3.0 RESULTS

3. 1 Bacteriological characteristic study

The cultural characteristics of the bacterial isolates on horse blood agar plates were observed after incubation period. The tested organisms were gray-white colour, entire margin, and small in size. The shape and texture were convex and shiny, respectively. In addition, all bacterial strains were subjected to Gram staining to ensure the identity of the test bacteria. When observed under microscope, the bacterial strains culture showed uniform arrangement of Gram-Positive Cocci occurred in clusters, pairs; long and short chains shape **(Figure 2).** Also, the bacterial strains showed beta haemolysis after overnight incubation at 37 ºC for 24 hours on Columbia Horse Blood Agar.

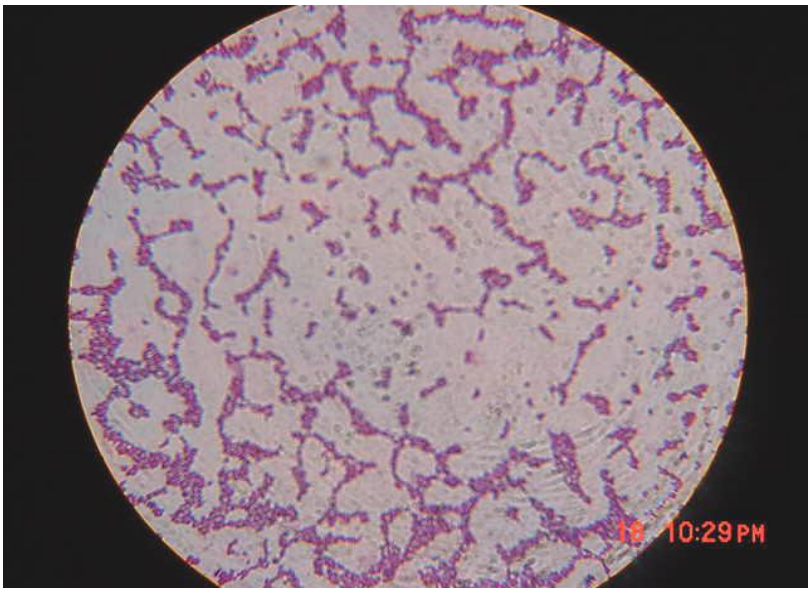

Figure 2: Gram stain of *S. aureus* bacteria from Columbia Horse Blood Agar (Magnification, 100x)

3.2 Antimicrobial susceptibility testing

3.2.1 Disc diffusion method

The results obtained from the disc diffusion method followed the same trend as the Minimum Inhibitory tests. The aqueous extracts of *Tinospora crispa* did not show any inhibition on all the tested organisms at the concentration used **(Figure 3).** On the other hand, using the same concentration, the ethanolic extracts of the plants inhibited the tested organisms at different rates **(Figure 4).** Hence, the ethanolic extracts of *Tinospora crispa* had antibacterial property on the tested organisms as compared to aqueous extract.

The appearance of zone inhibition that produced around the discs was observed and their diameters measured against a dark, non-reflective background. The zone diameter was measured in mm units. All the diameters of zone inhibition of the positive controls fall within susceptible ranges according to the interpretive standards for disc diffusion susceptibility testing by CLSI (Hindler & Jorgensen, 2007; Jorgensen & Turnidge, 2007), whereas all negative controls discs showed no zones of inhibition.

Figure 5 illustrates the mean diameter of zones of inhibitions obtained from the ethanolic extract of *T. crispa.* Ethanolic extract was found to show zones of inhibition against all MRSA strains, and *S. aureus* ATCC. All –ST/09 strains and *S. aureus* were tested at volume of 10, 30, 50 µl per disc of 100 mg/ml concentration of the extract. At 50 µl per disc of 100 mg/ml concentration of the extract, the highest volume used, the ethanolic extract gave mean diameters of zones that ranged from 11.3 mm (for *S. aureus* ATCC) to 13.5 mm (for MRSA ST/0904-30).

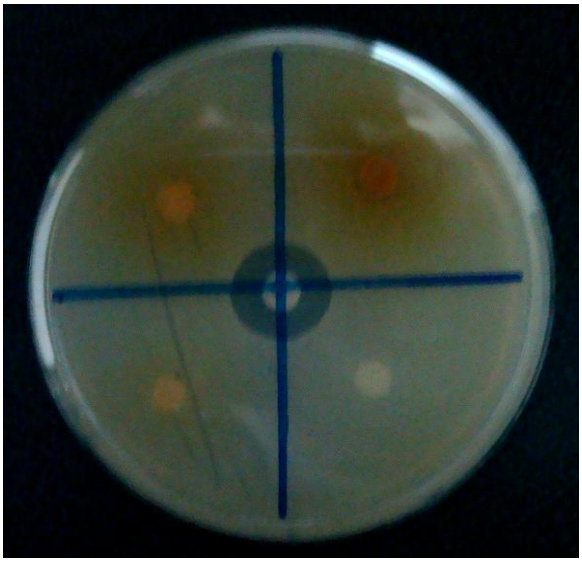

Figure 3: Plate showed the absence of any zone of inhibition by aqueous extract of *T. crispa*, and middle zone of inhibition given by vancomycin positive control.

