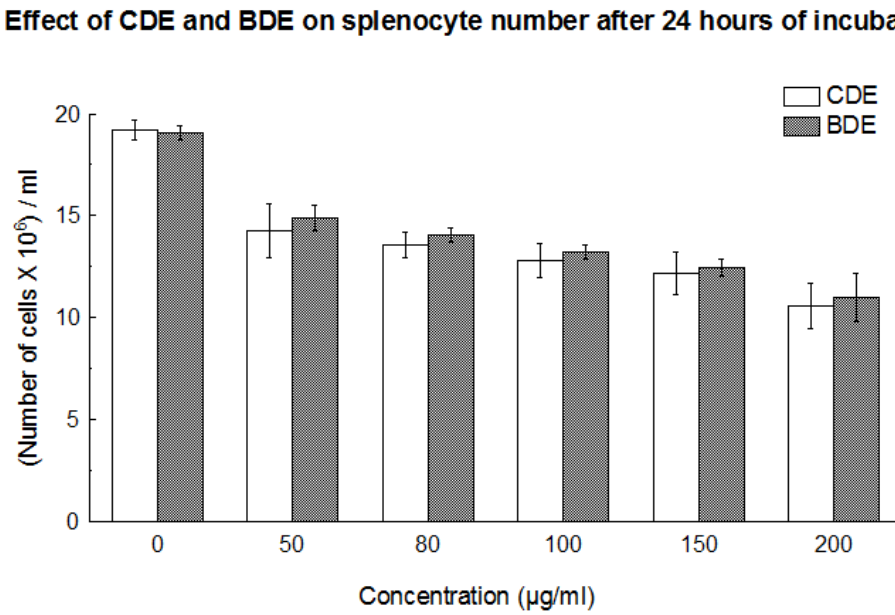


Figure 35: Result shows dose-dependent decrease in the number of splenocyte after 24 hours of incubation. There are no statistical differences between the CDE and BDE treated splenocyte at any of the dose (0-200 µg/ml). At 200 µg/ml of CDE and BDE, the no. of cell were $(10.56 \pm 1.09) \times 10^6$/ml and $(10.97 \pm 1.19) \times 10^6$/ml, respectively.

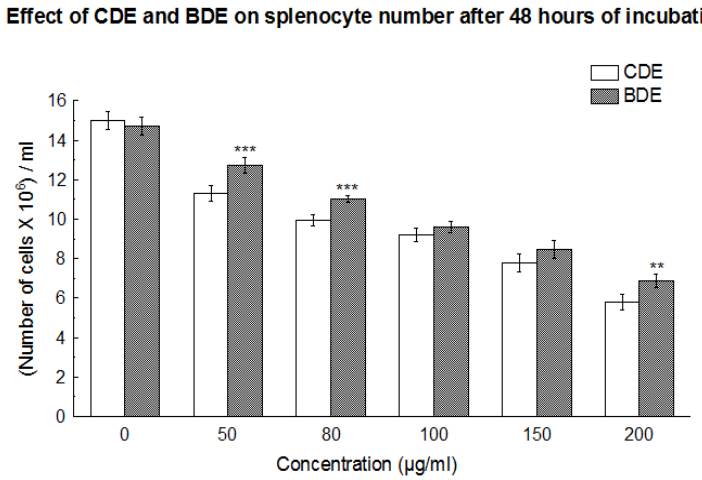


Figure 36: Result shows dose-dependent decrease in the number of splenocyte after 48 hours of incubation. ***$p < 0.001$ at 50µg/ml (CDE vs BDE) and at 80µg/ml (unboiled vs boiled), and **$p < 0.01$ at 200µg/ml (CDE vs BDE). At 200 µg/ml of CDE and BDE, the no. of cell were $(5.81 \pm 0.37) \times 10^6$/ml and $(6.88 \pm 0.34) \times 10^6$/ml, respectively.

