7.0 APPENDICES

APPENDIX A: Gram staining protocol

1. Smear the bacteria on glass slide.
2. Flood the slide with crystal violet solution for 1 minute, and then drain solution off with distilled water.
3. Flood slide with Lugol's iodine for 1 minute, and then drain solution off.
4. Rinse slide with acetone (decolourizer) for several seconds.
5. Drain solution thoroughly with distilled water until the excess colour goes off.
6. Flood slide with safranine for 1-2 minutes, and then wash off the stain with distilled water.
7. Leave slide to air-dry.
8. Observe slide microscopically under oil immersion.

APPENDIX B: Histology protocol

1. Specimen preparation
2. Automated Tissue Processing
3. Tissue blocking
4. Sectioning
5. Staining
6. Mounting

1. Specimen preparation

- Label biopsy tissue properly
- Fix organs (kidney and liver) by placing in 10% neutral buffered formalin solution immediately after excision for approximately 6 hours.
- Next, trim the tissues into small size and place in labelled cassettes.
- Then, place the cassettes in the 10% neutral buffered formalin again for further fixation overnight before processing.

2. Automated tissue processing (Leica TP1020)

Process	Solution	Period (hour)
Fixation	10% formalin I	1
	10% formalin II	1
Dehydration	70% ethanol	1
	95% ethanol I	1
	95% ethanol II	1
	95% ethanol III	1
	Absolute ethanol I	1
	Absolute ethanol II	1 ½
Cleaning	Alcohol : xylene (1:1)	1
	Xylene I	1 ½
	Xylene II	1 ½
Infiltration	Paraffin wax I	1 ½
	Paraffin wax II	1 ½

3. Tissue embedding (Leica HISTOEMBEDDER)

- Place and orientate the tissue in mould.
- Pour paraffin wax into mould.
- Place mould in cold plate area until paraffin wax solidifies
- Pry tissue block off of mould for sectioning.

4. Sectioning (Leica RM 2135)

- Section the tissue blocking by using a rotary microtome at a thickness of 5μm
- Place ribbon sections in water bath.
- Pick two best sections from water bath with separate, clean slides.
- Adherence the sections by placing glass slide on hot plate.

5. Staining

a. Bring section to water:

Process	Solution	Period (minutes)
Dewaxing	Xylene I	3
	Xylene II	3
Rehydration	Absolute alcohol	2
	95% alcohol I	2
	95% alconol II	2
	70% alconol	2
Bring section to water	Running tap water	3

b. Staining with Haematoxylin & Eosin:

Process	Solution	Period
Staining	Haematoxylin stain	10 minutes
	Running tap water	until excess colour goes off
Differentiation	0.5% acid alcohol	2-3 dips
	Running tap water	2-3 minutes
	2% sodium acetate	2 seconds
	Running tap water	2-3 minutes
	80% alcohol	2-3 dips
Staining	Eosin stain	5 minutes
Dehydration	95% alcohol I	5 seconds
	95% alcohol II	2 minutes
	Absolute ethanol I	2 minutes
	Absolute ethanol II	2 minutes
Cleaning	Xylene I	2 minutes
	Xylene II	2 minutes
	Xylene III	2 minutes

6. Mounting with DPX

- Mount slides with DPX mounting media and cover the section with cover slip.
- Wipe slide to remove excess xylene and then observed under light microscope**.**

APPENDIX C: Data tables

Table (i) Mean diameters of zone in agar plate against nine bacteria strains by ethanolic extract of *Tinospora crispa*

Bacteria strain	Mean diameters (mm)[a] of inhibition zones in			
	All used 10, 30, and 50 µl of 100 mg/ml			
	10µl	30µl	50µl	Vancomycin (30µl) [b]
MRSA ST/0903-22	10.7	11.3	12	19.3
MRSA ST/0903-23	11.3	12.0	12.3	19.3
MRSA ST/0903-25	12.7	13.3	13.3	20
MRSA ST/0904-28	11.3	11.7	12	17.6
MRSA ST/0904-29	12.0	12.7	12.7	18
MRSA ST/0904-30	13.0	13.3	13.5	19.6
MRSA ST/0904-31	13.0	13.3	13.3	20
MRSA ST/0904-32	12.0	12.7	13	19.3
S.aureus ATCC 25923	7.7	10.3	11.3	18.3

(-) Indicate absence of any observable inhibition zone.

