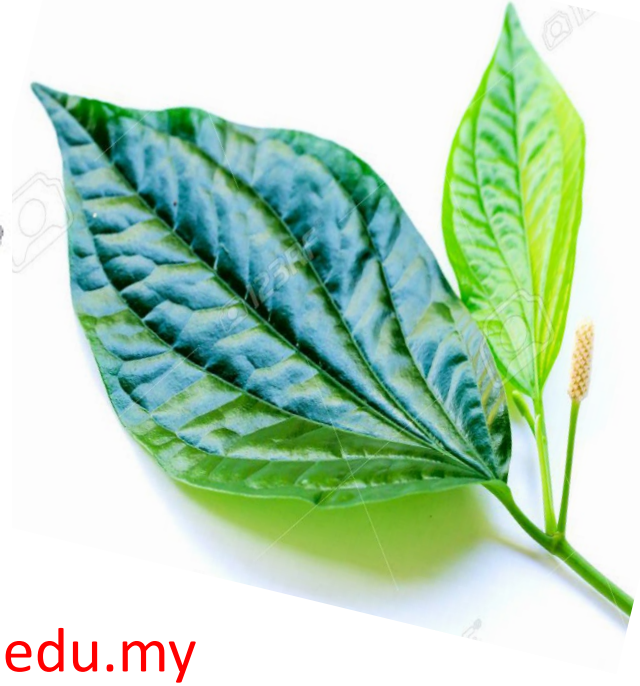
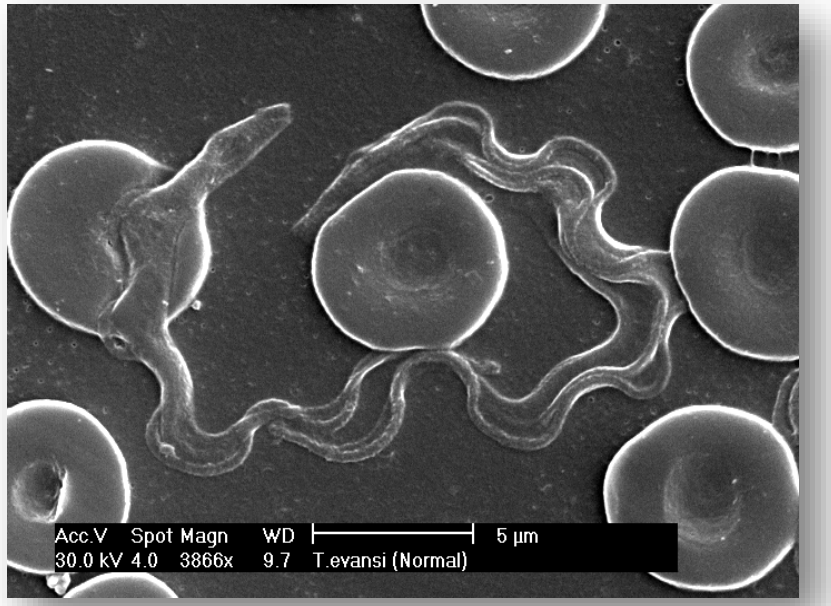
Empowering Light and Electron Microscopic Approach Towards Promising In-vivo Anti-Trypanosomal Activity of *Piper sarmentosum* Leaf





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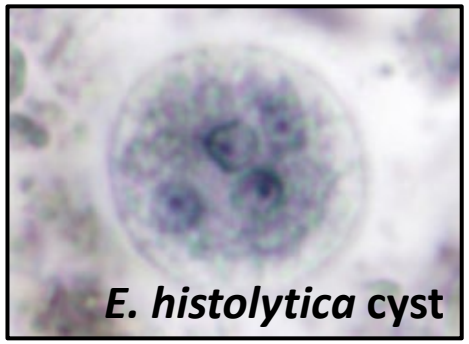
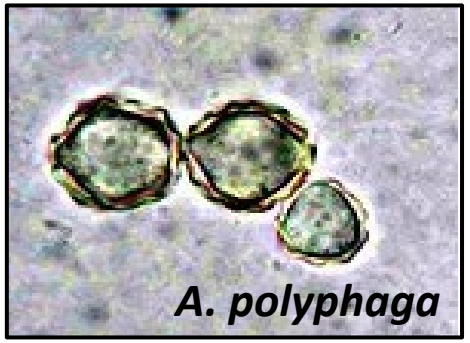
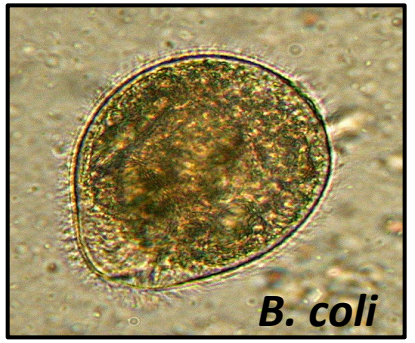
Light Microscope (LM) in Parasitology Research

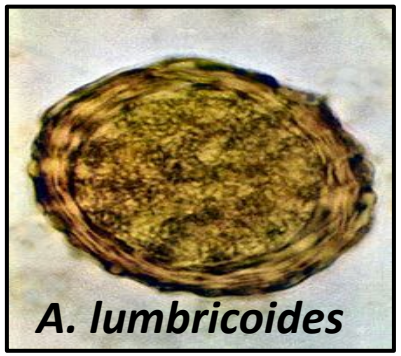
- As quantitative method to measure or detect the presence of parasite
- Largely applied to determine a various group of parasite in same specimen (eg: protozoa, helminthes and cilliates in stool sample)
- Purpose of applying LM technique for unstained parasite:
  - Observation of parasite's movement or mobility
  - Detection of special external structures on parasite cells
  - Unique morphology to determine the genus or perhaps the species
- Purpose of applying LM technique for stained parasite:
  - Differentiate between parasite's and host's cell structure
  - Some parasite organelles are colourable to particular dye
  - Determine the final stage of parasite development or survival