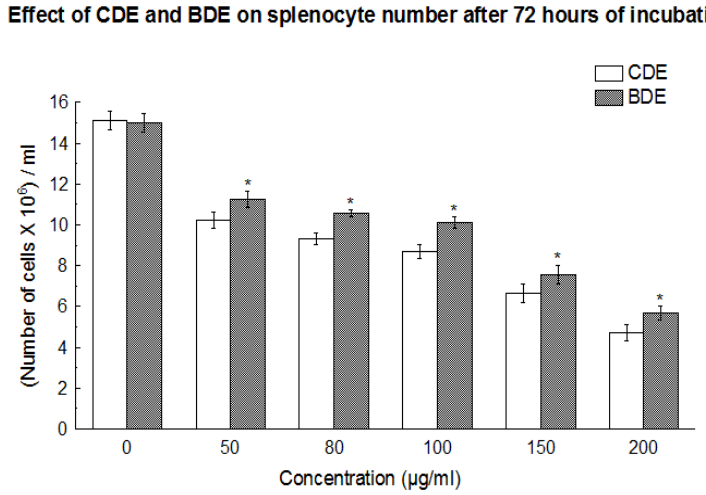


Figure 37: Result shows dose-dependent decrease in the number of splenocyte after 72 hours of incubation. *$p < 0.05$ at all the doses (CDE vs BDE). At 200 µg/ml of CDE and BDE, the no. of cell were $(4.69 \pm 0.37) \times 10^6$/ml and $(5.69 \pm 0.34) \times 10^6$/ml, respectively.

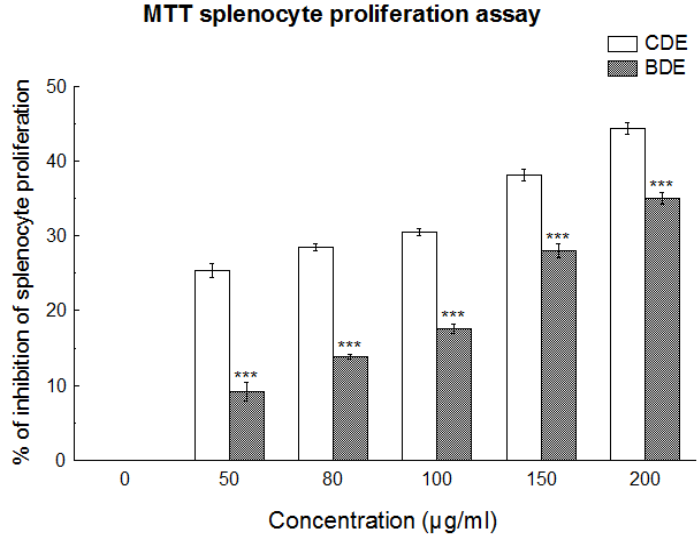


Figure 38: MTT assay showing the effect of CDE and BDE on splenocytes. All data are expressed as mean ± S.D. (n = 6). ***$p < 0.001$ for CDE vs BDE at each concentration (0-200 µg/ml). IC_{50} values of the CDE and BDE are 221.82 ± 3.97 µg/ml and 412.96 ± 12.14 µg/ml, respectively.

