Sphaeranthus indicus : As natural immunomodulator

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Herbs as immunomodulator

Herbal drugs are known to possess immunomodulatory properties and generally act by stimulating both specific (cell-mediated) and non specific (humoral) immunity.

Prunus amygdalus (Almond) and *Buchanania lanzan* (Chirronji), *Euryale ferox* (Tel makhana), and *Zingiber officinale* (Sonth), *Ocimum basilium* (tulsi), *Ficus benghalensis.*

Plant profile

Botanical name : *Sphaerantus indicus* Linn.

Family **:** Asteraceae

Traditional uses

- The plant is bitter, stomachic, demulcent. It is used in epilepsy, hemicrania, jaundice, liver disorders and gastric disorders.
- The roots are diuretic, expectorant, febrifuge, and antihelmintic.
- The leaf is good for skin diseases and is considered as nerve tonic.
- The flowers are highly esteemed as alternative, depurative and used as blood purifier in skin diseases[3].

Chemical Constituents

Sterols: β-Sitosterol and stigmasterol.

Long chain compounds: n-phenylurethane, n-pentacosane.

Sterol glycoside: β-D-glucoside of (24S)-24-ethylcholesta-4, 22-dien-3-β-ol.

Flavonoids: 7-Hydroxyfrullanolide (7HF), 11α,13-dihydro-3α,7α-dihydroxyfrullanolide, 11α,13-dihydro-7α,13-dihydroxyfrullanolide and 11α,13-dihydro-7α-hydroxy-13-methoxyfrullanolide.

Amino acids: glycine, alanine, valine, leucine, cysteine.

Sugars: lactose, raffinose, D-galactose, maltose, D-fructose, D-arabinose, L-rhamnose, and D-glucose[3].

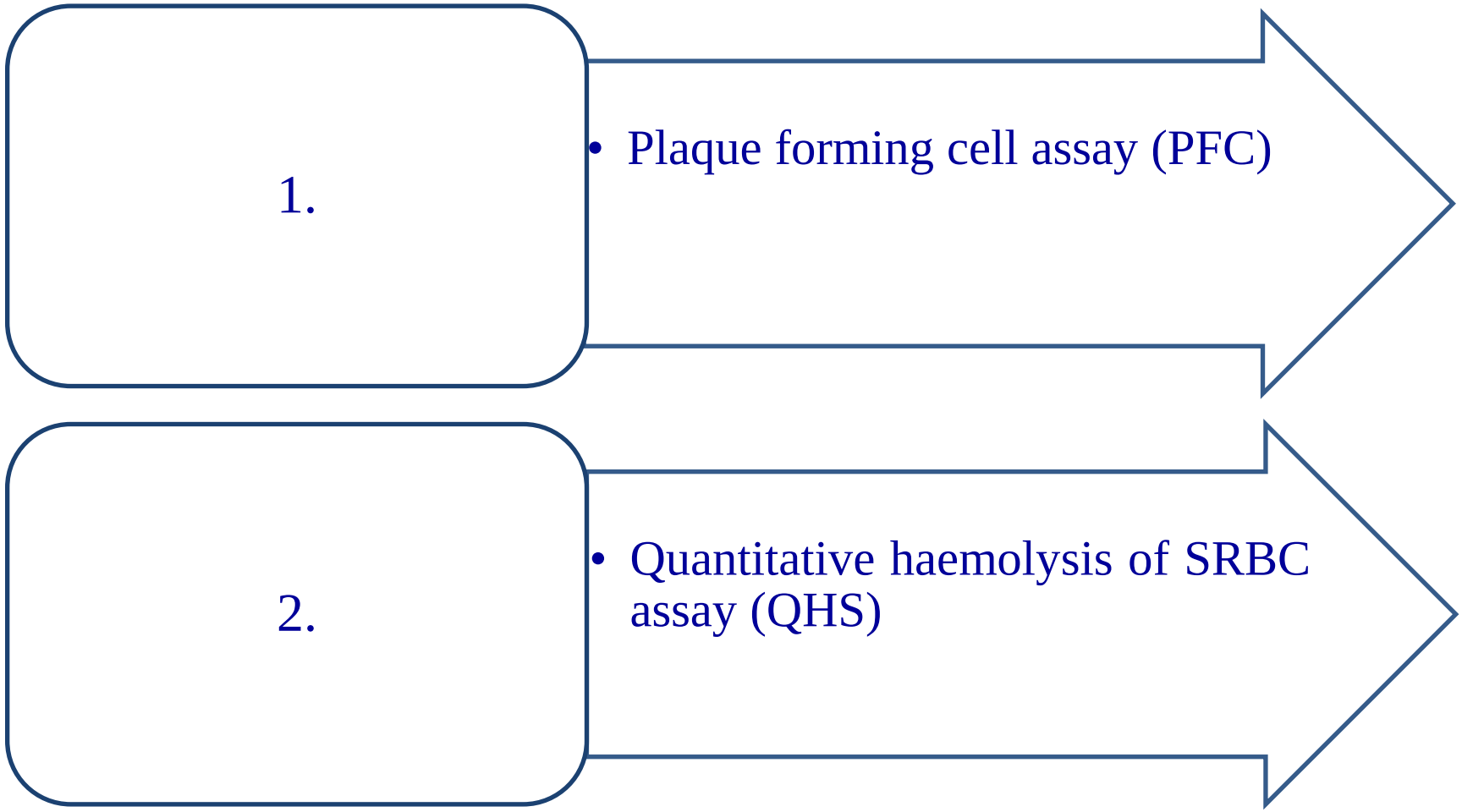
- Authentication of crude plant material of *Spheranthus indicus*
- Extraction of *Spheranthus indicus* whole plant
- Approval of protocol from animal ethical committee
- Screening of immunomodulatory activity for methanolic extract of *Spheranthus indicus*