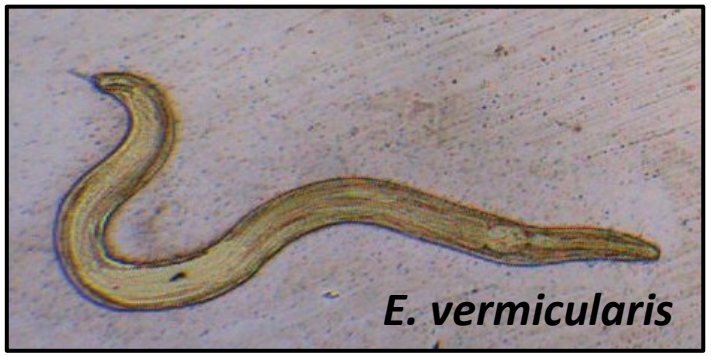
LM Observation of Unstained Parasites

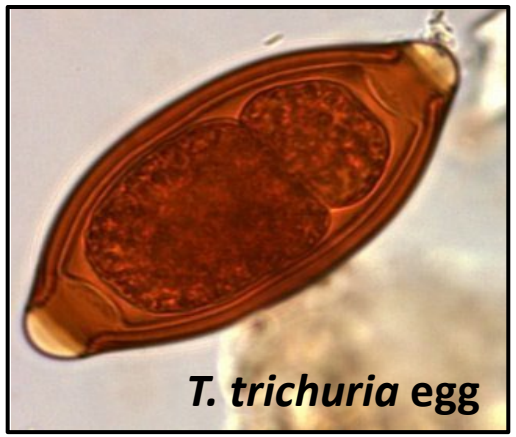




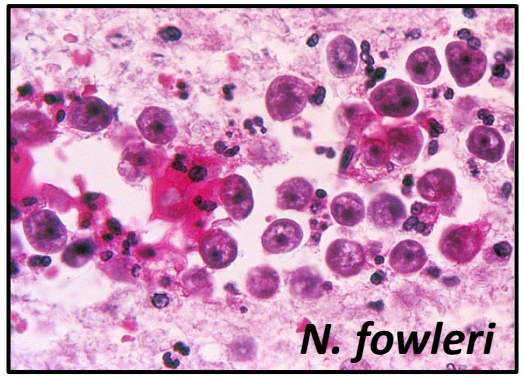


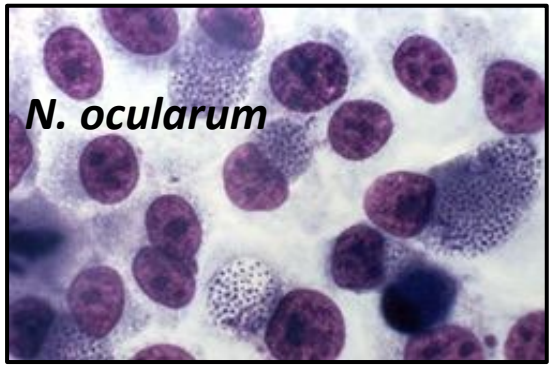


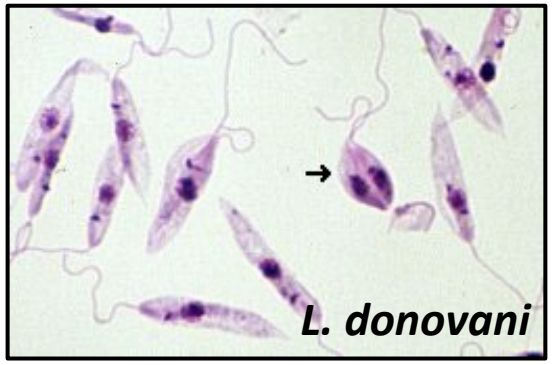


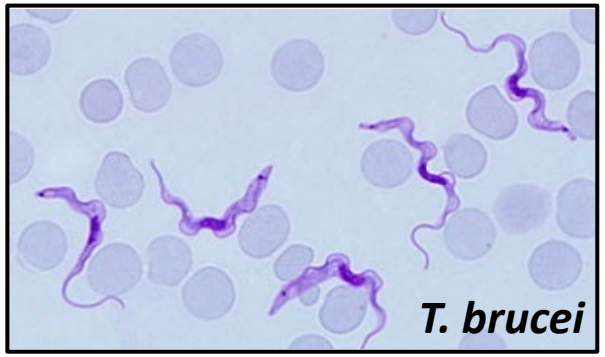


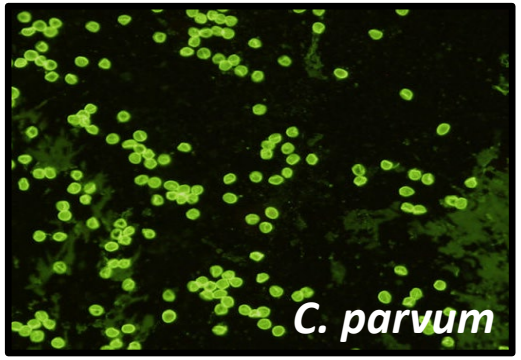
LM Observation of Stained Parasites

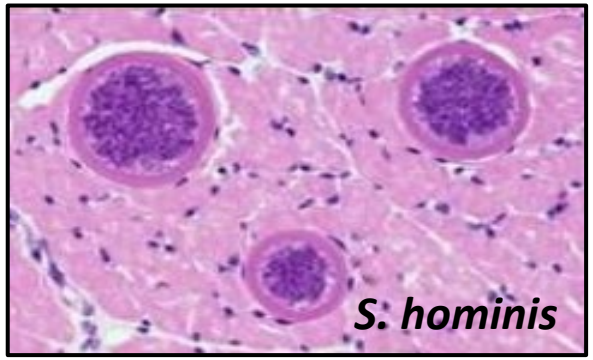


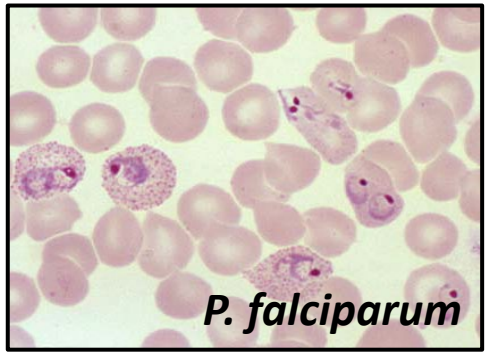


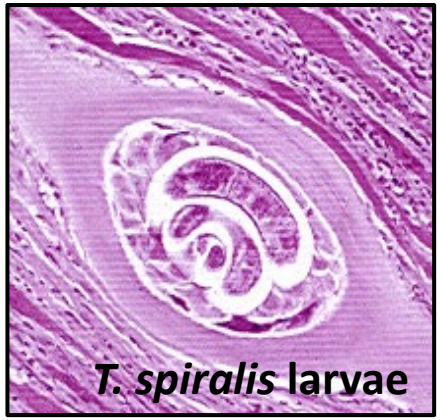


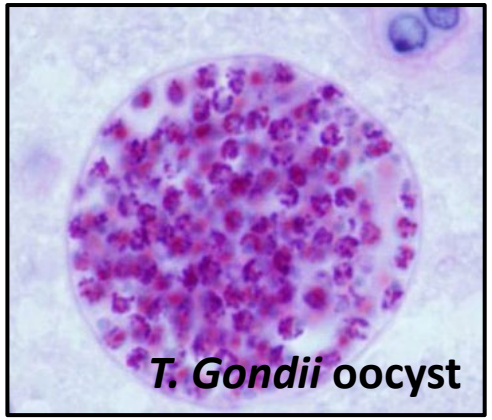


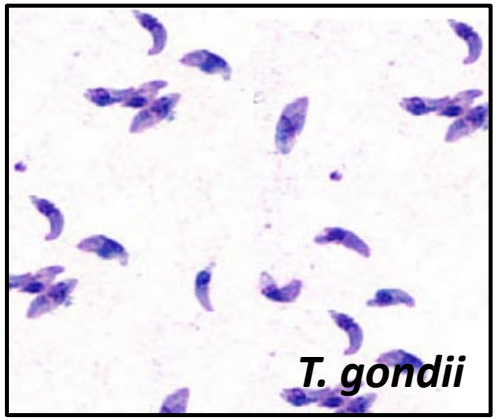


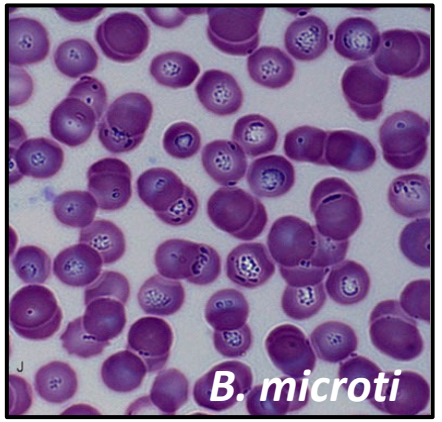








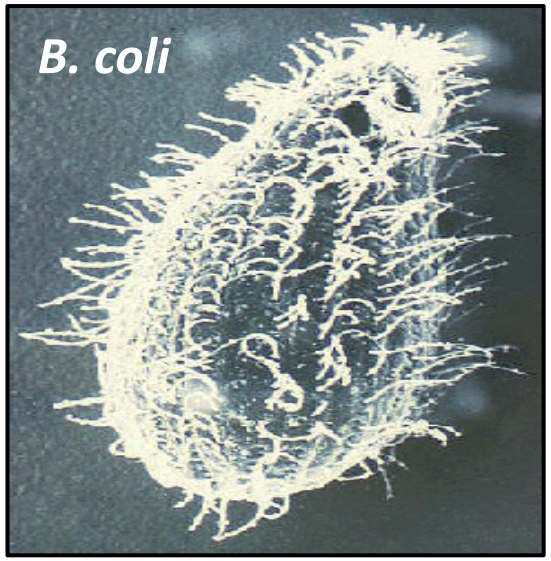


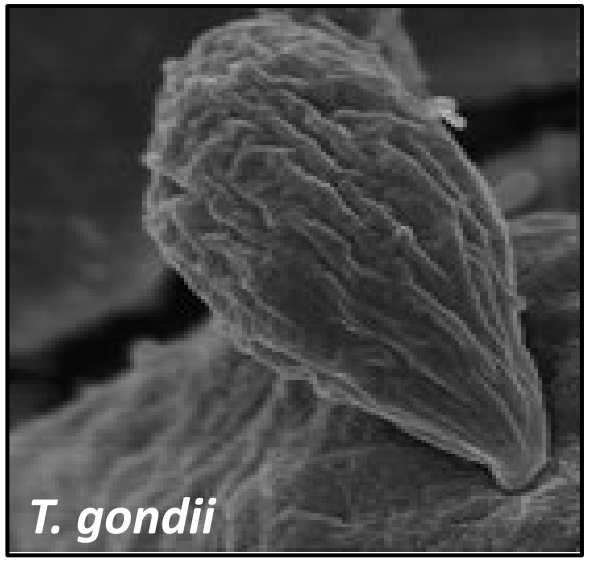


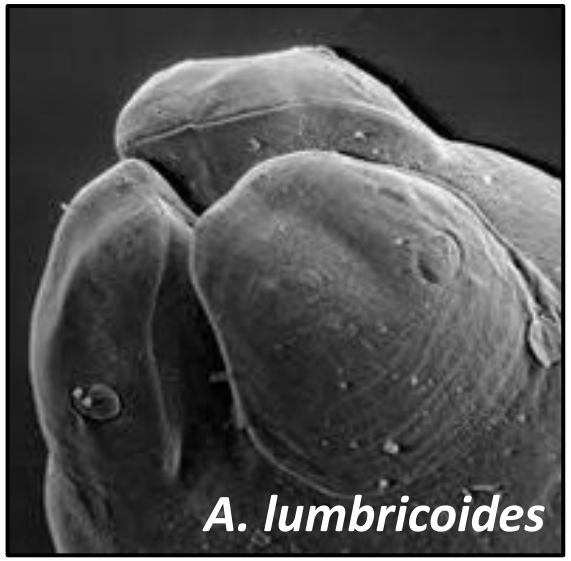
Electron Microscope in Parasitology Research

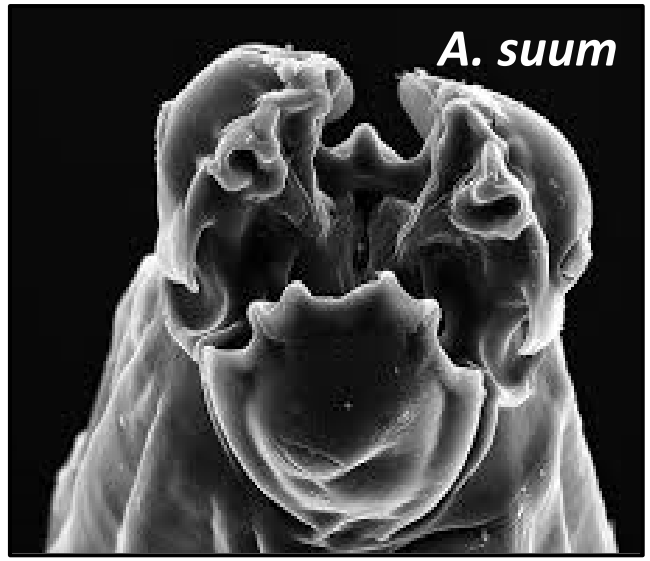
- As qualitative and descriptive analysis to support LM evidences
- Purpose of applying SEM technique:
  - Reveal interactions at the ultrastructural level of parasite
  - Investigate the different steps of parasite-host cell interaction
  - Determine the morphological changes of parasite and host's tissue
- Purpose of applying TEM technique:
  - Determine the mechanism of actions of parasite's infectivity
  - Observe multidimensional view of the biological processes during parasite development in the host.
  - Investigate individual events in individual cells by using different dye for organelles labelling via fluorescence microscopic observation