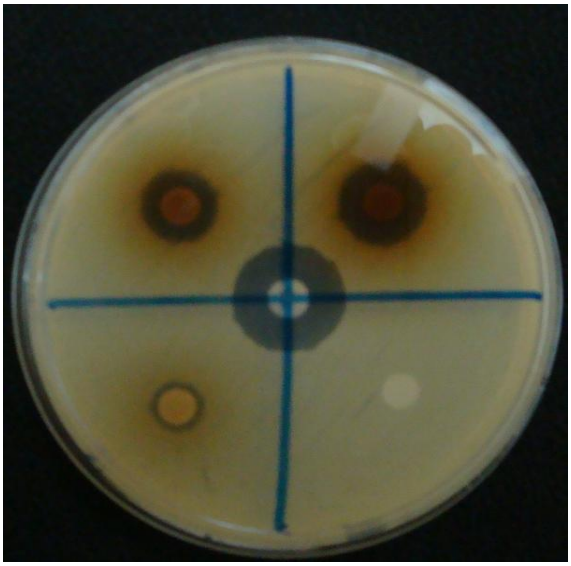

Figure 4: Plate showed clear, measureable inhibition zones of MRSA ST/0903-22 given by vancomycin positive control (middle zone) and discs loaded with different volumes of 100 mg/ml concentration of *T. crispa* ethanolic extract (top right: 50 µl; top left 30 µl; and bottom left: 10 µl of 100 mg/ml). The bottom right disc is the negative control.

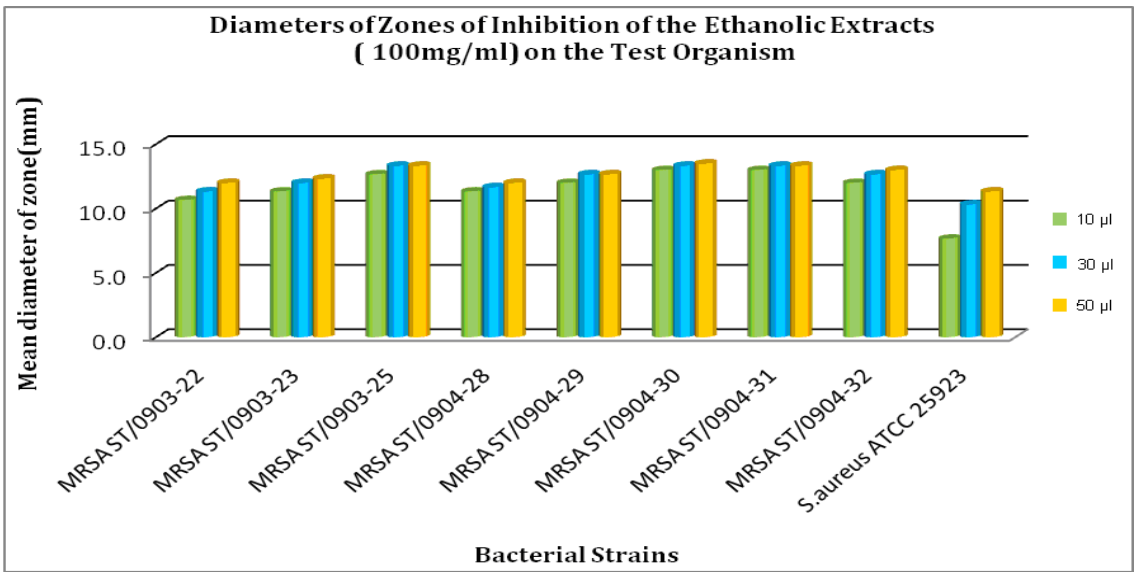

Figure 5: Mean diameters of zones of inhibitions where bacterial growth was inhibited by ethanol extract of *Tinospora crispa*

Mean values calculated from three replicate readings of the zones to the nearest millimetre (mm).

All –ST/09 strains denote MRSA strains. All tested at volume of 10 µl, 30 µl, and 50 µl per disc of 100 mg/ml concentration of the extract.

3.2.2 Minimum Inhibitory Concentration (MIC)

The bacterial strains showed measurable zones of inhibition to *Tinospora crispa* from the disc diffusion assay were further tested to determine their MIC values. Similarly, the extract which failed to inhibit the growth of bacteria was further tested to ensure that it has no antibacterial activity against tested organisms and to ensure the bacterial resistance as well. All MIC values obtained were tabulated in **(Table 1)** and a comparison of the results of each incubation time was analysed. From the results, the 48 hours incubation period gave lower MIC values in some strains for ethanol extract as shown in **(Figures 6).**

The result of the Minimum Inhibition Concentration (MIC) showed that the ethanolic extract exhibited varying inhibitory effects on the tested organisms. MRSA ST/0904-31 was the most susceptible strain to the ethanolic extract of *T. crispa*, unlike ST/0903-23, MRSA ST/0903-25 and MRSA ST/0904-32 which showed no variance for the two different periods (MIC 50.0 mg/ml). Furthermore, the extract inhibited the growth of four pure clinical isolate (MRSA ST/0903-22, MRSA ST/0904-28, MRSA ST/ 0904-29, MRSA ST/0904-30, MRSA ST/0904-31), and *S. aureus* ATCC 2592 with MIC 25.0 mg/ml after a 48 hour incubation period. **(Figure 6).**

In contrast, the results of the Minimum Inhibitory Concentration (MIC) showed that the aqueous extract of the plant failed to show inhibitory effect on majority of the test organisms at the test concentration used **(Table 1).**

Table 1: Minimum Inhibitory Concentration (MIC) of extract of *Tinospora crispa* on susceptible bacteria strain

Bacterial Name	24 hours [a]	48 hours [a]	24 hours [b]	48 hours [b]
MRSA ST/0903-22	50	25	-	-
MRSA ST/0903-23	50	50	-	-
MRSA ST/0903-25	50	50	-	-
MRSA ST/0904-28	50	25	-	-
MRSA ST/0904-29	50	25	-	-
MRSA ST/0904-30	50	25	-	-
MRSA ST/0904-31	25	25	-	-
MRSA ST/0904-32	50	50	-	-
S. aureus ATCC 25923	50	25	-	-