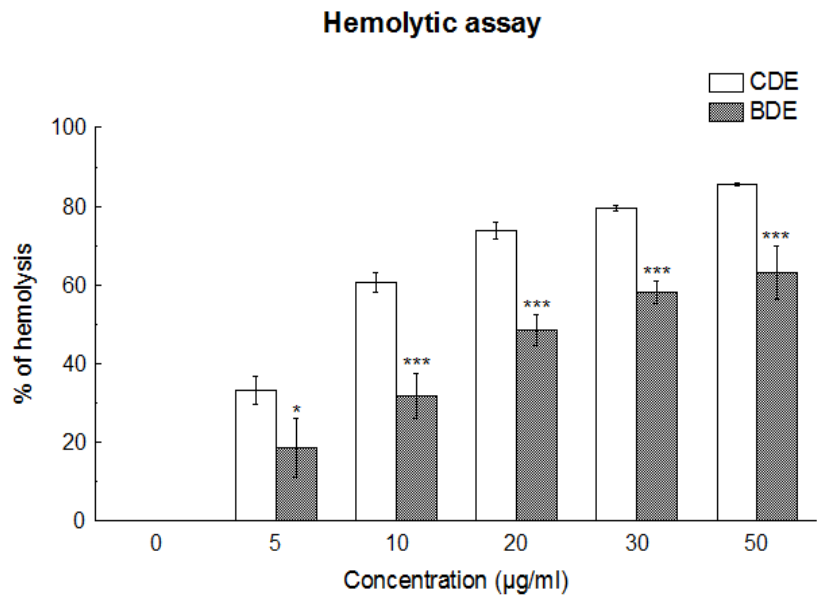


Figure 39: Comparison between the hemolytic activity of CDE and BDE on mouse erythrocytes. *p < 0.05 at 5µg/ml, ***p < 0.001 at 10, 20, 30 and 50 µg/ml. IC_{50} valus of CDE and BDE were 7.81 ± 0.36 µg/ml and 23.06 ± 3.14 µg/ml, respectively.

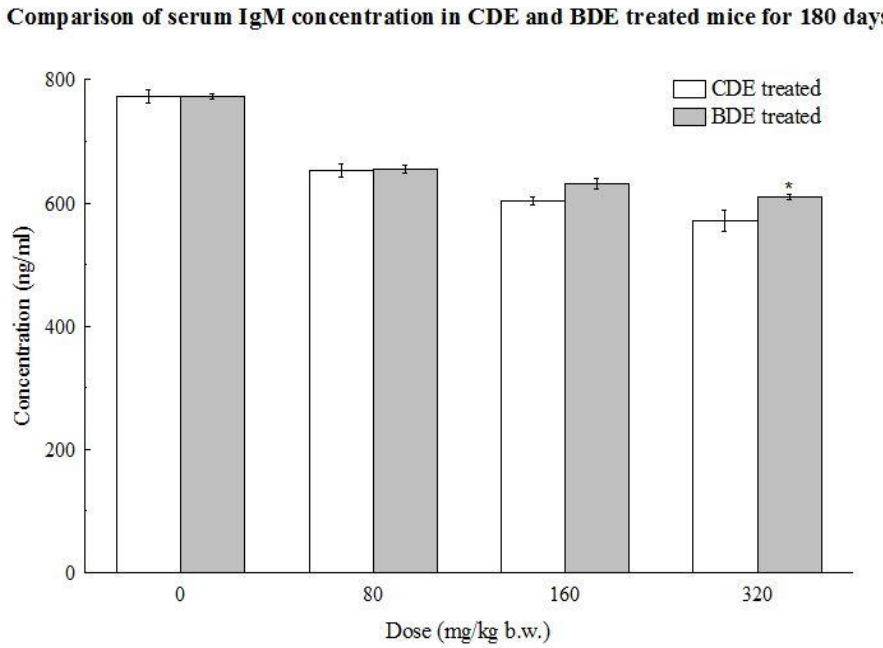


Figure 40: Comparison between serum IgM concentration of CDE and BDE. At 0 mg/kg bw of CDE and BDE treatment, the concentration of serum IgM were 772.66 ± 11.55 ng/ml and 772.74 ± 4.85 ng/ml, respectively, whereas, at 320 mg/kg bw of CDE and BDE treatment, the concentration of serum IgM decreased to 572.01 ± 16.66 ng/ml and 609.63 ± 4.99 ng/ml, respectively.