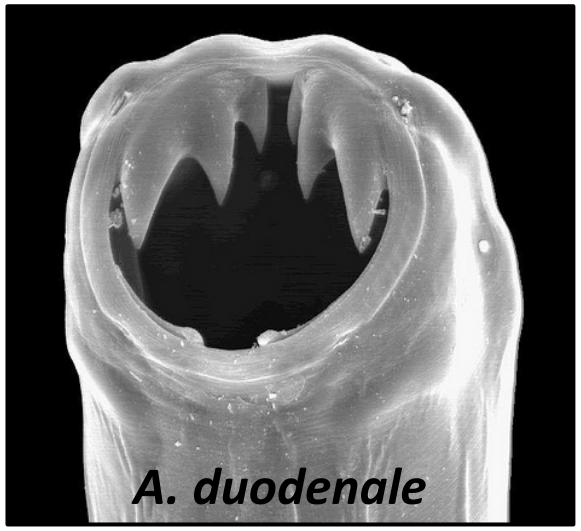
SEM Observation of Parasite

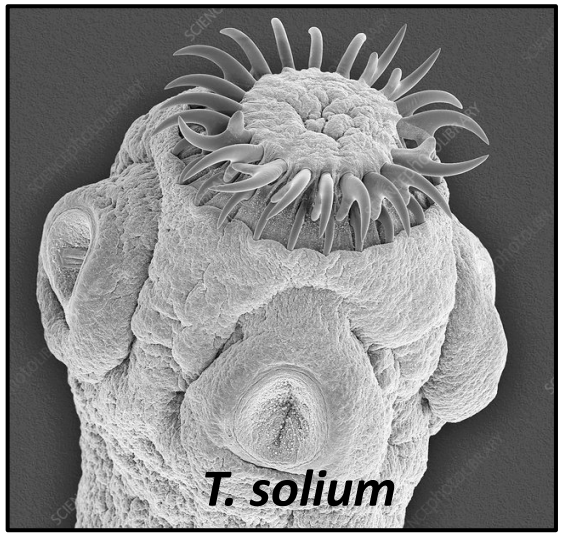


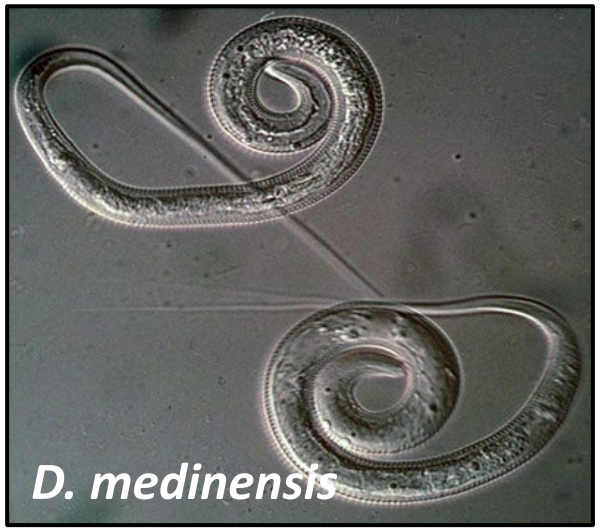


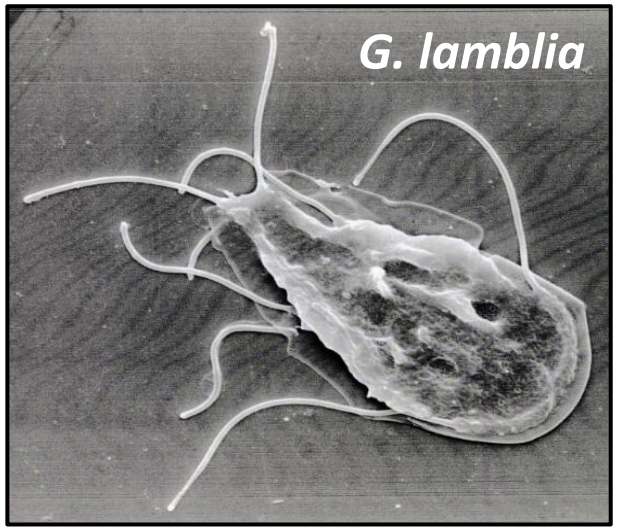




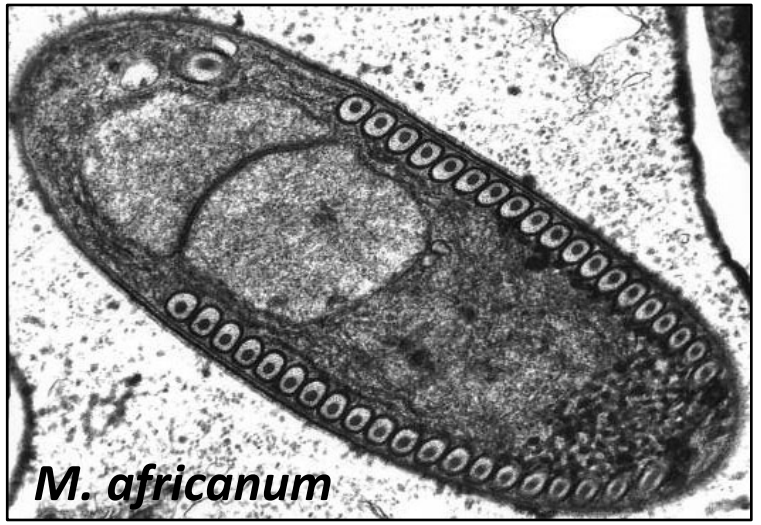


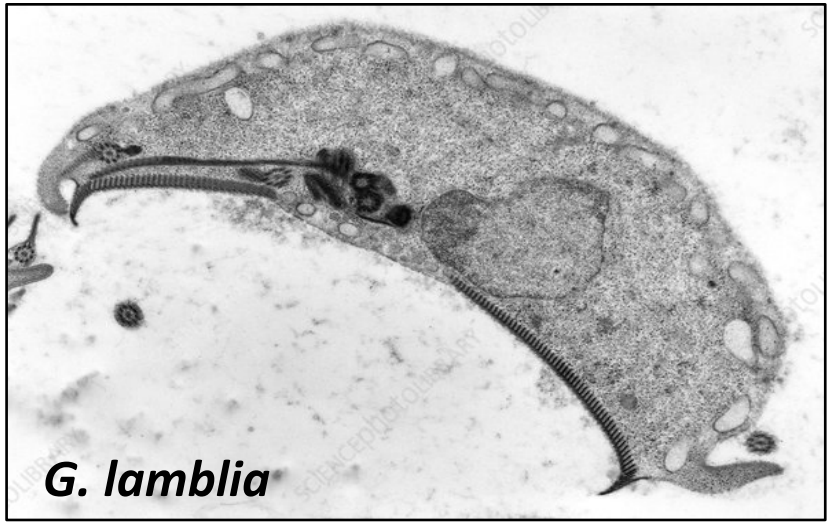


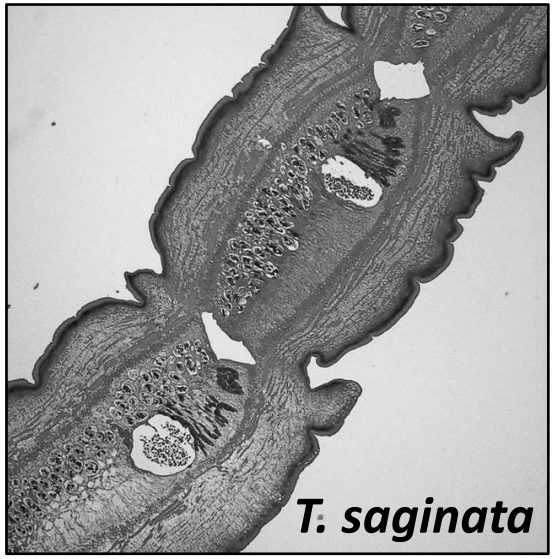


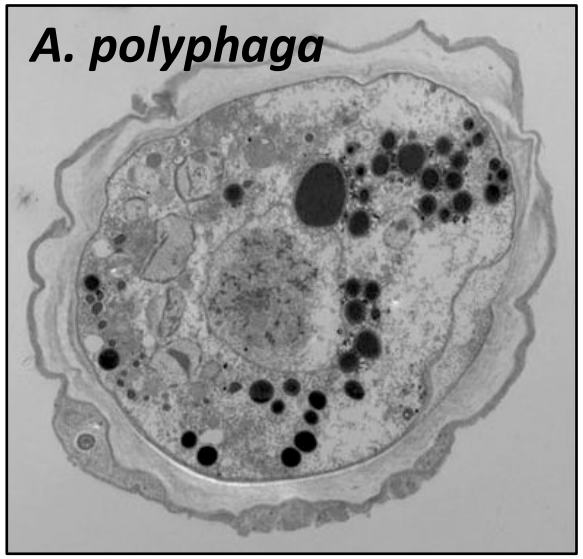


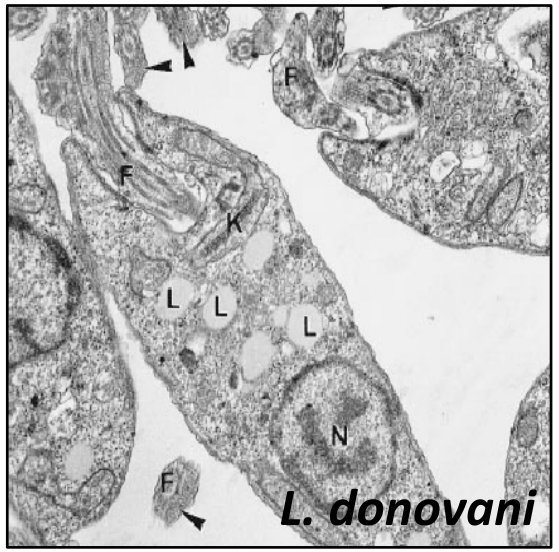
TEM Observation of Parasite

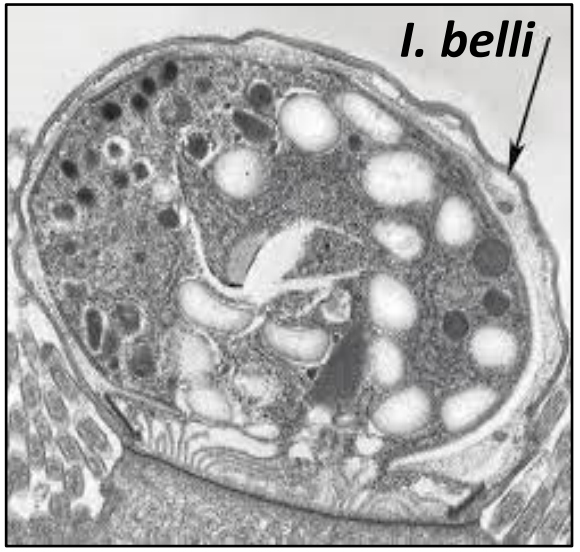








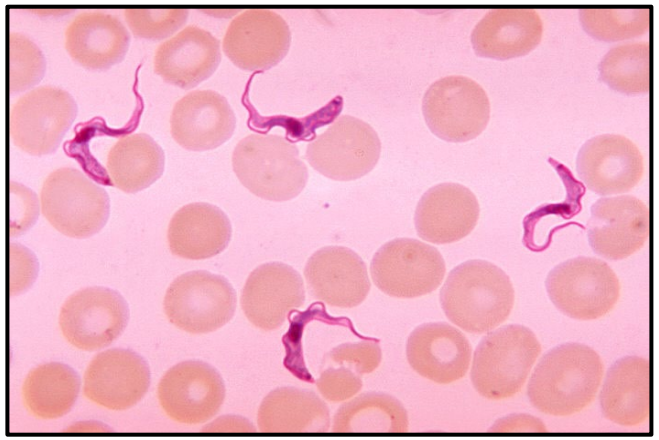




Trypanosoma evansi

- First discovered in in Punjab, India (1880), → haemoflagellated protozoa in both human and animals
- Caused atypical human trypanosomiasis (AHT) in human and Surra disease in mammals (mostly livestock)
- Zoonotic vector-borne disease → wide variety of vectors → worldwide
- Drug resistant in some regions → suramin, pentamidine, berenil
- Trans-host boundary : animal → human (first discovered in Assam, India 2008)



Vectors of *Trypanosoma evansi*

Hirudo medicinalis

Tabanus striatus

Desmondus rotundus

Triatoma infestants

Glossina morsitans

Anthomyia pluvialis

Piper sarmentosum

- A traditional herb & aromatic flowering plant locally known as “kaduk” in Malay
- Well-applied as the appetizer salad and cooked to be ate with rice among SEA population.
- Wildly & abundant in damp open areas, cleared riverbanks and under shady trees (Seyyedan *et al.*, 2013)
- Well growth on cultivated land in India, Sri Lanka & Southeast Asian region (Hussain *et al*., 2009).
- Variety of phytochemical constituents & groups identified from various parts of the plants → phenylpropanoids, α-asarone, asaricin, myricetin, sarmentamide A & B, piperitone, naringenin, spathulenol, farnesol, quercetin, etc…

Piper sarmentosum: The Testimonial

Significant antimicrobial activities (Chan & Wong, 2014):
- Antifungal
- Antiamoebic
- Antituberculosis
- Anti-dengue

Antiplasmodial & antileishmanial activities of Pellitorine compound (Souza Oliveira *et al*, 2018)

The flowers used to treat several chronic diseases (Shim & Gam, 2012):
- Hypertension
- Diabetes mellitus
- Asthma
- Atherosclerosis

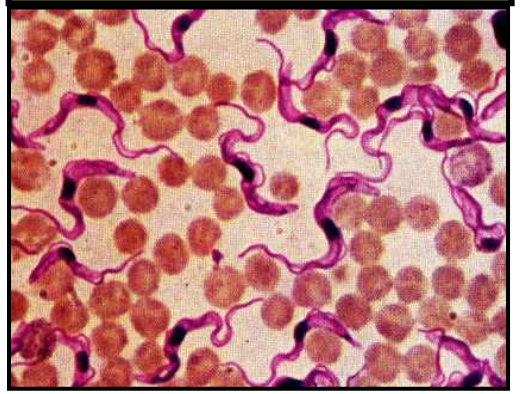
Wide range of pharmacological properties (Syed Ab Rahman *et al*, 2014):
- Wound healing
- Antioxidant
- Anti-infammatory
- Anti-osteoperosis

Antibacterial activities (Sanusi *et al*, 2017): *E. coli, MRSA, B. cereus, V. cholera* & *S. typhi*

Herbal remedies for many illnesses (Atiax *et al,* 2011):
- Feet dermatitis
- Toothache
- Headaches
- Coughs

Flow Chart

T. evansi stock



T. evansi administered *i.p.* (5 × 10^3 *T. evansi* / mice)

Orally administered of 0.1 mL 10 mg/kg bw of freeze-dried *P. sarmentosum* aqueous exract

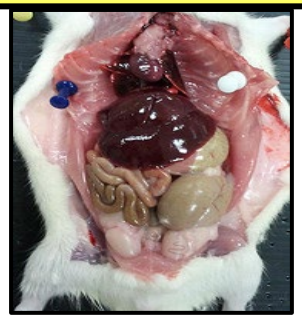
Thin stained blood smear for inhibition rate measurement

Thin unstained blood smear (morphological changes)

Physical observation of symptoms and mice survival

Blood biochemistry and renal function tests

Vital organ histology for toxicity assessment

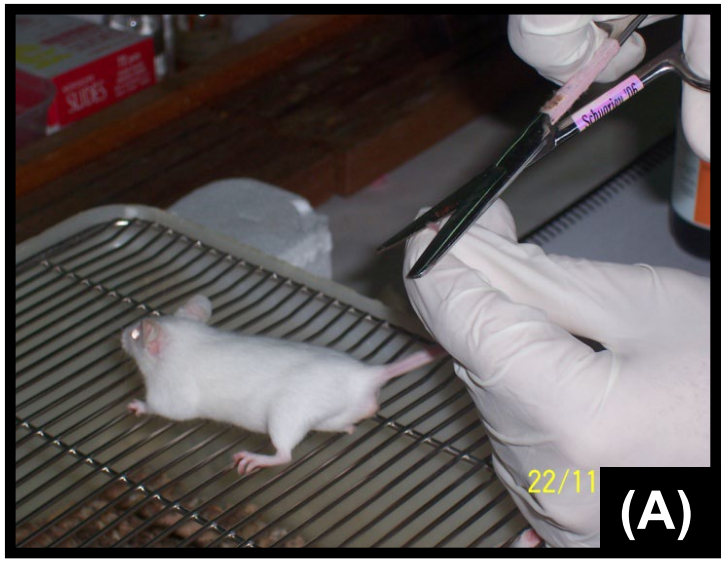


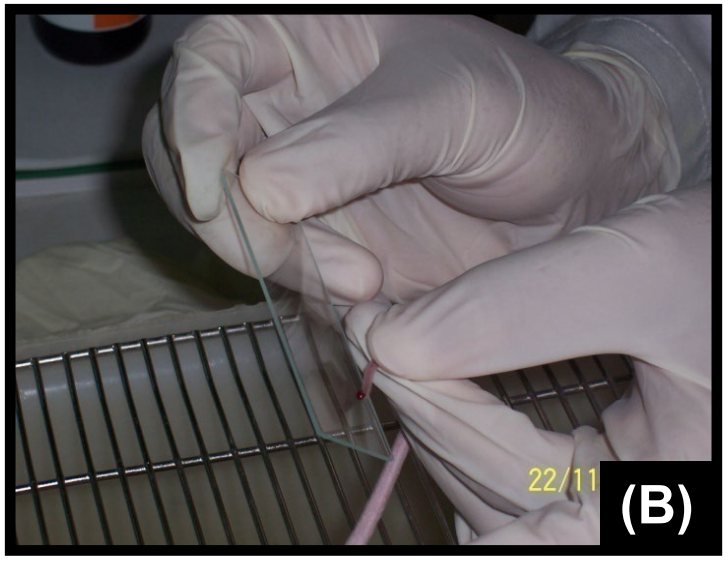
Experimental Design

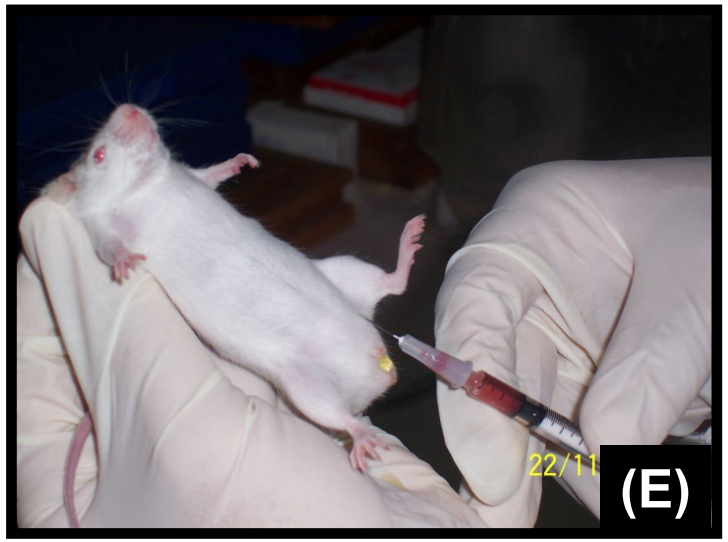
GROUP	REGIMENS	CODE	DESCRIPTION	*P. sarmentosum* DOSAGE
TREATMENT	PREVENTIVE	PRE14	14 days pre-infection	0.1 mL 10 mg/kg bw sdH_2O-extract
		PRE7	7 days pre-infection	0.1 mL 10 mg/kg bw sdH_2O-extract
		PRE3	3 days pre-infection	0.1 mL 10 mg/kg bw sdH_2O-extract
	CURATIVE	CUR3	3 days post-infection	0.1 mL 10 mg/kg bw sdH_2O-extract
		CUR5	5 days post-infection	0.1 mL 10 mg/kg bw sdH_2O-extract
		CUR7	7 days post-infection	0.1 mL 10 mg/kg bw sdH_2O-extract

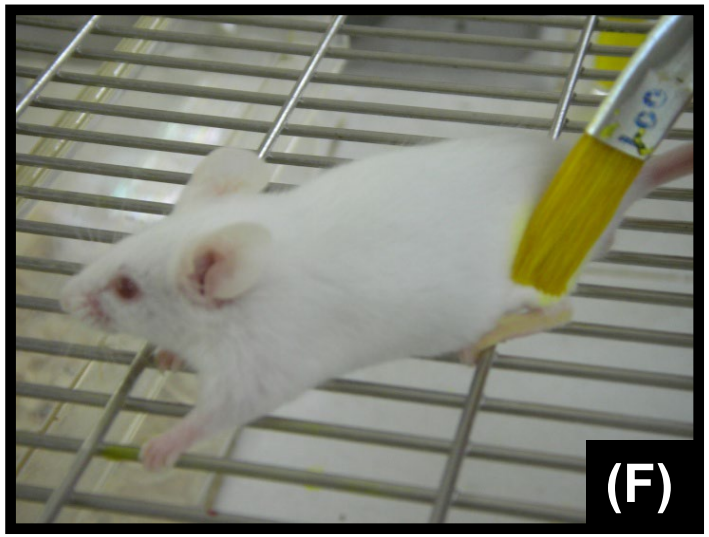
GROUP	REGIMENS	CODE	DESCRIPTION	CONTROL DOSAGE
CONTROL	POSITIVE	POS	Berenil (Sigma-Aldrich KL)	0.01 mL 3.5 mg/kg bw Berenil
	NEGATIVE	NEG	0.9 % Normal Saline	0.1 mL 0.9% normal saline (NS)
	LETHAL	LTN	Infection without treatment	5×10^3 *T. evansi* / mice (i.p.)