(-) No inhibition demonstrated,

[a] Ethanol extract of *T. crispa*

[b] Aqueous extract of *T. crispa*

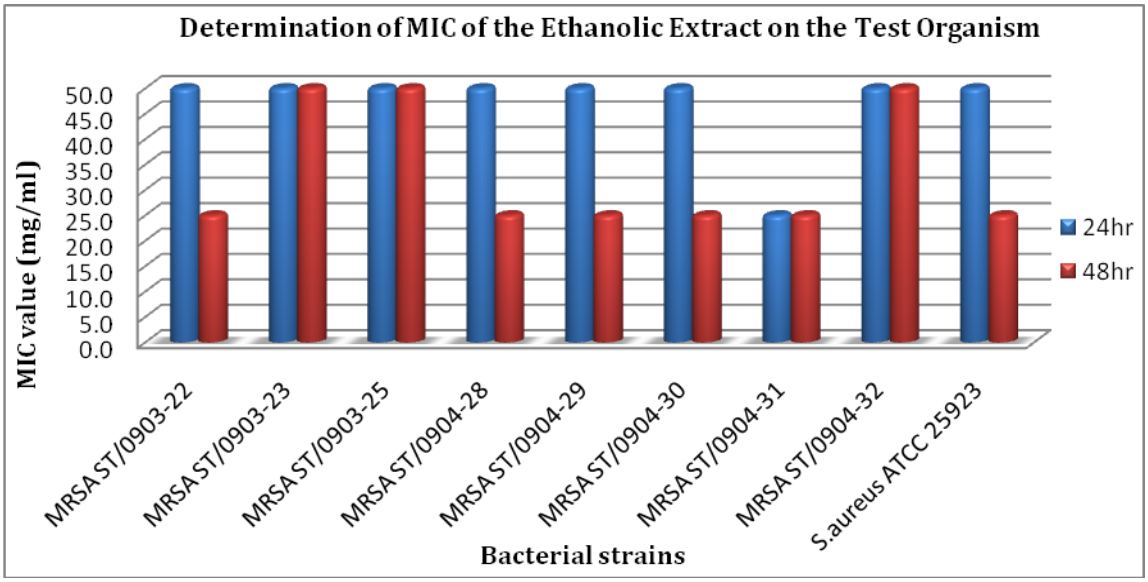

Figure 6: Effect of incubation period (24 and 48 hours) on *Tinospora crispa* ethanol extract by determination of MIC value. All –ST/09 strains denote MRSA strains

3.3 Antibacterial effects of several plant solvents

Several plant solvents were examined on tested organisms to ensure the absence of antibacterial effects by using set-up procedures similar to that of the MIC assay, at cell counts of approximately 5 x 10^5 CFU/ml. As a result, sterile distilled water and 10% (v/v) Tween 20 did not inhibit the growth of bacteria after 24 and 48 hours of incubation. However, the growth of the tested organisms was completely inhibited in all tubes by 99.9 % DMSO and by absolute ethanol as well.

3.4 Acute toxicity study

3.4.1 Mortality and behavioural changes

Within four hours after administration of the water and ethanolic extracts, no mortality occurred in all group. However, mortality occurred in all rats of 4 g/kg group after 72 hours administration of the aqueous extract and one male in 2 g/kg group after 96 hours administration of the aqueous extract **(Table 2)**. In addition, no mortality occurred in all other rats until the scheduled termination. No behavioural evidence of sign of toxicity was observed throughout the study period in all groups.

Table 2: Occurrence of mortality in acute toxicity study of *Tinospora crispa* extract

Dose	Extract type of *Tinospora crispa*	
	Aqueous	Ethanol
4 g/kg	**6/6***	0/6
2 g/kg	**1/6***	0/6
Vehicle	0/6	0/6

*Number of animals showing mortality after 72 and 96 hours of extract administration

3.4.2 Analysis of body weight

The changes of body weight of animals after the 14 days were recorded. **Figure 7 and 8** illustrated that there was no significant observed in any of rats groups when compared to their respective vehicle groups.

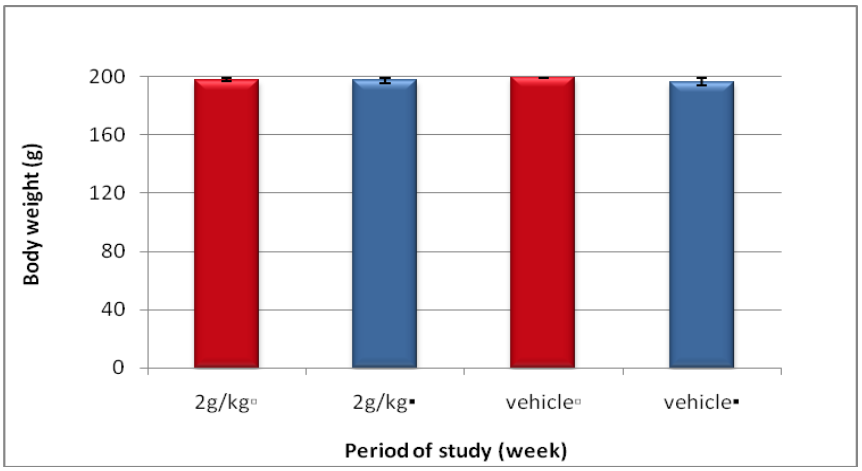

Figure 7: Mean body weights of rats tested with aqueous extract of *Tinospora crispa.*

▫Body weight at first day of experiment.

▪Body weight on 15th day of experiment.

No significant weight changes were observed in all groups.

Values are expressed as mean ± S.E.M.