5.2.2. Comparative analysis of the effect of CDE and BDE on some reproductive functions

5.2.2.1. Hypo-osmotic swelling test of mouse sperm

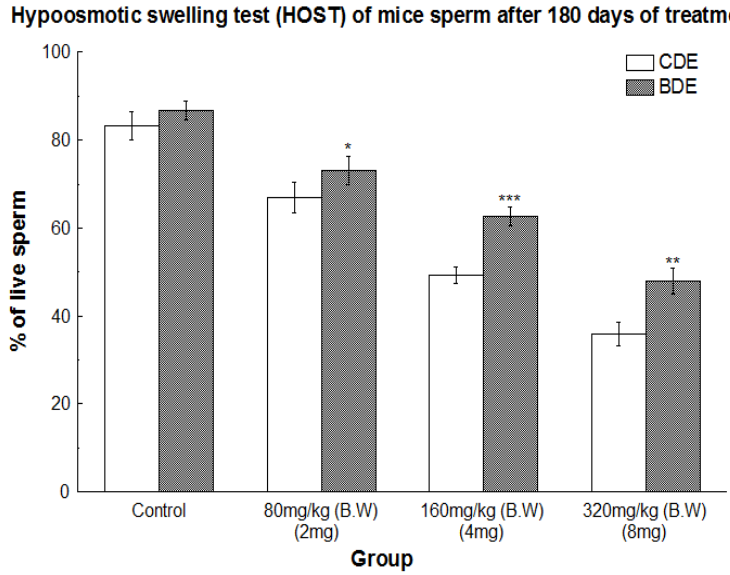


Figure 41: Comparison of the percentage of live sperm between CDE and BDE. Data represents dose-dependent decrease in both CDE and BDE treated mice. All data are expressed as mean ± S.D. (n = 6). *p < 0.05, ***p < 0.001 and **p < 0.01 vs 80mg/kg body weight, 160mg/kg body weight and 320mg/kg body weight, respectively. At 320 mg/kg bw of CDE and BDE, the percentages of live sperm were 35.83% and 48%, respectively.

5.2.2.2. Comparison of the sperm MTT assay between CDE and BDE treated mice

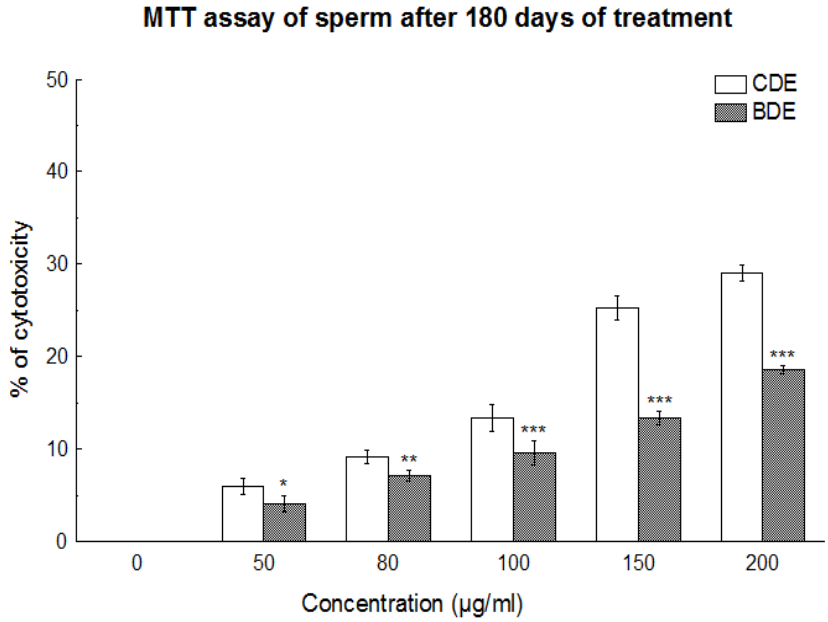


Figure 42: MTT assay showing the effect of CDE and BDE on sperm. All data are expressed as mean ± S.D. (n = 6). *p < 0.05, **p < 0.01 and ***p < 0.001 for CDE vs BDE of each concentration (0-200 µg/ml). IC_{50} values of the CDE and BDE were 537.75 ± 19.13 µg/ml and 941.86 ± 37.63, respectively.