Parasite Administration And Mice Tagging

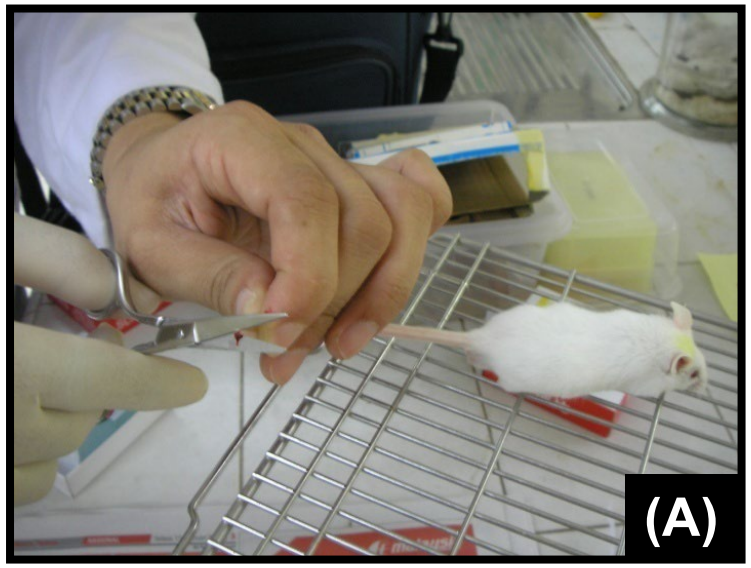


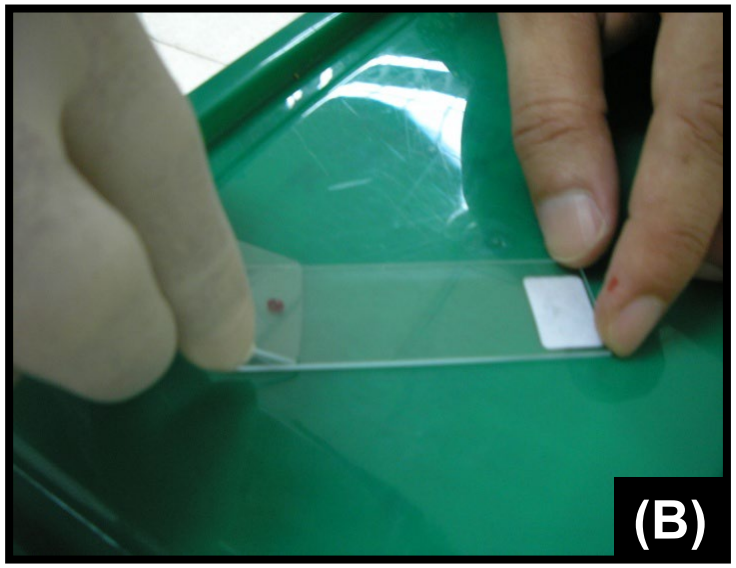


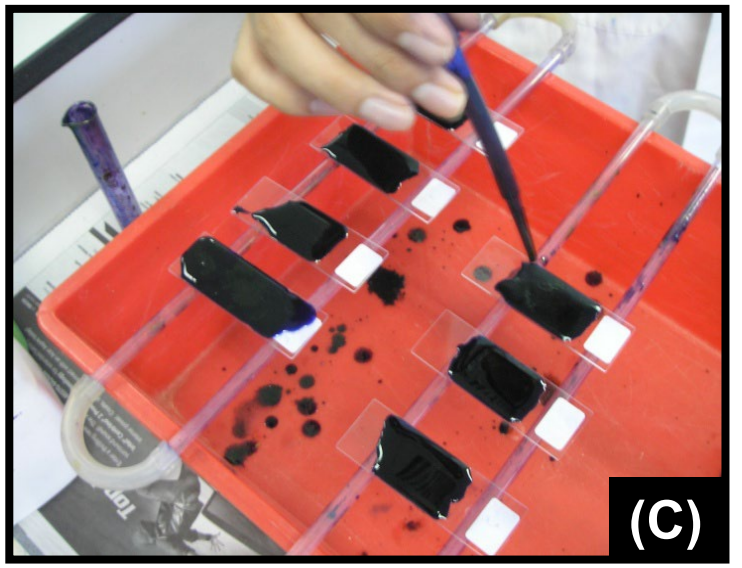


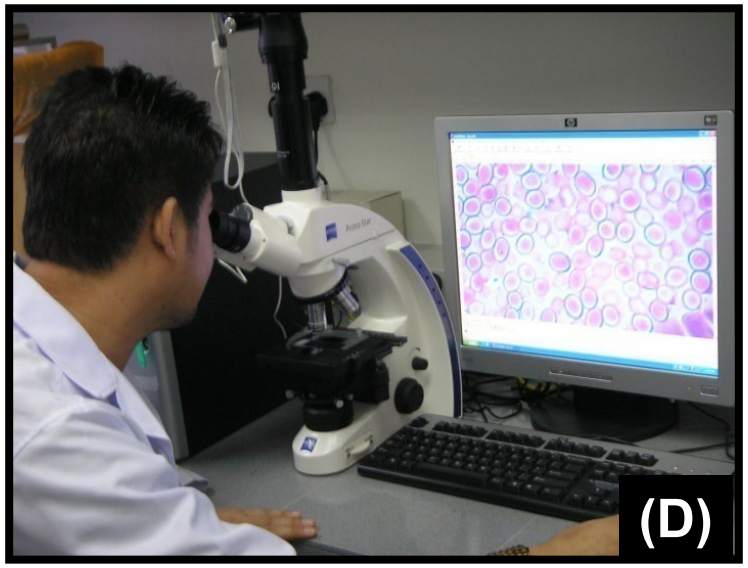


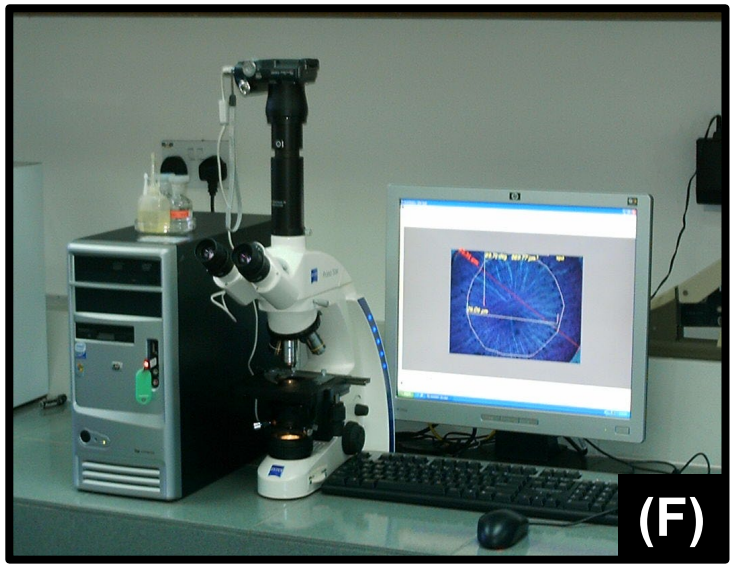
Giemsa Staining And Microscopic Observation



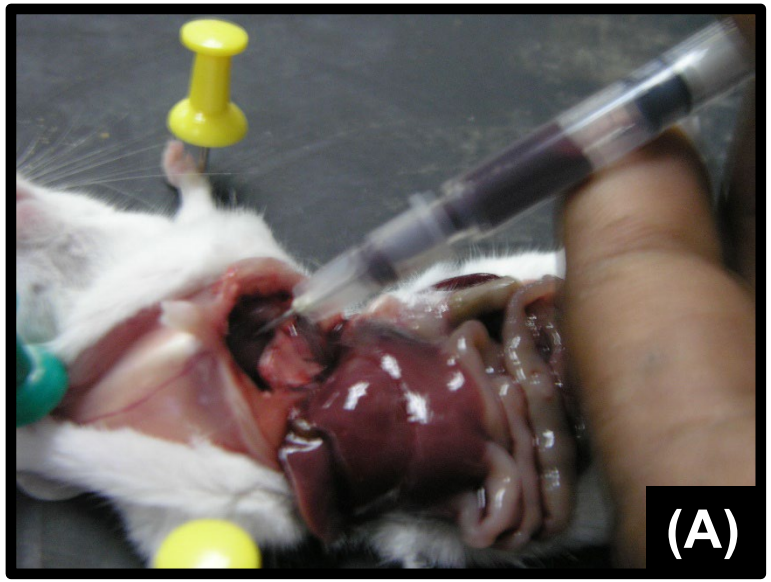






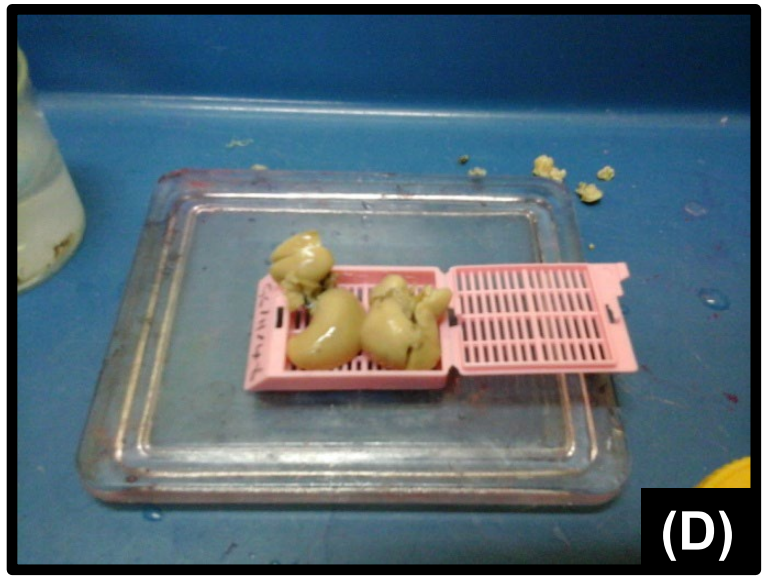


Biochemical Test And Histology Of Liver & Kidney


(A)


(B)


(C)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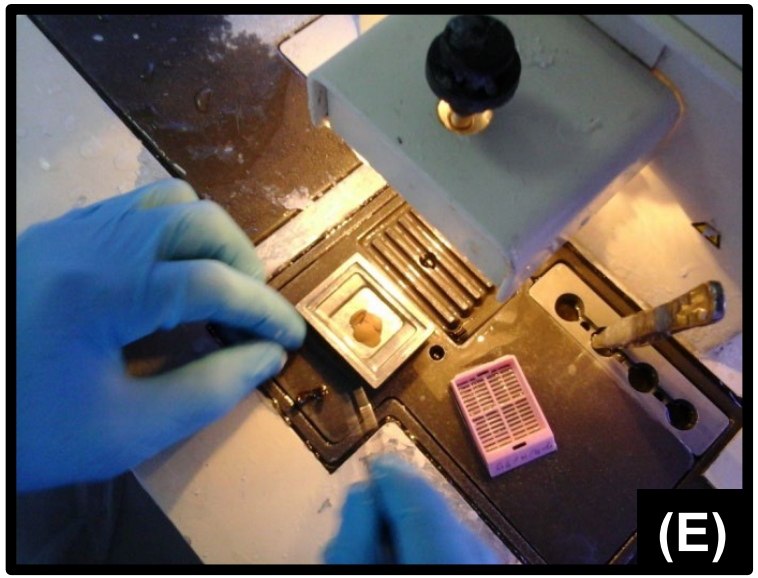
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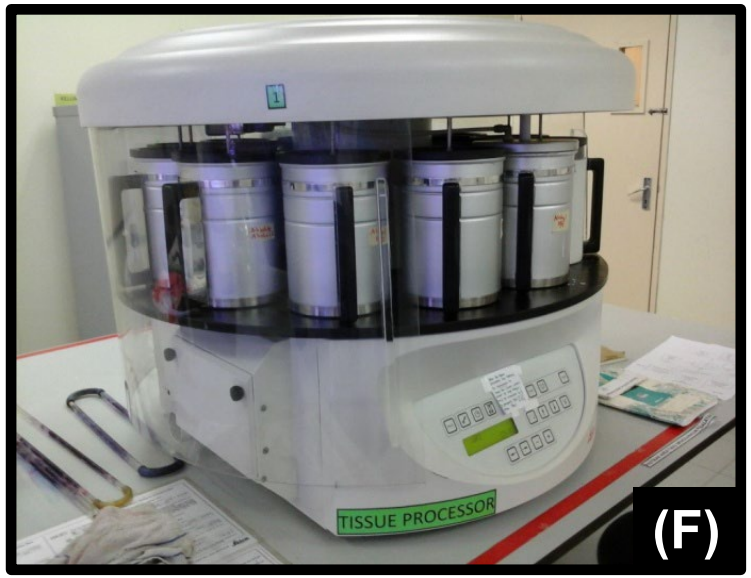
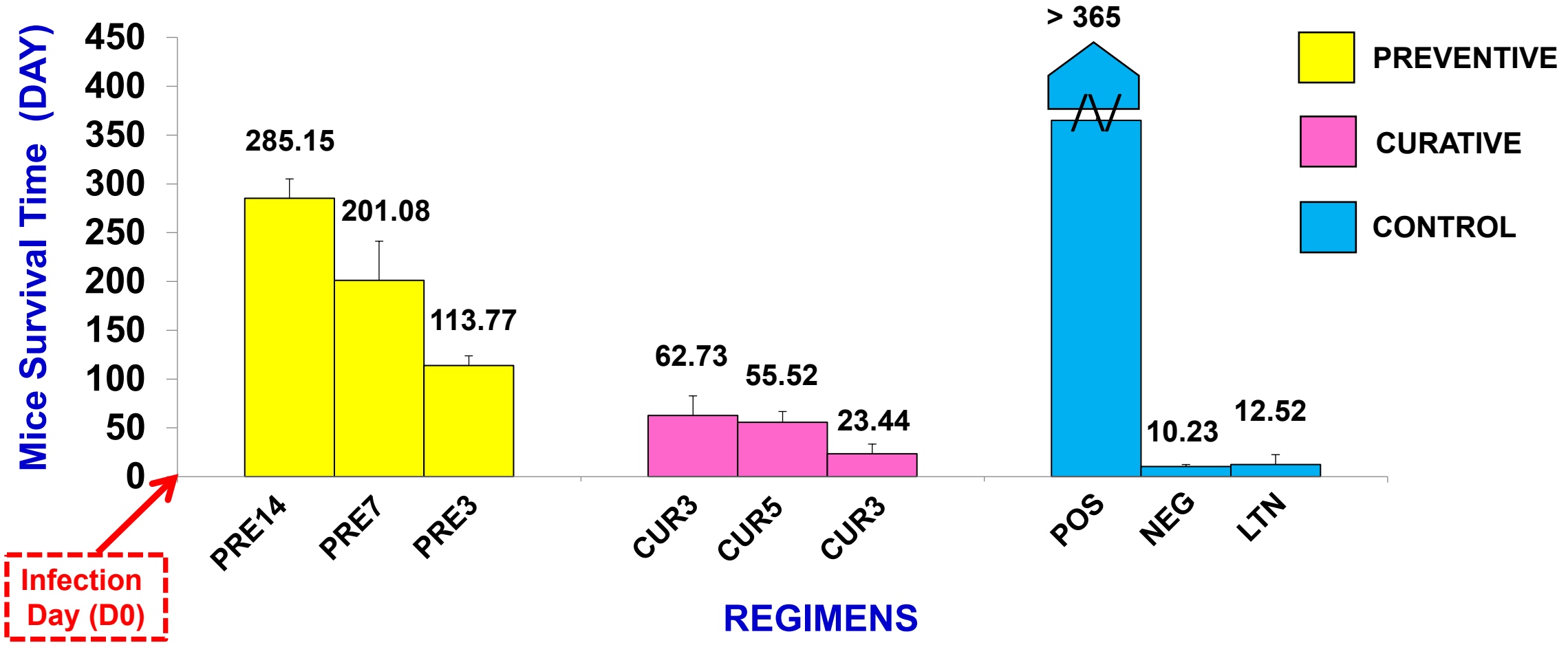