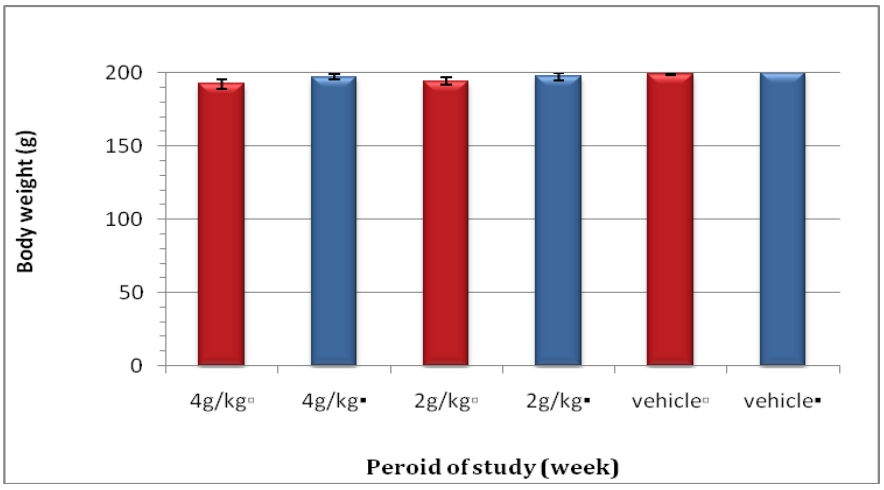

Figure 8: Mean body weights of rats tested with ethanol extract of *Tinospora crispa.*

▫Body weight at first day of experiment.

▪Body weight on 15th day of experiment.

No significant weight changes were observed in all groups.

Values are expressed as mean ± S.E.M.

3.4.3 Liver and renal function analysis

The parameter of liver function that had been tested was serum total protein, albumin, globulin, alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatise. The levels of each of these enzymes were analysed for all groups and thereafter indications of liver function were compared to their vehicle groups. Creatinine and urea levels of all groups were determined as markers of kidney function.

In rat groups given the aqueous extract of *Tinospora crispa*, no significant increases were observed in levels of all tested parameters in all groups (**Figures 9** to **11**). Likewise, Levels of all tested parameters were not significantly increased between males or females groups as compared to the control groups. However, all rat groups given extract at high dose of 4g/kg died within 72 hours after administration.

Also, in rat groups given the ethanol extracts instead, no significant changes were found in all tested parameters levels of all rat groups as compared to their respective vehicle groups (**Figures 12** to **14**). Moreover, there was no a significant decrease or increase in levels of all parameters of male and as well as female groups in both doses.

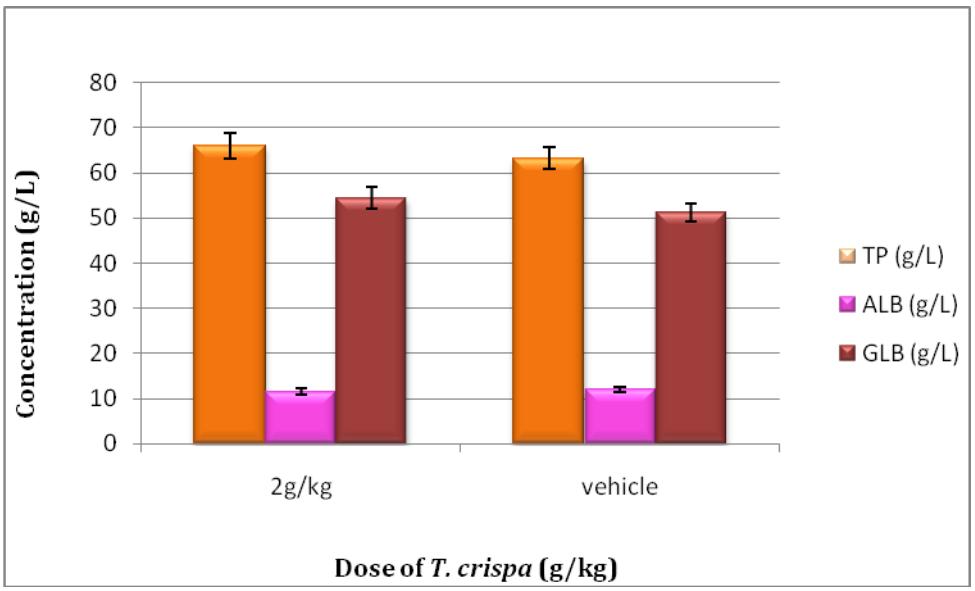

Figure 9: Total protein (TP), albumin (ALB), and globulin (GLB) levels in rats treated with aqueous extract of *T. crispa*

Values are expressed as mean ± S.E.M.

No significant difference as compared to vehicle group ($p<0.05$)

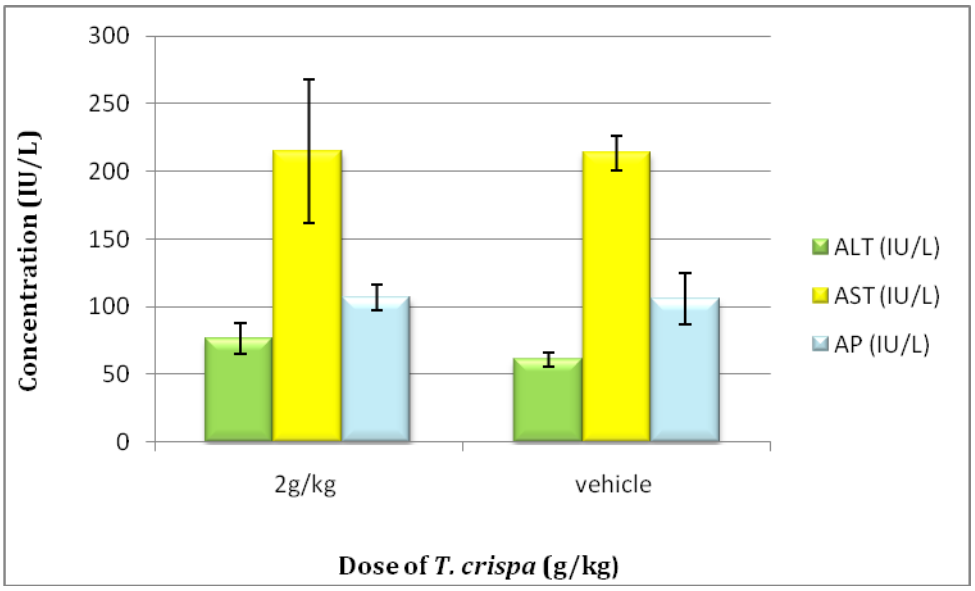

Figure 10: Levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (AP) in rats treated with aqueous extract of *T. crispa*

Values are expressed as mean ± S.E.M.