5.2.2.3. Comparative effect of CDE and BDE on body weight, relative organ weight and different biochemical parameters of male reproductive organs

Table 19: Effect of CDE and BDE on body weight and relative weight of male reproductive organs after chronic dose (180 days)

Parameters	CDE (180 days)				BDE (180 days)			
	0 mg/kg bw	80 mg/kg bw	160 mg/kg bw	320 mg/kg bw	0 mg/kg bw	80 mg/kg bw	160 mg/kg bw	320 mg/kg bw
Body weight (g)	25.13 ± 0.18	24.86 ± 0.24	23.53 ± 0.15	21.12 ± 0.11*	24.83 ± 0.32	23.03 ± 0.93	22.85 ± 0.77	22.27 ± 1.13
Testis weight (g/100 g body weight)	0.95 ± 0.08	0.94 ± 0.07	0.91 ± 0.07*	0.86 ± 0.06***	0.95 ± 0.01	0.93 ± 0.01	0.93 ± 0.01	0.91 ± 0.01**
Epididymis (g/100 g body weight)	0.33 ± 0.01	0.27 ± 0.02**	0.22 ± 0.01**	0.18 ± 0.01***	0.33 ± 0.02	0.33 ± 0.01	0.27 ± 0.01**	0.24 ± 0.01***
Seminal vesicles (g/100 g body weight)	0.48 ± 0.01	0.42 ± 0.01*	0.40 ± 0.01**	0.35 ± 0.01***	0.48 ± 0.01	0.43 ± 0.02*	0.42 ± 0.02**	0.37 ± 0.02***
Prostate gland (g/100 g body weight)	0.24 ± 0.01	0.23 ± 0.01	0.22 ± 0.01**	0.17 ± 0.01***	0.24 ± 0.01	0.22 ± 0.01*	0.21 ± 0.01**	0.19 ± 0.01***

*$p < 0.05$ when compared with control (Significantly different).

**$p < 0.01$ when compared with control (Significantly different).

***$p < 0.001$ when compared with control (Significantly different).

Table 20: Effect of CDE and BDE on different biochemical parameters of male reproductive organs after 180 days of treatment

Parameters	CDE (180 days)				BDE (180 days)			
	0 mg/kg bw	80 mg/kg bw	160 mg/kg bw	320 mg/kg bw	0 mg/kg bw	80 mg/kg bw	160 mg/kg bw	320 mg/kg bw
Total Protein (serum) (mg/dl)	7.84 ± 0.49	6.12 ± 0.16***	5.68 ± 0.32***	5.35 ± 0.35***	7.85 ± 0.83	6.78 ± 0.41**	6.06 ± 0.30***	5.64 ± 0.55***
Total Protein (testis) (mg/g)	0.58 ± 0.04	0.50 ± 0.01***	0.48 ± 0.03***	0.39 ± 0.02***	0.56 ± 0.03	0.52 ± 0.03*	0.48 ± 0.02***	0.41 ± 0.01***
Total Protein (epididymis) (mg/g)	0.25 ± 0.04	0.18 ± 0.02***	0.12 ± 0.02***	0.06 ± 0.01***	0.28 ± 0.02	0.19 ± 0.01***	0.13 ± 0.01***	0.08 ± 0.01***
Chelesterol (testis) (mg/g)	3.85 ± 0.13	3.52 ± 0.08**	3.43 ± 0.03***	3.22 ± 0.02***	3.82 ± 0.08	3.53 ± 0.12**	3.43 ± 0.07**	3.27 ± 0.06***
α-glucosidase (Epididymis) (mU/g)	4.76 ± 0.06	4.53 ± 0.03**	4.41 ± 0.08***	4.32 ± 0.05***	4.77 ± 0.06	4.56 ± 0.06**	4.40 ± 0.05***	4.32 ± 0.06***
Fructose (µM/g) (Seminal vesicle)	4.56 ± 0.11	4.46 ± 0.05	4.31 ± 0.07**	4.26 ± 0.04***	4.52 ± 0.08	4.42 ± 0.04	4.35 ± 0.06*	4.30 ± 0.04**
Glycogen (mg/g) (testis)	39.58 ± 1.58	28.66 ± 2.25***	23.19 ± 1.20***	20.27 ± 0.53***	40.58 ± 1.38	31.46 ± 2.85***	27.19 ± 3.28***	20.27 ± 3.53***
Sialic acid (µM/100 g tissue) (epididymis)	62.12 ± 3.24	51.71 ± 0.93***	40.03 ± 1.14***	28.06 ± 0.81***	63.59 ± 2.24	54.36 ± 3.36***	40.25 ± 3.35***	31.36 ± 2.54***
Prostate citric acid (mg/g)	37.02 ± 1.52	21.75 ± 1.51***	17.37 ± 2.56***	11.33 ± 0.99***	36.58 ± 2.27	25.48 ± 3.35***	21.36 ± 3.69***	18.47 ± 1.36***
Acid phosphatase (µM/min/g of testicular tissue)	30.07 ± 5.12	17.94 ± 0.88***	15.34 ± 1.44***	14.29 ± 0.05***	33.72 ± 0.38	21.70 ± 0.60***	18.35 ± 1.02***	15.96 ± 1.57***