[a] Values of each represent the mean of three replicates.

[b] Zone diameters of positive controls.

DMSO was used as a negative control.

Table (ii) Body weights of rats in acute toxicity of aqueous extract of *Tinospora crispa*

Dose	Body weight (g) day 0	Body weight (g) day 15
4g/kg	198.00 ± 1.23	-
2g/kg	197.80 ± 1.01	197.40 ± 1.66 [a]
Vehicle	199.50 ± 0.50	196.50 ± 2.36

Values expressed as mean ± S.E.M., n = 6, [a] n = 5

No significant body weight changes observed in all rats groups

(-) No value due to the death of rats of this group within 72 hours

Table (iii) Body weights of rats in acute toxicity of ethanolic extract of *Tinospora crispa*

Dose	Body weight (g) day 0	Body weight (g) day 15
4g/kg	192.33 ± 3.06	197.16 ± 1.66
2g/kg	194.33 ± 2.59	197.33 ± 2.66
Vehicle	199 ± 0.70	199.75 ± 0.25

Values expressed as mean ± S.E.M., n = 6

No significant body weight changes observed in all rats groups

Table (iv) Liver function analysis of rats in acute toxicity study of aqueous extract of *Tinospora crispa*

Dose	TP (g/L)	ALB (g/L)	GLB (g/L)	ALT (IU/L)	AST (IU/L)	AP (IU/L)
4g/kg	-	-	-	-	-	-
2g/kg [a]	66.00 ± 2. 82	11.60 ± 0.67	54.40 ± 2.37	76.60 ± 11.66	215.00 ± 53.11	106.80 ± 9.51
Vehicle	63.28 ± 2.49	12.00 ± 0.57	51.25 ± 2.09	60.75 ± 5.17	213.75 ± 12.74	106.00 ± 19.10

Values expressed as mean ± S.E.M., n = 6, [a] n = 5

Parameters tested: TP: total protein; ALB: albumin; GLB: globulin; ALT: alanine aminotransferase; AST: aspartate aminotransferase and AP: alkaline phosphatise

(-) No value due to the death of rats of this group within 72 hours

Table (v) Liver function analysis of rats in acute toxicity study of ethanolic extract of *Tinospora crispa*

Dose	TP (g/L)	ALP (g/L)	GLB (g/L)	ALT (IU/L)	AST (IU/L)	AP (IU/L)
4g/kg	60.67 ± 1.80	12.00 ± 0.51	48.66 ± 1.47	70.50 ± 6.86	207.50 ± 14.63	164.66 ± 21.11
2g/kg	61.00 ± 1.12	12.33 ± 0.81	48.66 ± 0.98	69.16 ± 3.23	209.66 ± 7.25	166.50 ± 23.53
Vehicle	56.50 ± 1.93	10.75 ± 0.62	45.75 ± 1.43	59.00 ± 2.08	200.25 ± 9.25	121.75 ± 11.77

Values expressed as mean ± S.E.M., n = 6

Parameters tested: TP: total protein; ALB: albumin; GLB: globulin; ALT: alanine aminotransferase; AST: aspartate aminotransferase and AP: alkaline phosphatise

Table (vi) Renal function analysis of rats in acute toxicity study of *Tinospora crispa* extracts

Dose	Creatinine (umol/L)	Urea (mmol/L)
4g/kg[b]	-	-
2g/kg[b]	71.60 ± 12.93[a]	7.90 ± 0.51[a]
Vehicle	53.00 ± 1.87	9.05 ± 0.22
4g/kg[c]	51.00 ± 2.16	7.70 ± 0.62
2g/kg[c]	45.66 ± 1.35	7.10 ± 0.40
Vehicle	50.25 ± 2.42	7.35 ± 0.54

Values expressed as mean ± S.E.M., n = 6, [a] n = 5

[b] Aqueous extract of *Tinospora crispa*

[c] Ethanolic extract of *Tinospora crispa*

No significant body weight changes observed in all rats groups

(-) No value due to the death of rats of this group within 72 hours