No significant difference as compared to vehicle group ($p<0.05$)

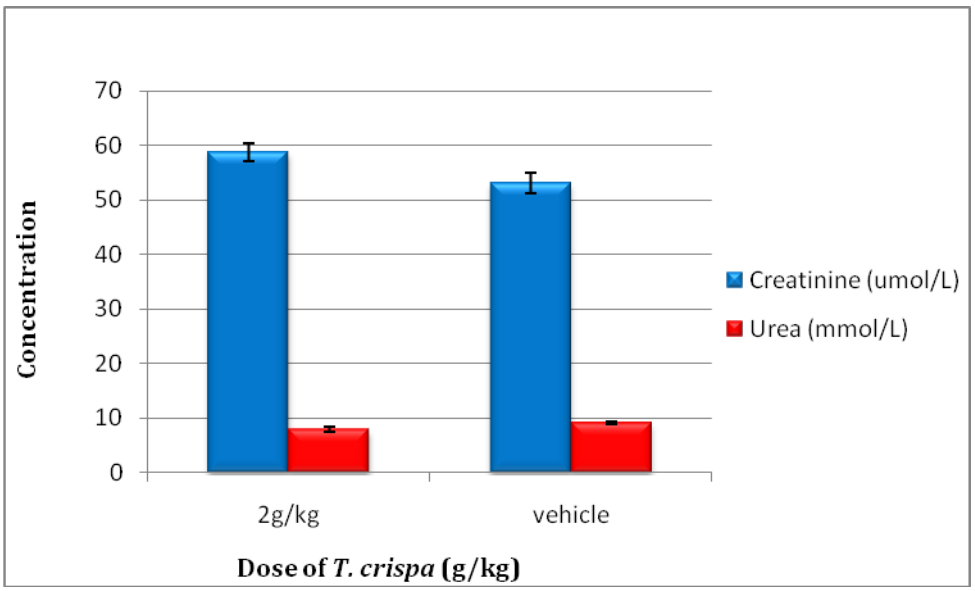

Figure 11: Creatinine and urea levels in rats treated with aqueous extract of *T. crispa*

Values are expressed as mean ± S.E.M.

No significant difference as compared to vehicle group ($p<0.05$)

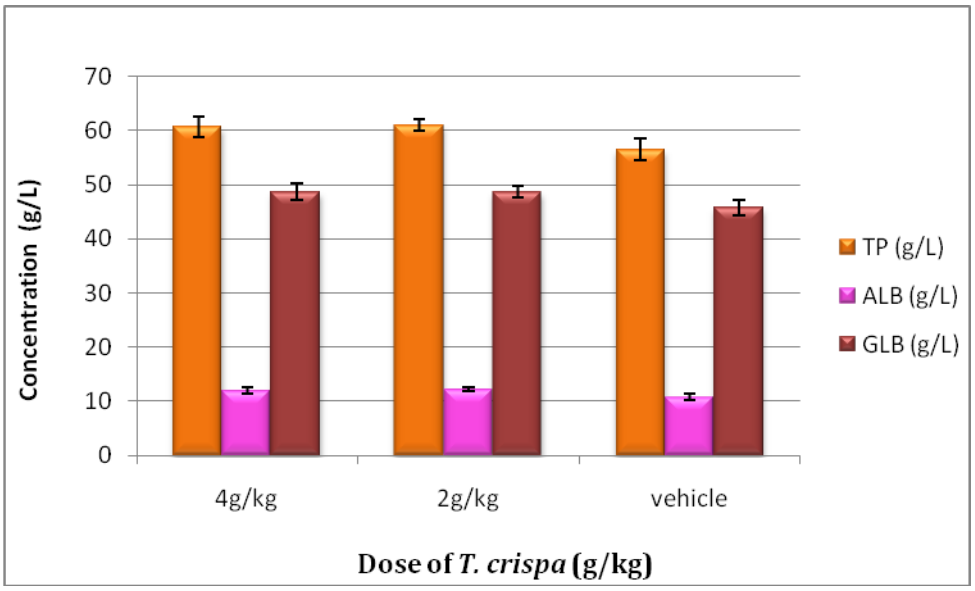

Figure 12: Total protein (TP), albumin (ALB), and globulin (GLB) levels in rats treated with ethanolic extract of *T. crispa*

Values are expressed as mean ± S.E.M.

No significant difference as compared to vehicle group ($p<0.05$)