*$p < 0.05$ when compared with control (Significantly different).

**$p < 0.01$ when compared with control (Significantly different).

***$p < 0.001$ when compared with control (Significantly different).

5.2.3. Comparison of the effect of CDE and BDE on the cholinergic nervous system

5.2.3.1. Effect of CDE and BDE on acetylcholinesterase activity

In vivo acetylcholinesterase activity of CDE and BDE had been determined and the results were illustrated in Figure 43. The data represents that both CDE and BDE inhibit the acetylcholinesterase activity *in vivo* in a dose-dependent manner.

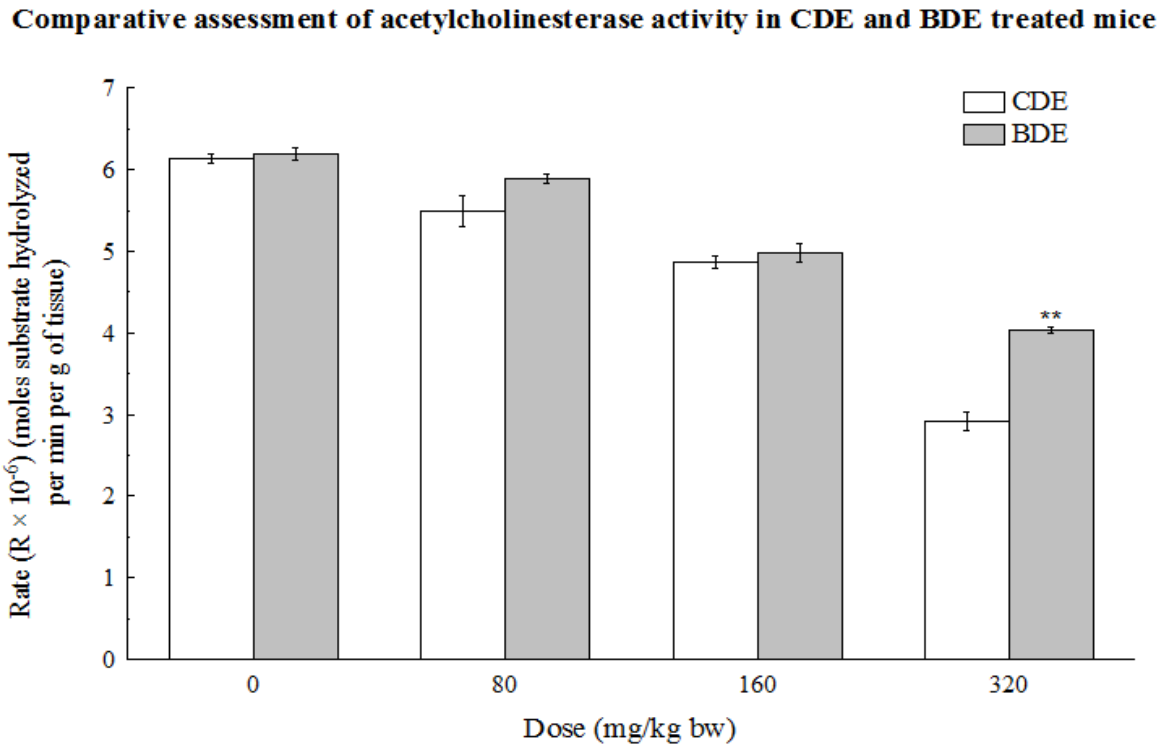


Figure 43: Results indicated dose-dependent decrease in the rate of hydrolysis of acetylthiocholine iodide substrate by acetylcholinesterase. All data are expressed as mean ± S.D. (n = 6). **p < 0.01 for CDE vs BDE at 320 mg/kg bw.

5.2.4. Comparative effect of CDE and BDE on different biochemical parameters of liver and kidney function

Table 21: Comparison of the biochemical parameters in serum of mice fed with CDE and BDE for 180 days

Parameters	CDE (180 days)				BDE (180 days)			
	0 mg/kg bw	80 mg/kg bw	160 mg/kg bw	320 mg/kg bw	0 mg/kg bw	80 mg/kg bw	160 mg/kg bw	320 mg/kg bw
AST (Units/ml)	20.67 ± 0.04	22.80 ± 0.39***	23.88 ± 0.06***	25.84 ± 0.09***	20.90 ± 0.04	21.92 ± 0.09***	23.49 ± 0.09***	25.70 ± 0.05***