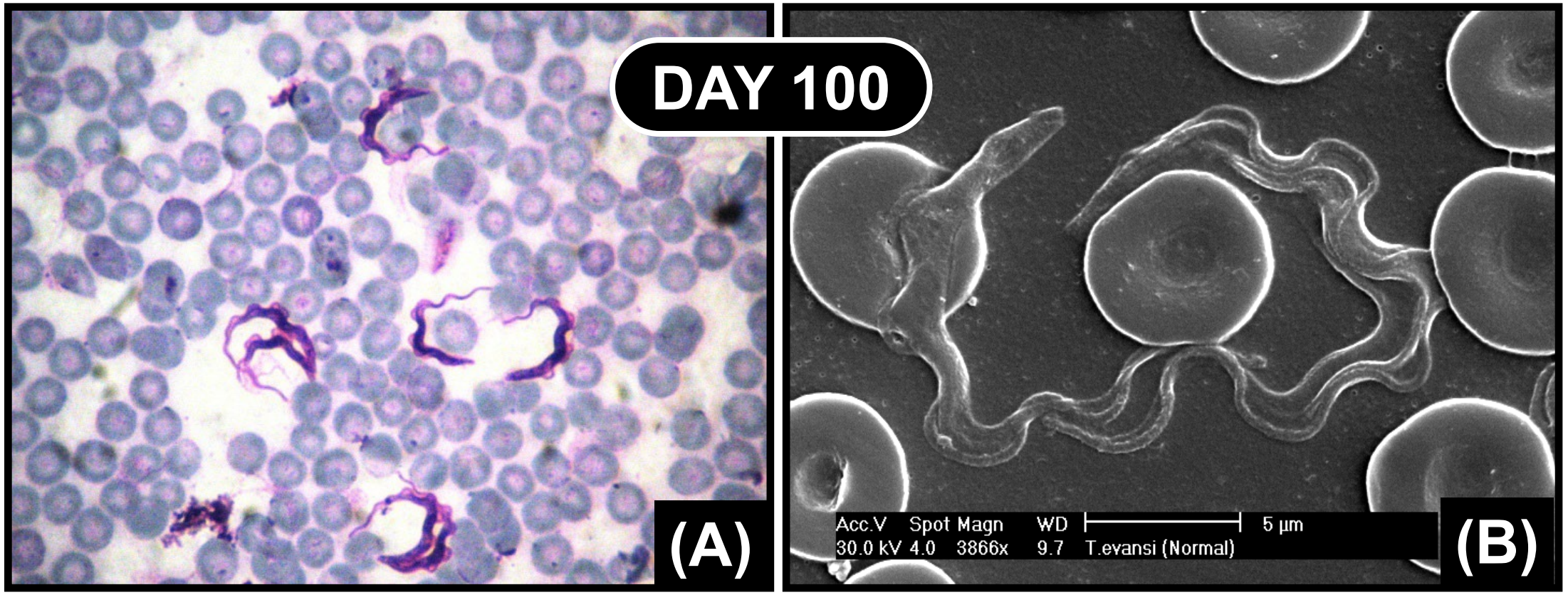
(F)

Mice Survival Time (Day)

Mice Survival Time(day) of the mice treated with 0.1 mL 10 mg/kg bw sdH_2O-*P. sarmentosum* extract at 5 × 10^3 *T. evansi* / mice (i.p.) as compared with 3 regimes of control

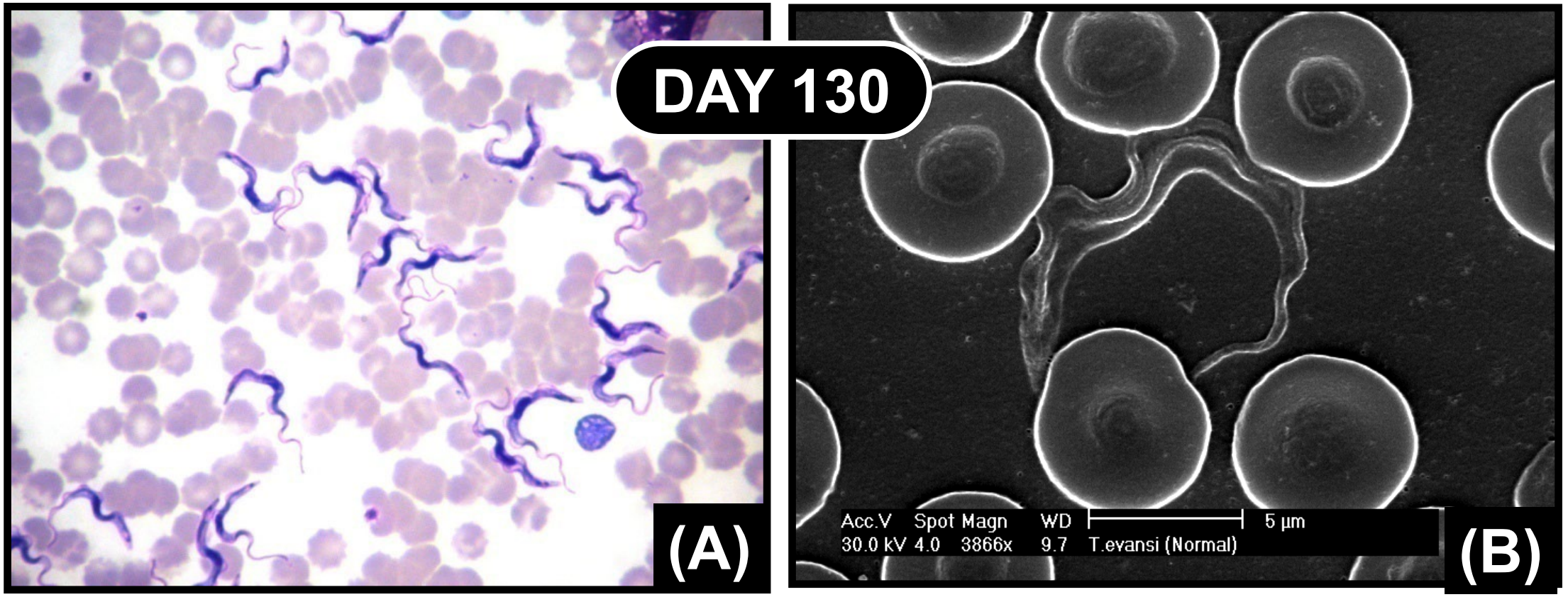


Parasite Survival In PRE14 Mice Group : 100th Day



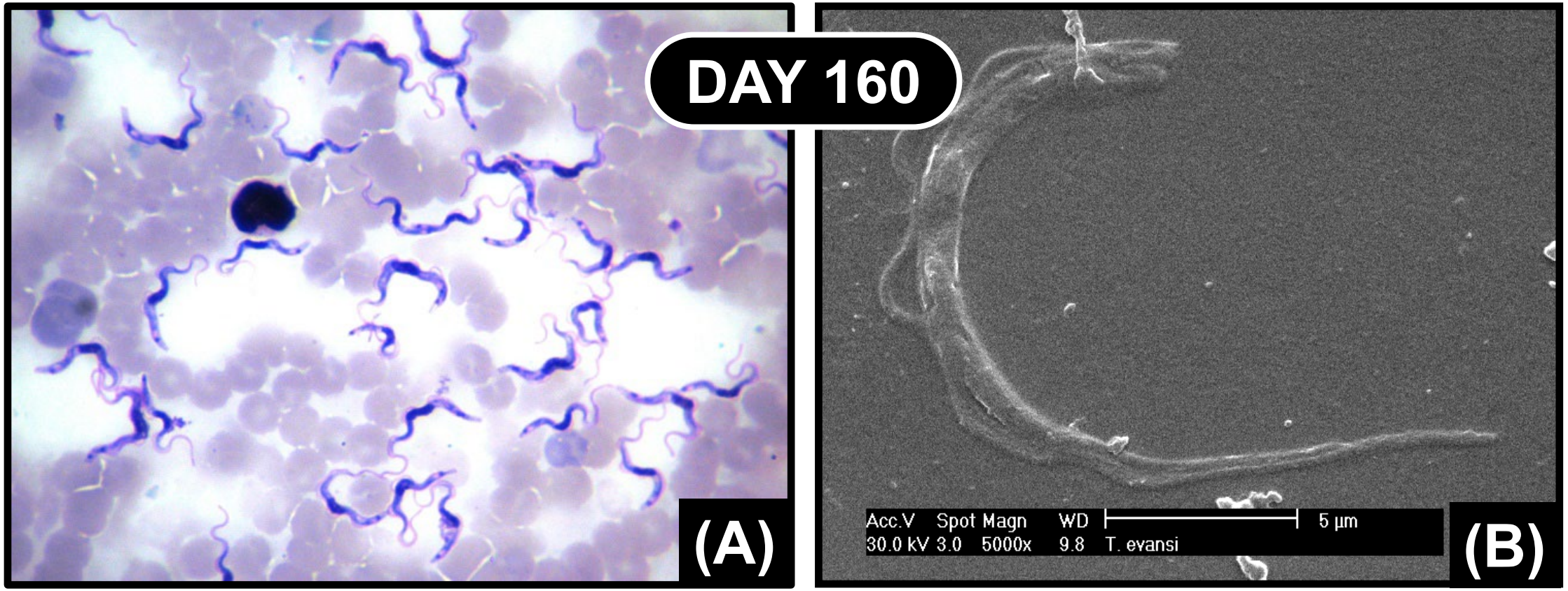
Thin blood smear of the mice from PRE14 mice group treated with 0.1 mL 10 mg/kg bw sdH_2O-*P. sarmentosum* extract as observed on day 100 post-infection under x100 mag. of light microscope (Olympus CX22, UK) (A) and x3866 mag. of SEM (Phillips XL30, UK) (B)

Parasite Survival In PRE14 Mice Group : 130th Day



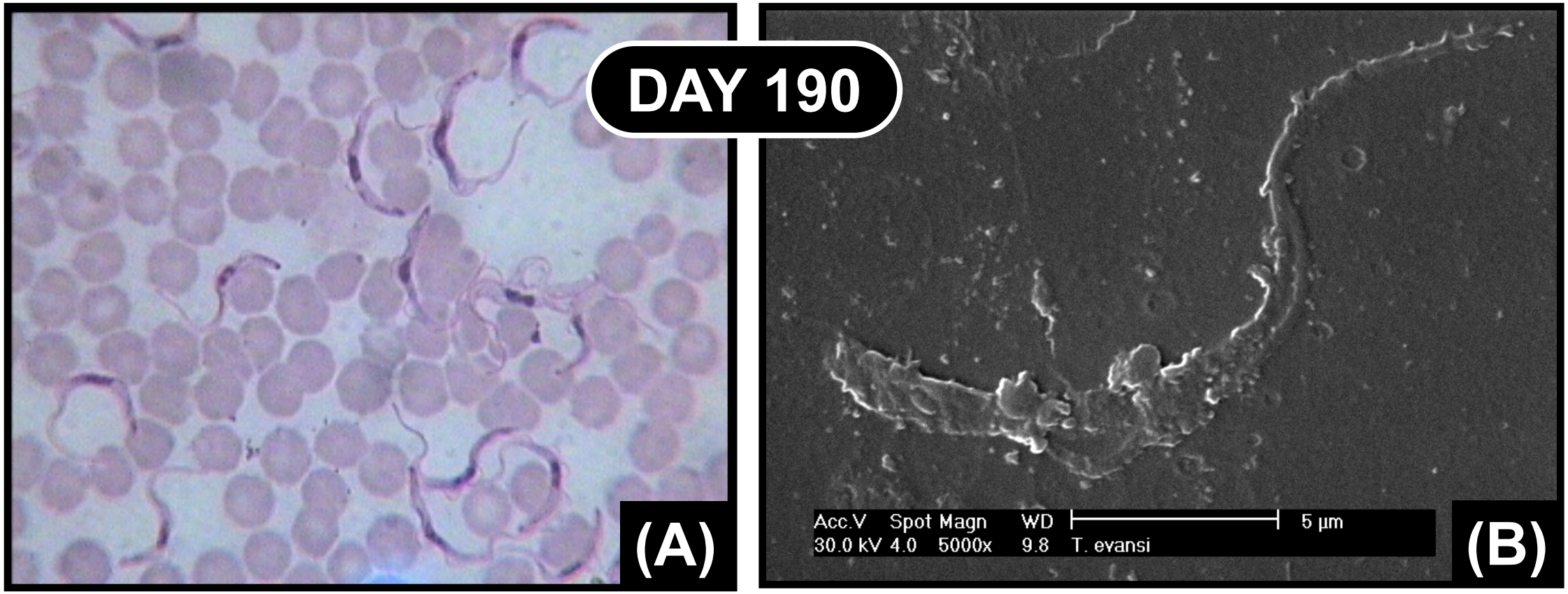
Thin blood smear of the mice from PRE14 mice group treated with 0.1 mL 10 mg/kg bw sdH_2O-*P. sarmentosum* extract as observed on day 130 post-infection under x100 mag. of light microscope (Olympus CX22, UK) (A) and x3866 mag. of SEM (Phillips XL30, UK) (B)

Parasite Survival In PRE14 Mice Group : 160th Day



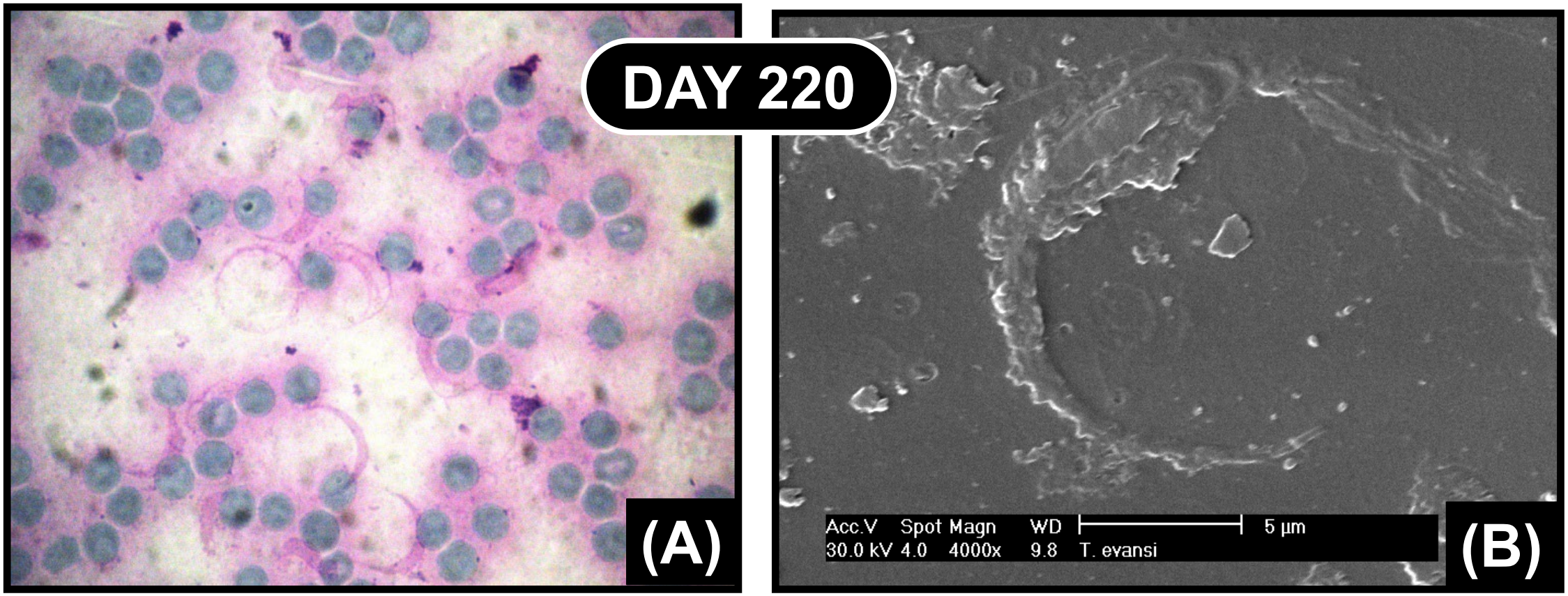
Thin blood smear of the mice from PRE14 mice group treated with 0.1 mL 10 mg/kg bw sdH_2O-*P. sarmentosum* extract as observed on day 100 post-infection under x160 mag. of light microscope (Olympus CX22, UK) (A) and x5000 mag. of SEM (Phillips XL30, UK) (B)