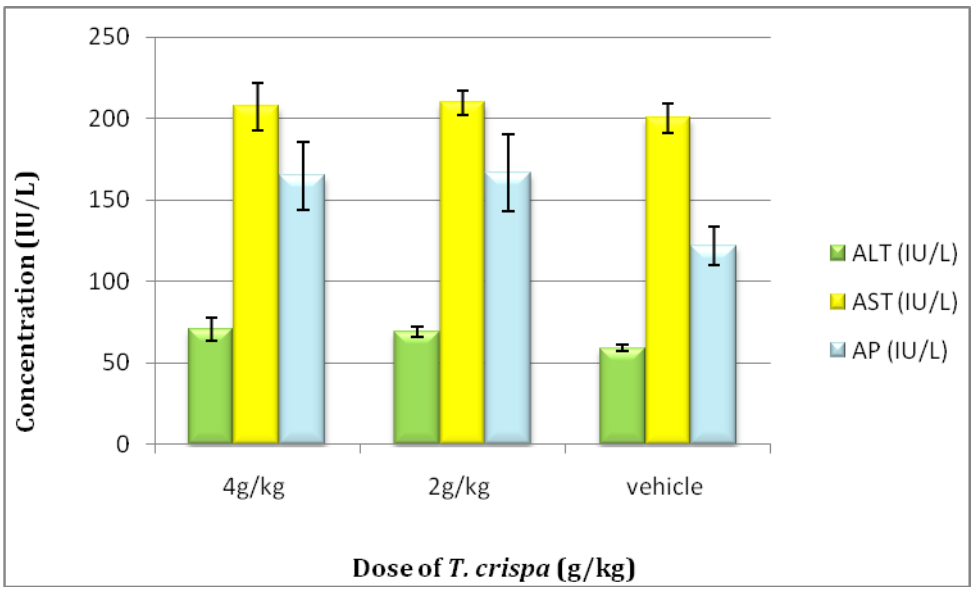

Figure 13: Levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (AP) in rats treated with aqueous extract of *T. crispa*
Values are expressed as mean ± S.E.M.
No significant difference as compared to vehicle group ($p<0.05$)

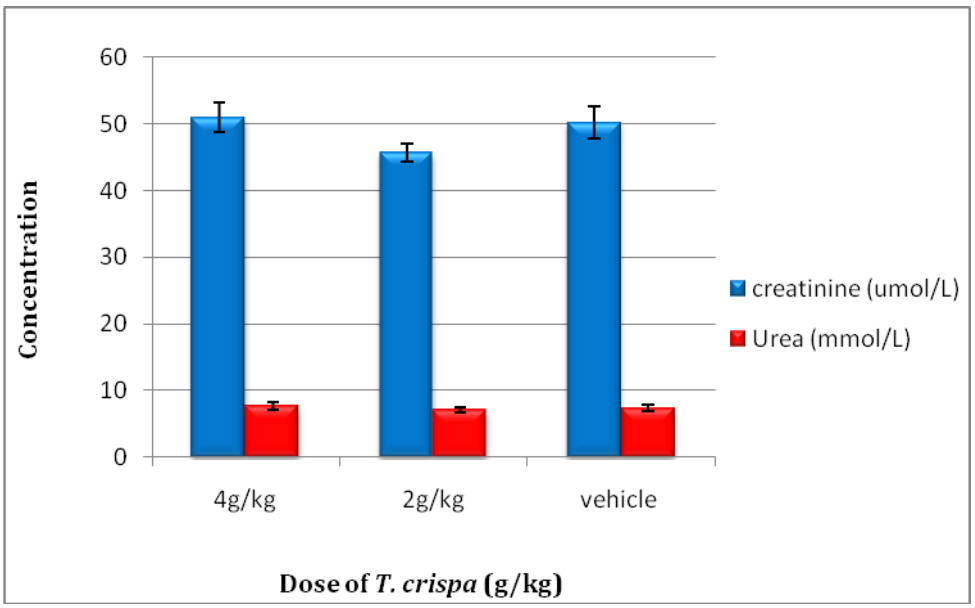

Figure 14: Creatinine and urea levels in rats treated with ethanolic extract of *T. crispa*
Values are expressed as mean ± S.E.M.
No significant difference as compared to vehicle group ($p<0.05$)

3.4.4 Gross necropsy and histology

Gross necropsy was performed before the livers and kidneys were excised. No gross pathological changes were observed in the organs of all animals. Generally, in histological examination, livers and kidneys of most dosage group animals exhibited normal architecture with the absence of pathological lesions. Liver lobules showed uninucleated and binucleated hepatocytes that radiate from the central vein to the periphery (**Figure 15 to 17**). Kidneys showed normal, distinct glomeruli and renal tubules (**Figure 18 to 20**). On the other hand, some of examined organs showed slightly changes in their architecture but were not significant when compared to their vehicle groups.

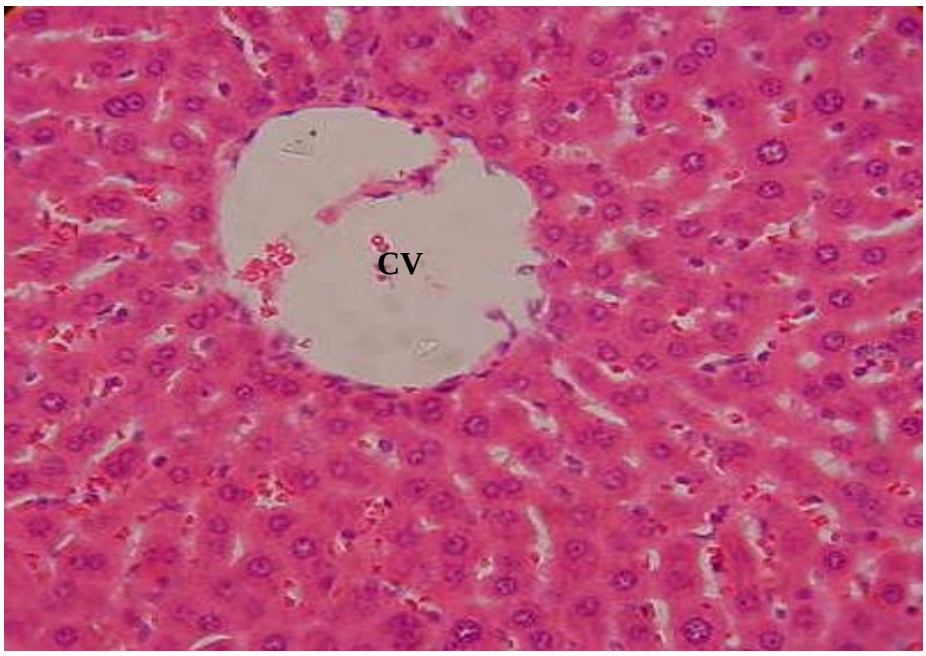

Figure 15: Histological section of liver parenchyma in rats treated with 10% (v/v) Tween 20 (vehicle control) showed normal architecture and no toxic effect to hepatic cells (H & E stain 40x). Microphotograph shows: central vein (CV).