ALT (Units/ml)	20.72 ± 0.10	22.87 ± 0.11***	23.93 ± 0.10***	25.62 ± 0.10***	20.71 ± 0.12	22.02 ± 0.09***	23.19 ± 0.08***	24.48 ± 0.09***
LDH (Units/L)	126.26 ± 0.59	129.60 ± 0.87***	138.73 ± 0.89***	143.81 ± 0.87***	124.87 ± 1.20	128.86 ± 1.30***	137.78 ± 1.04***	142.02 ± 1.06***
ALP (K.A. Units)	10.21 ± 0.11	12.18 ± 0.10***	13.31 ± 0.12***	14.84 ± 0.10***	10.21 ± 0.09	11.45 ± 0.14***	12.44 ± 0.09***	13.27 ± 0.05***
Total ACP (K.A. Units)	1.27 ± 0.10	2.99 ± 0.12***	3.68 ± 0.14***	4.49 ± 0.11***	1.27 ± 0.12	2.27 ± 0.12***	3.27 ± 0.10***	4.34 ± 0.14***
Prostatic ACP (K.A. Units)	0.22 ± 0.01	0.27 ± 0.01***	0.32 ± 0.01***	0.39 ± 0.01***	0.22 ± 0.01	0.26 ± 0.01***	0.30 ± 0.01***	0.33 ± 0.01***
γ-glutamyl transferase (Units/L)	13.33 ± 0.57	15.57 ± 1.20**	16.68 ± 0.74***	18.87 ± 1.10***	13.80 ± 0.49	15.25 ± 1.09*	16.02 ± 0.69***	17.67 ± 0.85***
Total bilirubin (mg/dl)	0.51 ± 0.01	0.81 ± 0.02***	0.97 ± 0.02***	1.72 ± 0.02***	0.51 ± 0.01	0.81 ± 0.02***	0.92 ± 0.02***	1.62 ± 0.02***
Urea (mg/dl)	17.64 ± 0.51	25.16 ± 0.83***	31.38 ± 0.94***	39.41 ± 1.13***	18.38 ± 0.73	24.69 ± 1.58***	30.62 ± 1.46***	37.33 ± 1.64***
Creatinine (mg/dl)	1.24 ± 0.06	1.66 ± 0.07***	1.85 ± 0.06***	2.48 ± 0.08***	1.24 ± 0.04	1.57 ± 0.13***	1.77 ± 0.11***	2.07 ± 0.13***

*$p < 0.05$ when compared with control (Significantly different).

**$p < 0.01$ when compared with control (Significantly different).

***$p < 0.001$ when compared with control (Significantly different).