Parasite Survival In PRE14 Mice Group : 190th Day



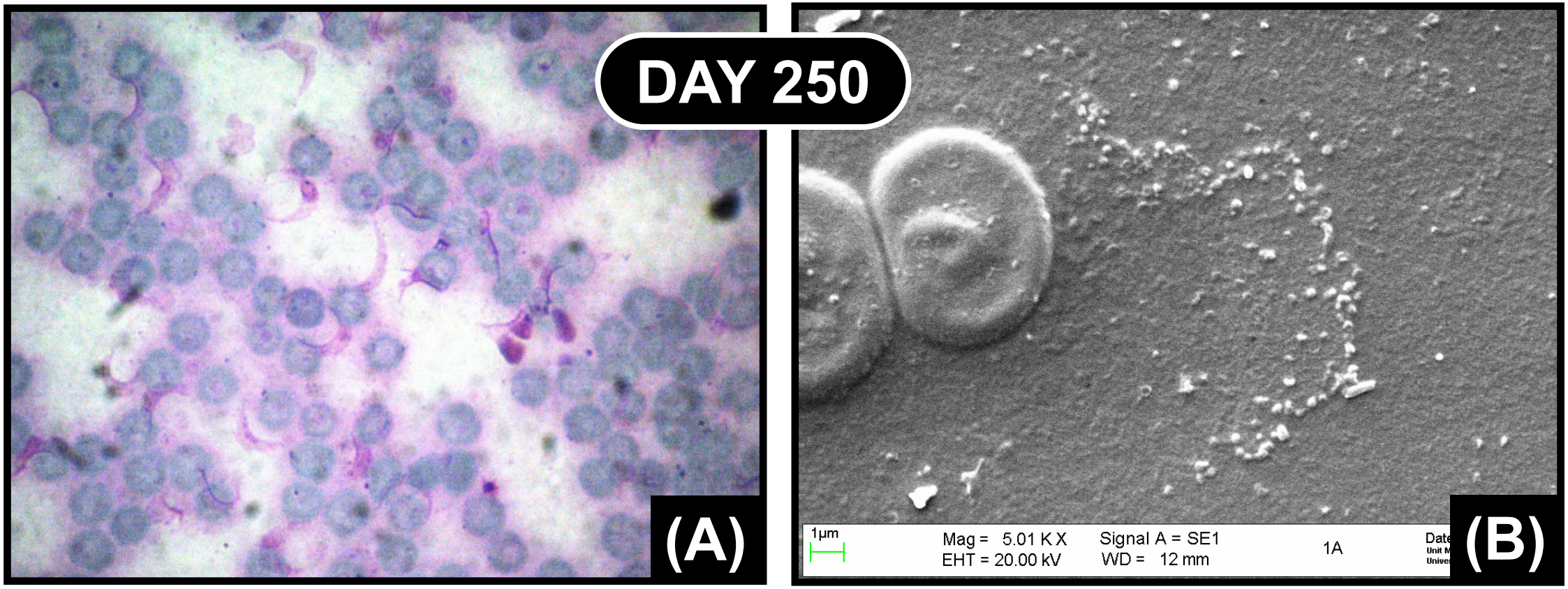
Thin blood smear of the mice from PRE14 mice group treated with 0.1 mL 10 mg/kg bw sdH_2O-*P. sarmentosum* extract as observed on day 190 post-infection under x100 mag. of light microscope (Olympus CX22, UK) (A) and x5000 mag. of SEM (Phillips XL30, UK) (B)

Parasite Survival In PRE14 Mice Group : 220th Day



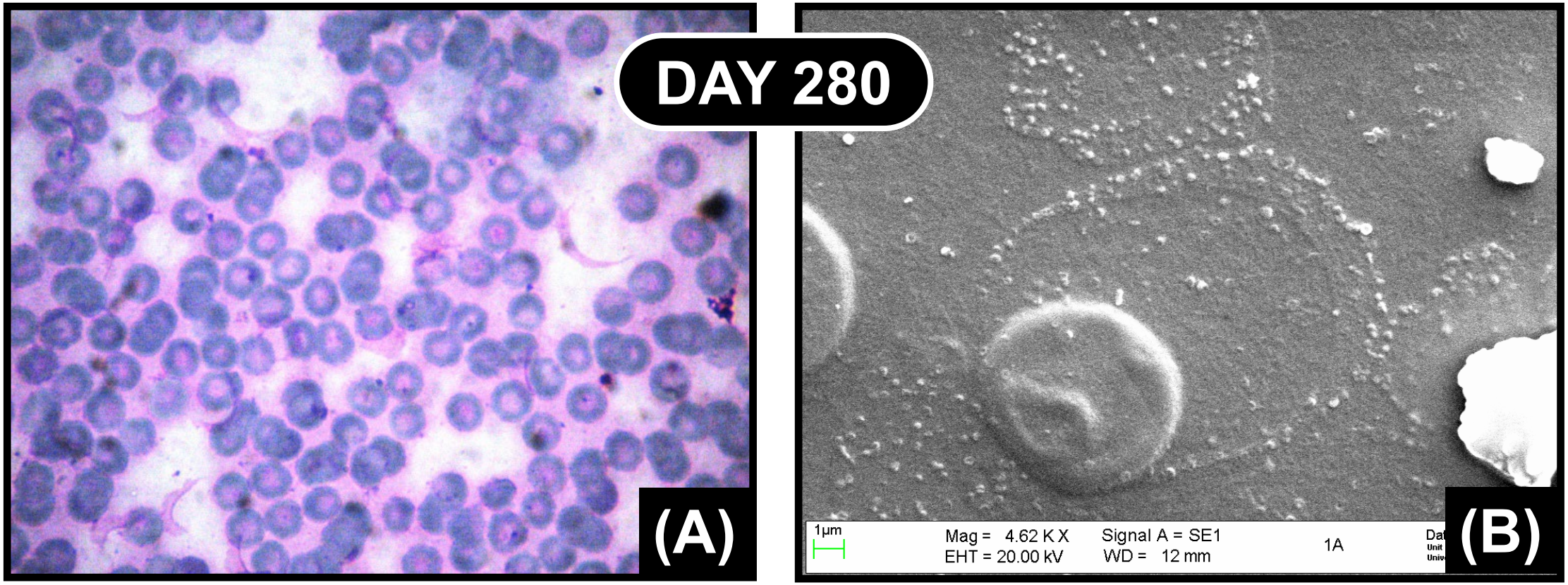
Thin blood smear of the mice from PRE14 mice group treated with 0.1 mL 10 mg/kg bw sdH_2O-*P. sarmentosum* extract as observed on day 220 post-infection under x100 mag. of light microscope (Olympus CX22, UK) (A) and x4000 mag. of SEM (Phillips XL30, UK) (B)

Parasite Survival In PRE14 Mice Group : 250th Day



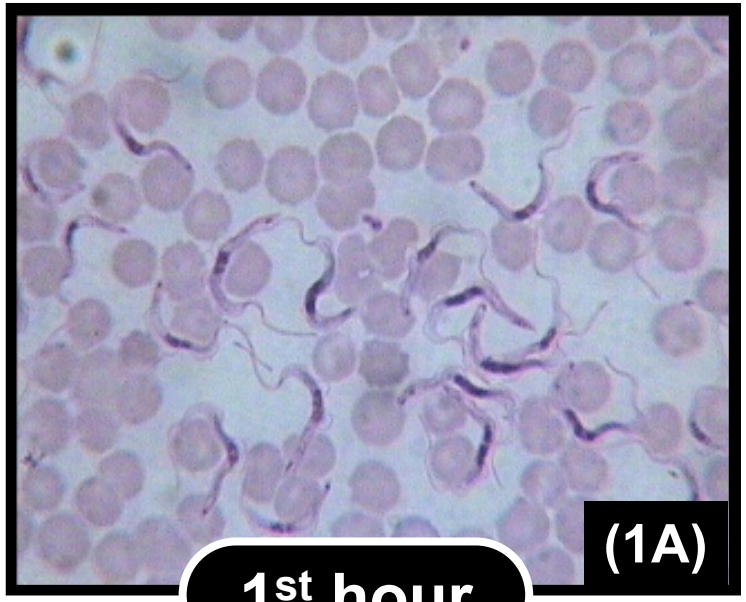
Thin blood smear of the mice from PRE14 mice group treated with 0.1 mL 10 mg/kg bw sdH_2O-*P. sarmentosum* extract as observed on day 250 post-infection under x100 mag. of light microscope (Olympus CX22, UK) (A) and x5000 mag. of SEM (Leo 1450VP, Japan) (B)

Parasite Survival In PRE14 Mice Group : 280th Day

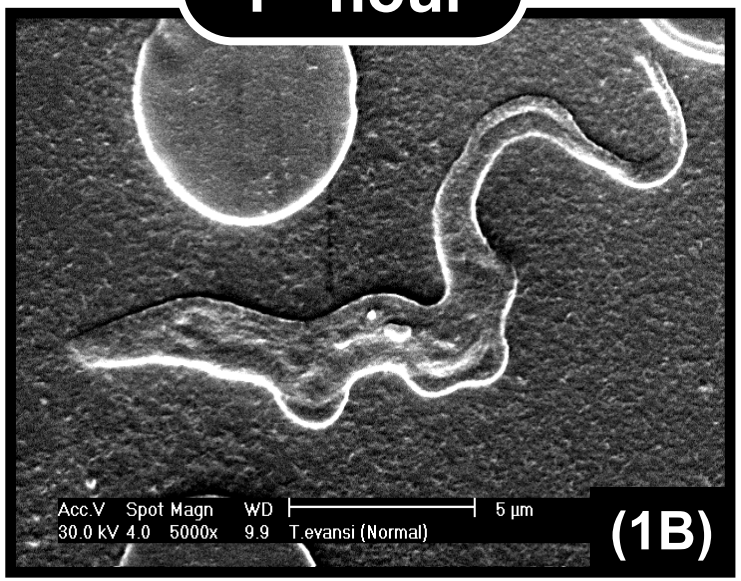


Thin blood smear of the mice from PRE14 mice group treated with 0.1 mL 10 mg/kg bw sdH_2O-*P. sarmentosum* extract as observed on day 280 post-infection under x100 mag. of light microscope (Olympus CX22, UK) (A) and x4600 mag. of SEM (Leo 1450VP, Japan) (B). These images was captured 5 days before the mice in PRE14 group were dead.

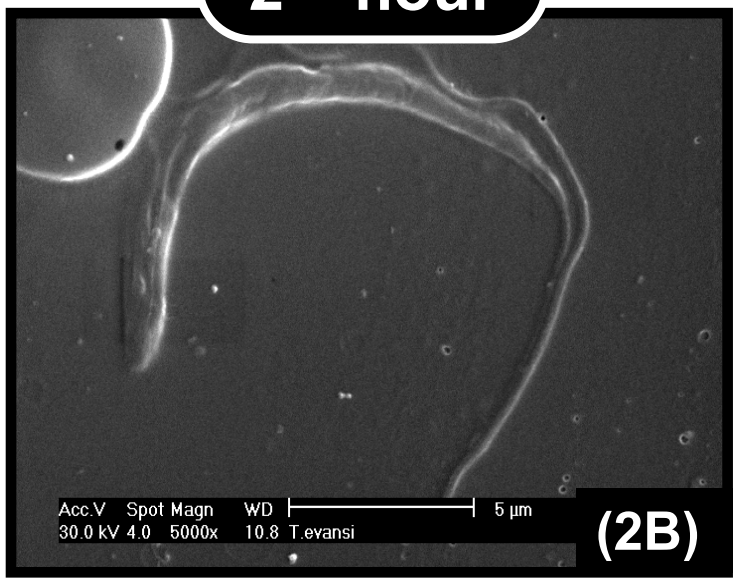
Parasite Growth in Berenil-Treated Group (POS)