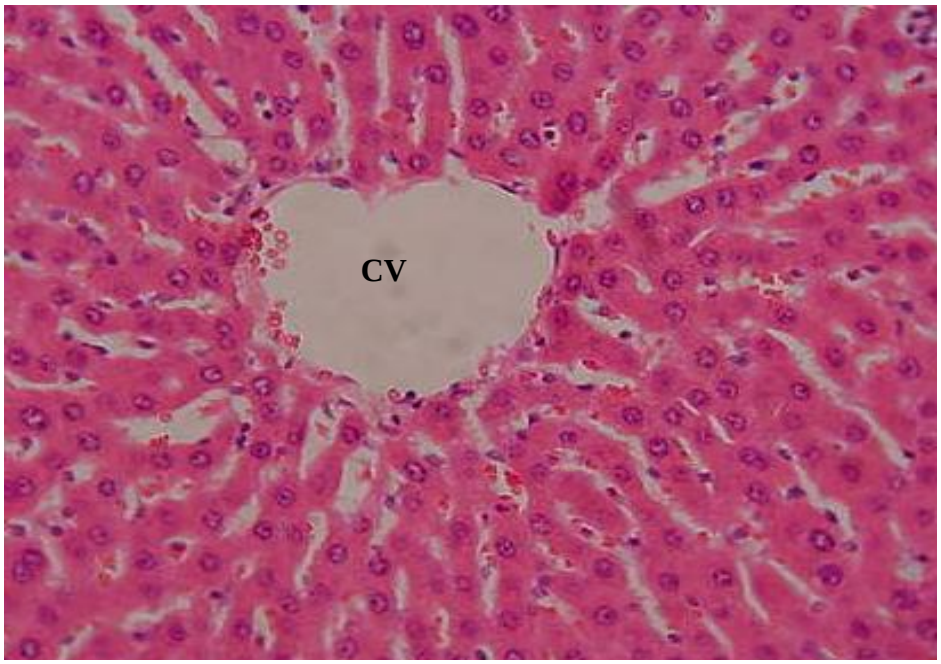

Figure 16: Histological section of liver parenchyma in rats treated with low dose aqueous extract of *T. crispa* (2 g/kg) showed normal architecture and no toxic effects to hepatic cells (H & E stain 40x). Microphotograph shows: central vein (CV).

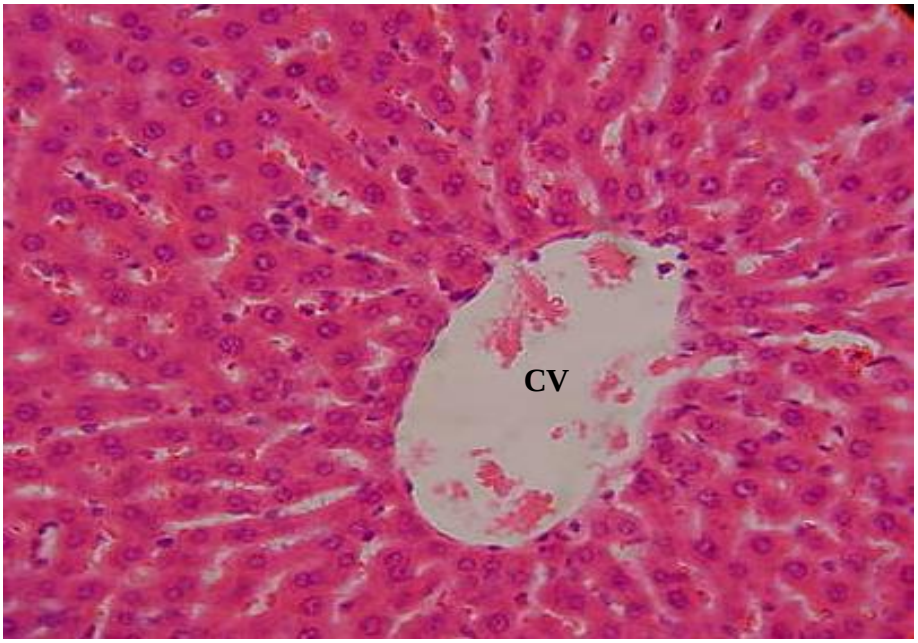

Figure 17: Histological section of liver parenchyma in rats treated with high dose ethanol extract of *T. crispa* (4 g/kg) showed normal architecture and no toxic effects to hepatic cells (H & E stain 40x). Microphotograph shows: central vein (CV).