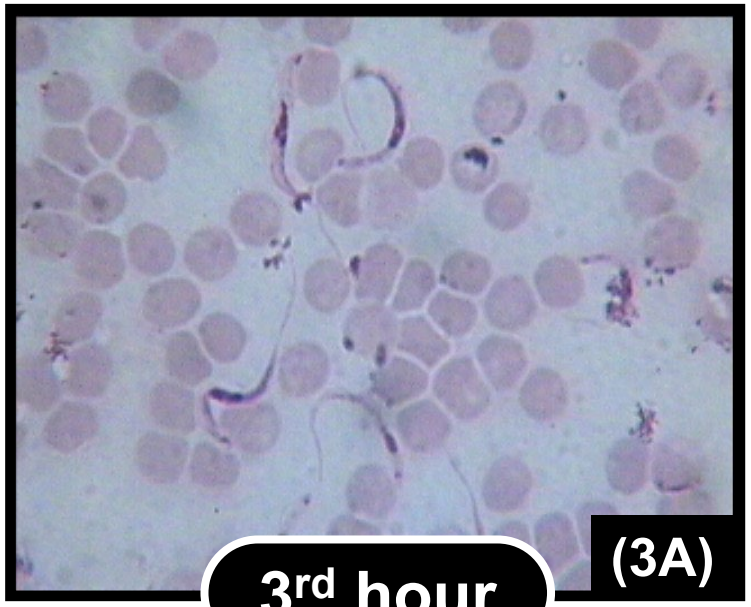


1st hour

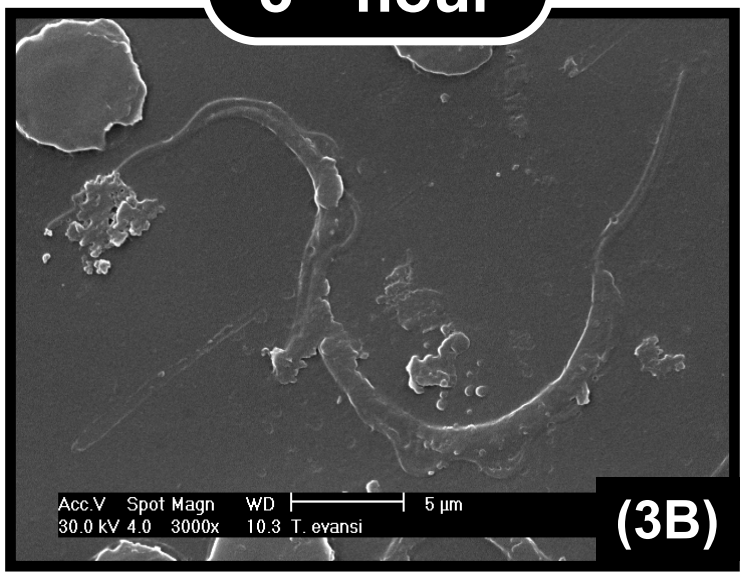


2nd hour

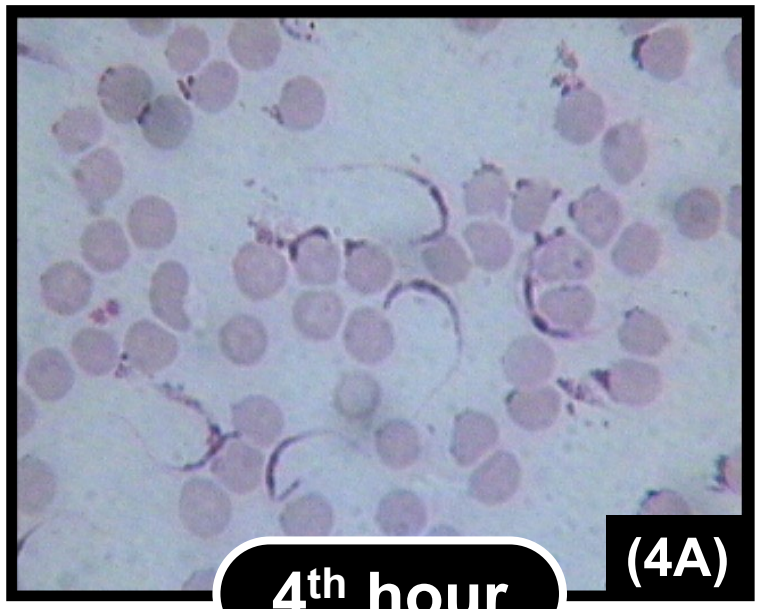




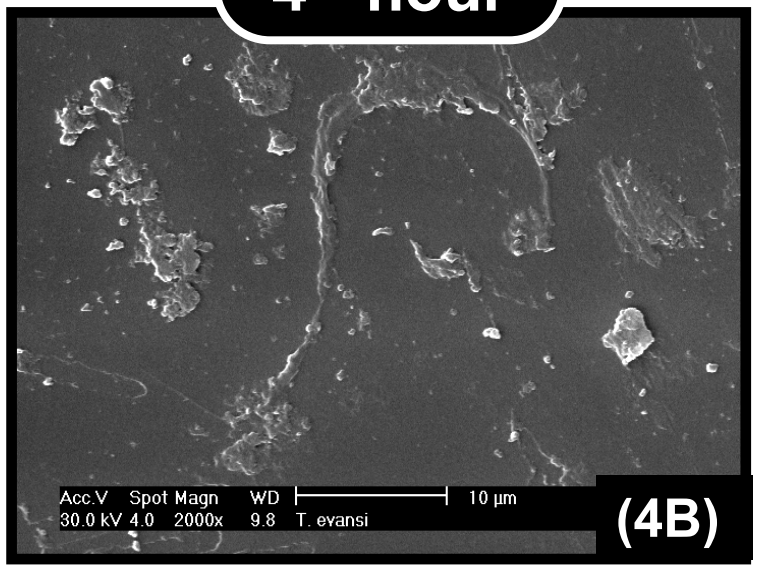
3rd hour



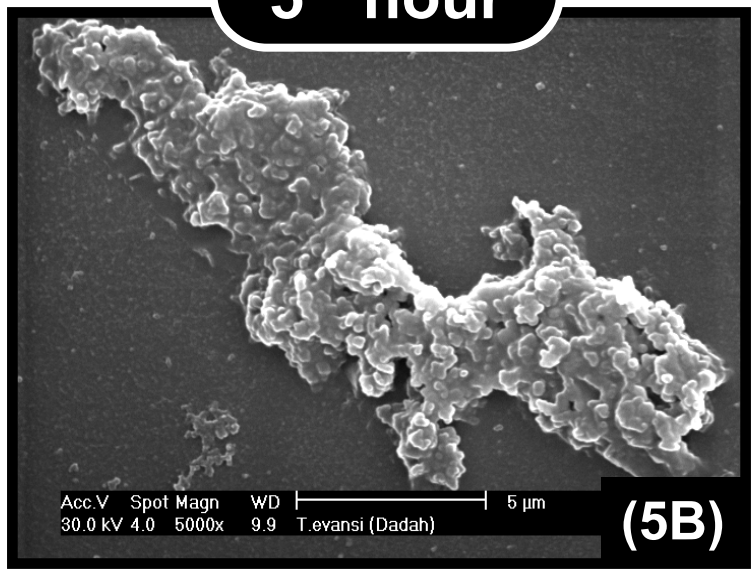
Parasite Growth in Berenil-Treated Group (POS)

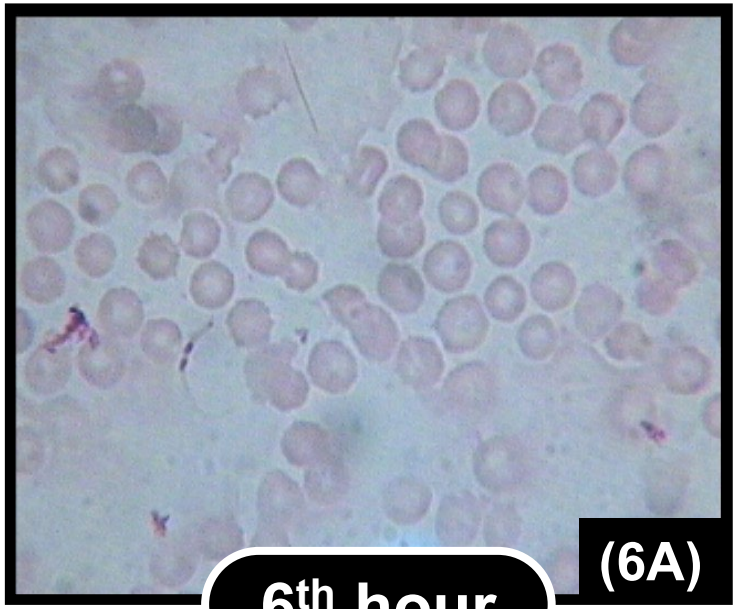


4th hour

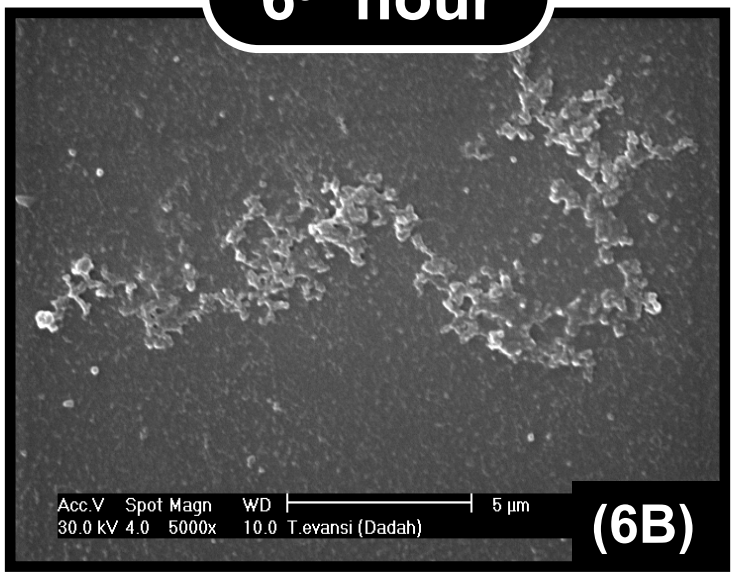


5th hour

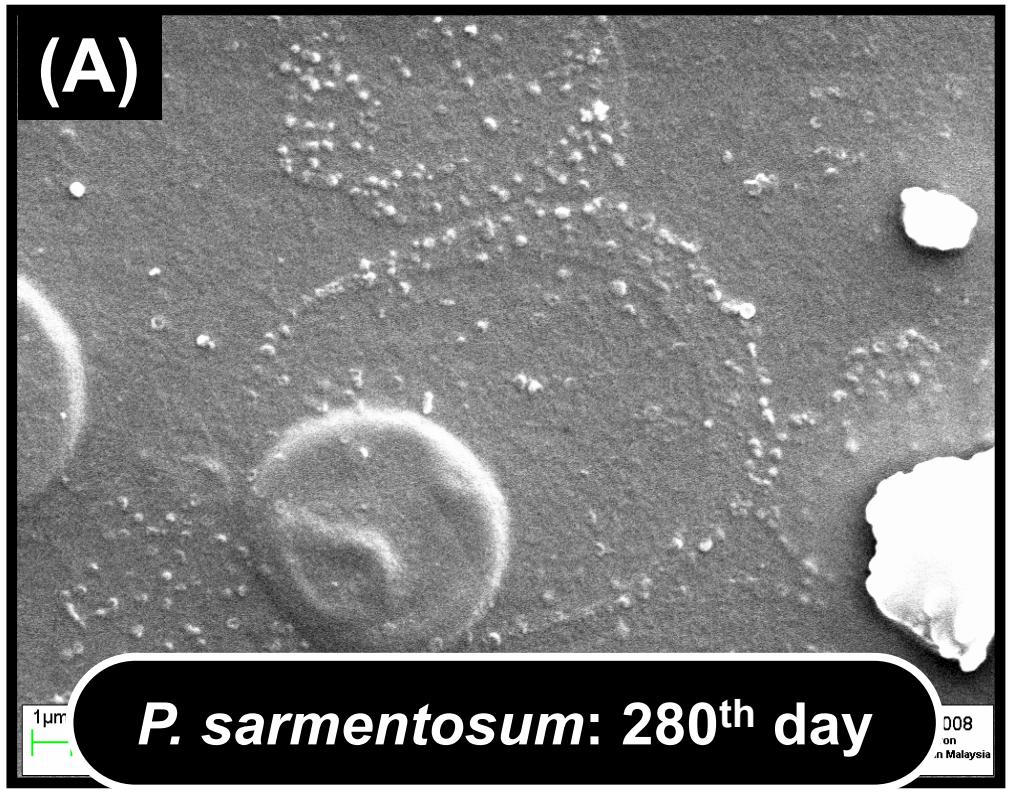


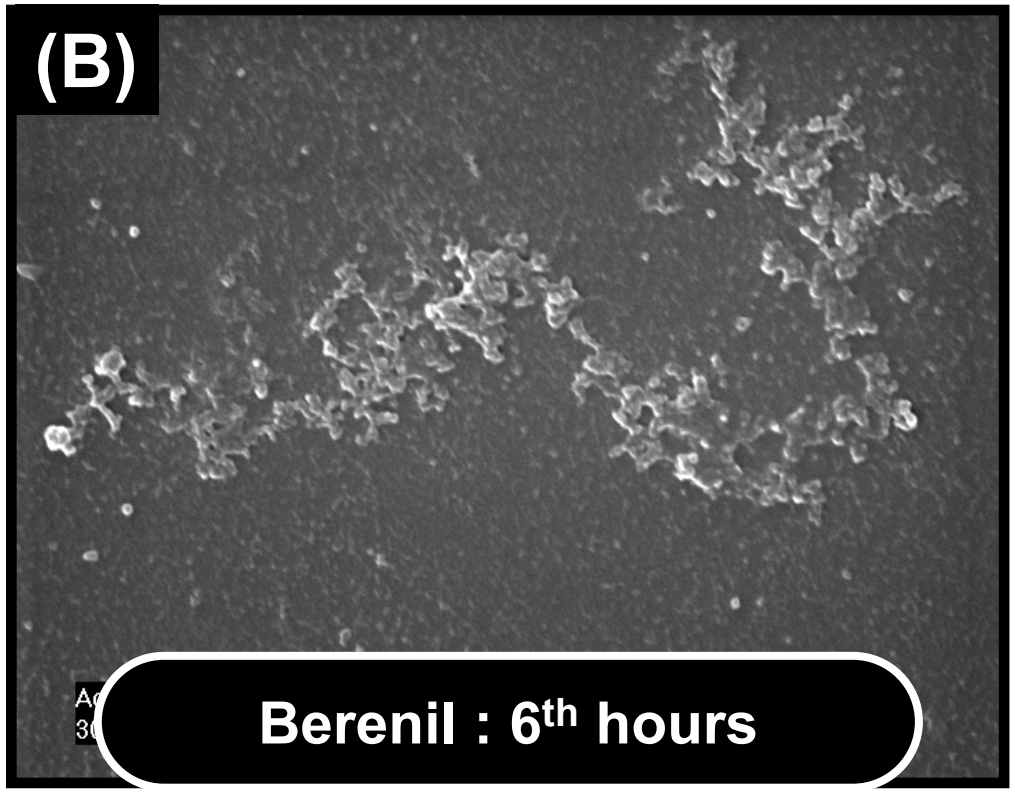


6th hour



P. Sarmentosum vs Berenil





SEM micrograph of the morphological changes of *T. evansi* in PRE14 mice treated with 0.1 mL 10 mg/kg bw of sdH_2O-*P. sarmentosum* extract on 280th day post- infection (x4600 mag.) (A) and in POS mice at 6th hours post-treatment with 0.01mL 3.5 mg/kg bw Berenil (x5000 mag.) (B)