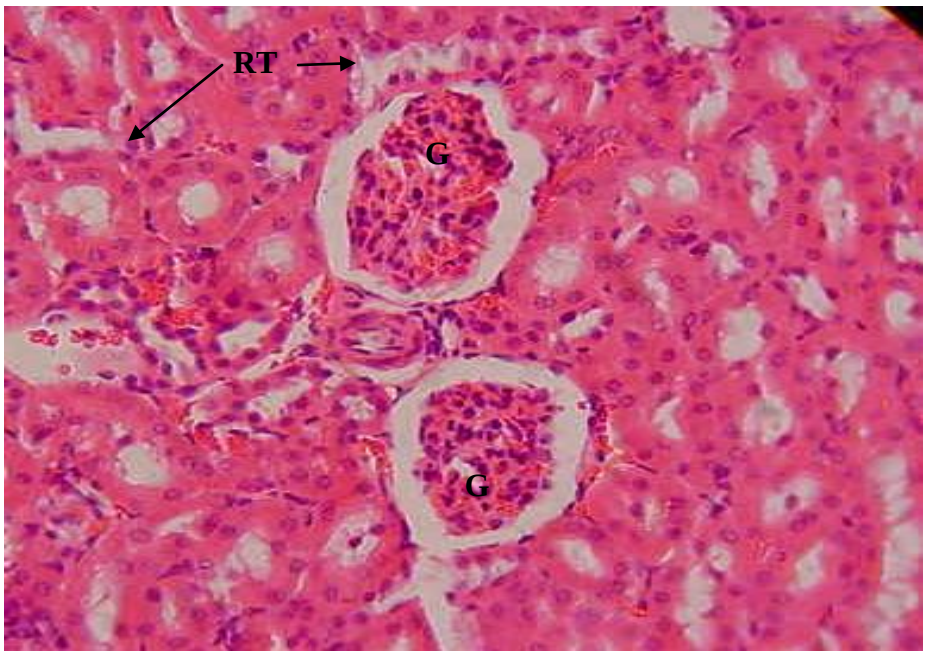

Figure 18: Histological section of kidney in rats treated with 10% (v/v) Tween 20 (vehicle control) showed normal tissue structure and no toxic effect to kidney cells (H & E stain 40x). Microphotograph shows: renal tubules (RT) and glomeruli (G).

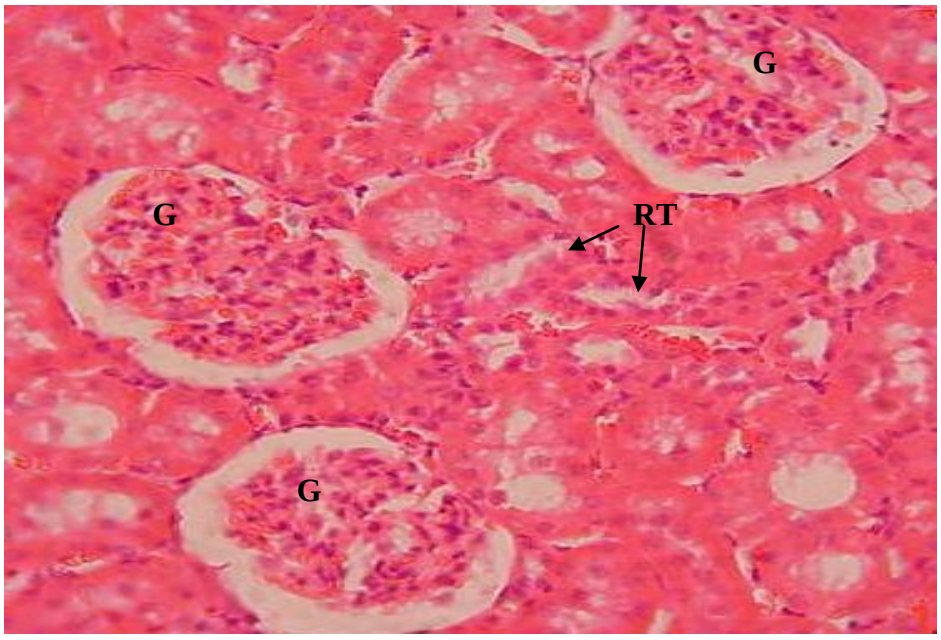

Figure 19: Histological section of kidney in rats treated with low dose of aqueous extract of *T. crispa* (2 g/kg) showed normal tissue structure and no toxic effect to kidney cells (H & E stain 40x). Microphotograph shows: renal tubules (RT) and glomeruli (G).

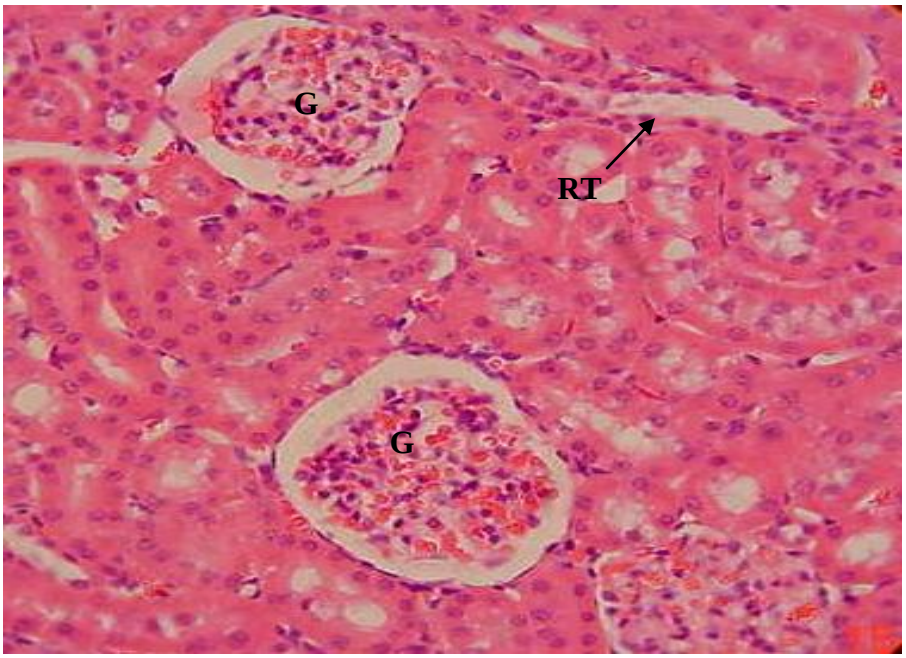

Figure 20: Histological section of kidney in rats treated with high dose of ethanol extract of *T. crispa* (4 g/kg) showed normal tissue structure and no toxic effect to kidney cells (H & E stain 40x). Microphotograph shows: renal tubules (RT) and glomeruli (G).