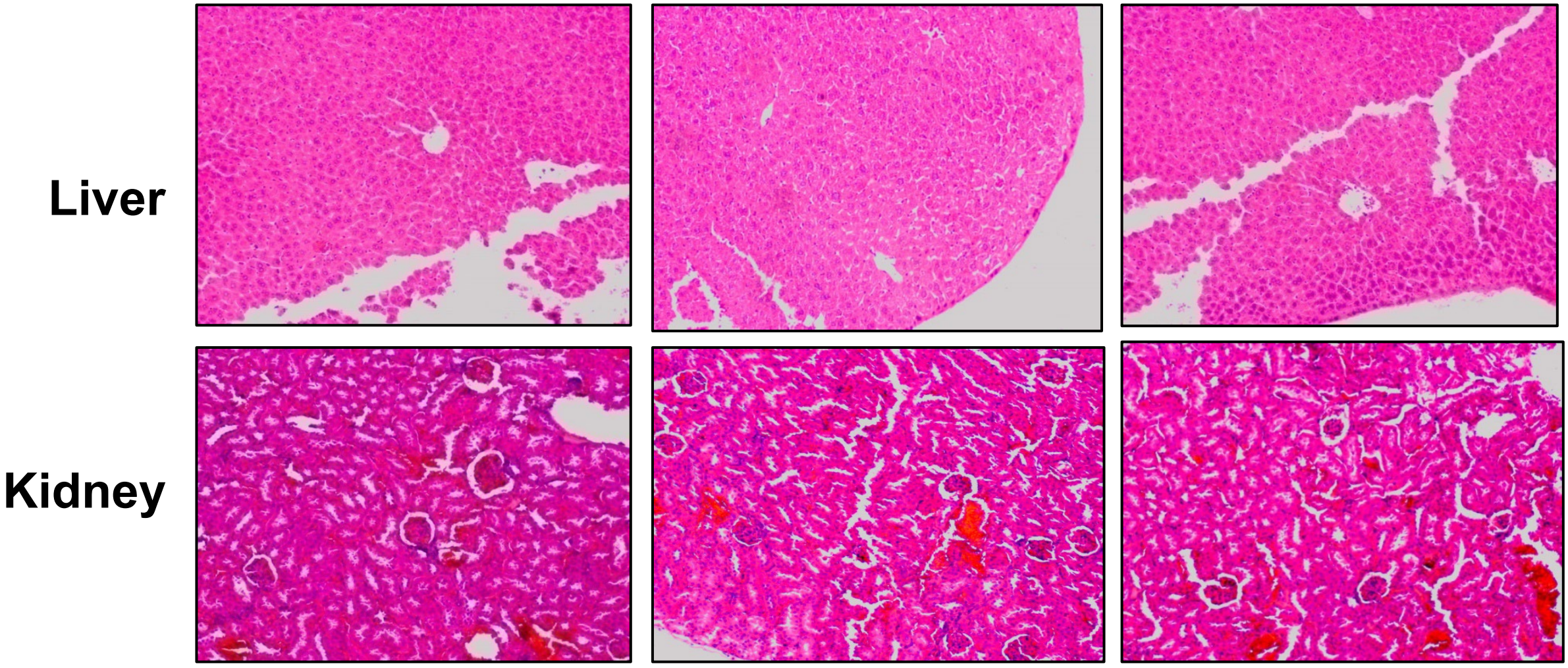
Biochemical Test For Toxicity Assessment

Test	TA	TB	TC	TD	CN	CI	NR	Unit
ALT (*)	41.81 ± 2.14	45.20 ± 1.13	67.57 ± 2.91	90.03 ± 2.02	41.03 ± 3.91	44.83 ± 1.11	**40 – 93**	IU/L
AST (*)	133.13 ± 2.04	125.93 ± 2.12	167.76 ± 2.27	187.01 ± 2.09	111.62 ± 1.19	134.43 ± 4.01	**92 – 206**	IU/L
ALP (*)	62.76 ± 2.33	59.4 ± 2.97	69.2 ± 2.90	68.03 ± 2.10	61.46 ± 2.46	58.32 ± 2.97	**54 – 115**	IU/L
STP (*)	6.12 ± 2.32	7.21 ± 3.81	7.93 ± 2.01	8.83 ± 3.90	6.40 ± 1.01	6.80 ± 3.06	**5.8 – 9.5**	g/dL

TA : Sub-acute regime – Daily treatment (28 days)
TB : Sub-acute regime – Daily treatment (28 days) 2 hours post-infection
TC : Sub-chronic regime – Daily treatment (90 days)
TD : Sub-chronic regime – Daily treatment (90 days) 2 hours post-infection
CN : Control regime – Normal mice without infection and treatment
CI : Control regime – Infected mice on D0
ALT : Alanine aminotransferase
AST : Aspartate transaminase
ALP : Alkaline phosphatase
STP : Serum total protein

(*) *All values were expressed as mean ± standard errors (se)*
(*) *All NR values were referred from Research Animal Resources, University of Minnesota, USA*

Organ Histology For Toxicity Assessment



Conclusion

- Tryptophol toxin and stochastic genetic modification of VSA is still the best ‘weapon’ for *T. evansi* survival (Otto *et al.* 2010).

- New wave of infection & periodic changes of antigenic variation → changes in parasitemia peaks → longer survival time of the parasite & chronic infection on host (Salleh *et al*. 2009)

- The action of pellitorine ($C_{13}H_{25}ON$) molecule in *P. sarmentosum a*gainst –thiol group of parasite enzymes in which crucial for parasite proliferation (Souza Oliveira *et al*, 2018).

- Bioactive compound of Sarmentamide A in *P. sarmentosum* inhibited the important enzymes (alcohol dehydrogenase, cysteine proteinase and thioredoxin reductase) for the stability of the redox reaction in fungal cells such as *A. fumigatus* & *C. albians* (Tuntiwachwuttikul *et al*., 2006)

Future Plans

Various solvents of *P. sarmentosum* extract

Mechanism of action

In-vitro anti-trypanosomal screening

Concentration- & time-dependant alteration

Clinical & molecular approaches

Screening against *T. cruzi* and *T. brucei*

Absolute Hypothesis

EAT KADUK..!

NO HARM TO EAT AS MUCH AS YOU CAN

Start to Put Kaduk in Your Meal



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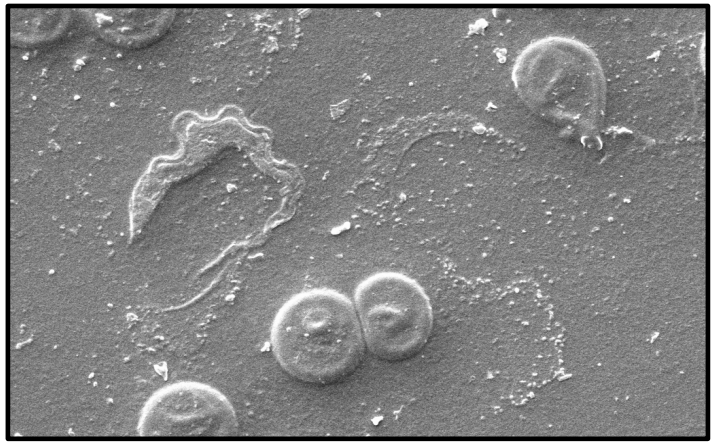
KEMENTERIAN PENDIDIKAN MALAYSIA

THANK YOU



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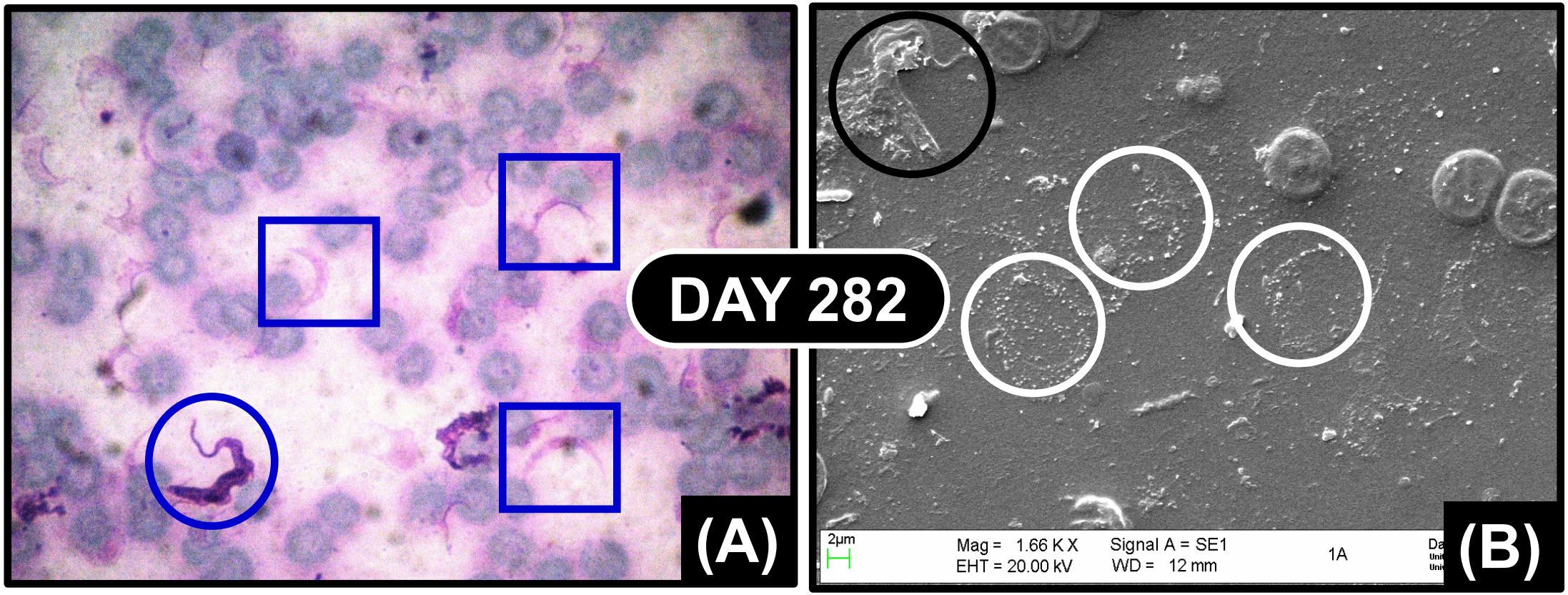
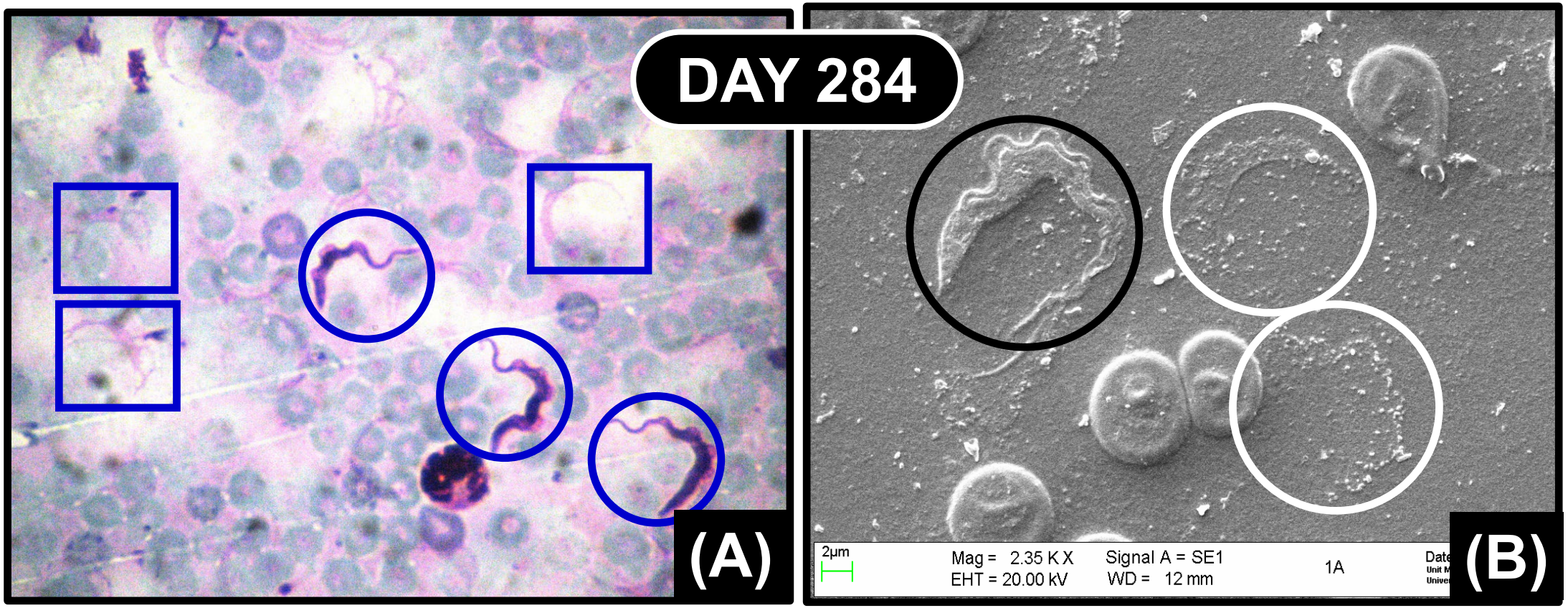
Parasite Survival In PRE14 Mice Group : 282nd Day



Reemerged of *T. evansi* which survived in PRE14 group mice on day 282 due to the action of 'variable surface glycoprotein (VSA) stochastic genetic modification' as observed under x100 mag. of light microscope (A) and x1600 mag. of SEM (Leo 1450VP, Japan) (B). This scenario is believed to cause the mice in this group eventually dead.

Parasite Survival In PRE14 Mice Group : 284th Day



Reemerged of *T. evansi* which survived in PRE14 group mice on day 284 due to the action of 'variable surface glycoprotein (VSA) stochastic genetic modification' as observed under x100 mag. of light microscope (A) and x2300 mag. of SEM (Leo 1450VP, Japan) (B). Later the mice died on day 286

Rationale Of The Study

Reliability of Anti-Trypanosomial Drugs

- Resistant issues in India, Thailand & Indonesia
- Unaffordable → expensive in certain regions
- Wrong dosage & concentration → side effects

Economic Growth & Biotechnology Sector

- Biotechnology → main focus in the next decade
- Snake gourd → consumable & easily manipulated
- AHT & Surra → influenced productivity of human & livestock

Parasite Pre-Patent Period (Day)

Pre-Patent Period (day) of the mice treated with 0.1 mL 10 mg/kg bw sdH_2O-*P. sarmentosum* extract at 5 × 10^3 *T. evansi* / mice (i.p.) as compared with 3 regimes of control