Preliminary phytochemical studies

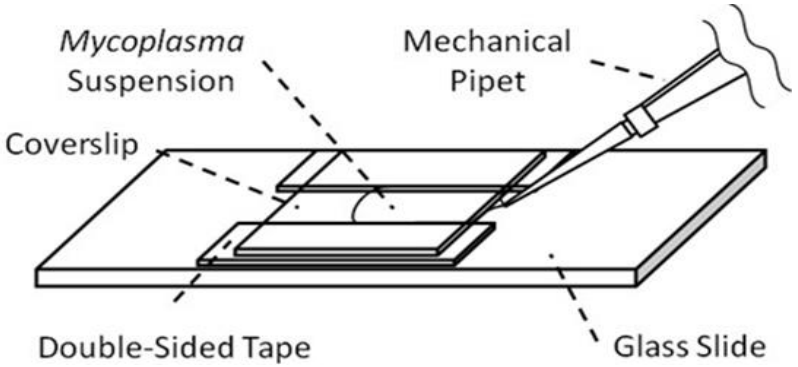
Chemical test	MeOH SI
Test for flavonoids	++
Test for phenols and tannins	++
Test for alkaloids	-
Test for steroids	+
Test for saponins	+
Test for glycosides	+
Test for carbohydrates	+
Test for amino acids and proteins	+
Test for terpenes	+

where + indicates (present) and - indicates (absent)

In vitro immunomodulatory assays for methanolic extract of *S. indicus*



Plaque forming cell (PFC) assay of methanolic extract of *S. indicus*



SRBC were prepared at a cell density of 1×10^{8} cells/mL in PBS.

1mL SRBC + 0.5 mL of diluted rabbit serum (1:10 diluted with normal saline) was added to 1 mL of spleen cell suspension (PBS).

Cuningham chambers were prepared using glass slide, coverslips and double- sided tape .

The chambers should be loaded with a known volume of assay mixture, sealed with petroleum jelly and incubated at 37°C for 1 h.

The plaques were counted under a light microscope and expressed as PFC per 10^{6} spleen cells[19].

Plaque forming cells for methanolic extract of *Sphaeranthus indicus* whole plant

Groups	Treatment	Dose	Plaque forming cells X 10^6
I	0.1 mL SRBC + Control	-	540 ±3.395
II	0.1 mL SRBC + Methanolic extract of *Sphaeranthus indicus*	100 mg/kg bd.wt.	655.33±4.957 **, b
III	0.1 mL SRBC + Methanolic extract of *Sphaeranthus indicus*	200 mg/kg bd.wt.	620.83±3.70**, a
IV	0.1 mL SRBC + Methanolic extract of *Sphaeranthus indicus*	400 mg/kg bd.wt.	563±1.366**, a
V	0.1 mL SRBC + Standard (levamisole)	50 mg/kg bd.wt.	678.833±1.701**

Values are expressed as mean± SEM, (n=6). All the groups were compared with control group and standard group (Dunnett's t- test). Significant values are expressed as control (=p<0.01), standard (a=p<0.01, b=P<0.05).**

Quantitative haemolysis of SRBC (QHS) assay of methanolic extract of *S. indicus*

Spleens should be removed and a cell suspension of 1×10^6 cells /mL was prepared in PBS.

1 mL of 0.2% SRBC + 1 mL of 10% rabbit serum were mixed with cell suspension and incubated for 1h at 37°C.

After centrifugation at 3000 rpm for 3 min, optical density of the supernatant was measured at 413 nm using a spectrophotometer[19].

QHS for or methanolic extract of *sphaeranthus indicus* whole plant

Groups	Treatment	Dose	O. D. X 10 X 10^6
I	0.1 mL SRBC + Control	-	0.661±0.0054
II	0.1 mL SRBC + Methanolic extract of *Sphaeranthus indicus*	100 mg/kg bd.wt.	$0.769±0.01004^{**, b}$
III	0.1 mL SRBC + Methanolic extract of *Sphaeranthus indicus*	200 mg/kg bd.wt.	$0.745±0.0017^{**, a}$
IV	0.1 mL SRBC + Methanolic extract of *Sphaeranthus indicus*	400 mg/kg bd.wt.	$0.707±0.0127^{**, a}$
V	0.1 mL SRBC + Standard (levamisole)	50 mg/kg bd.wt.	$0.88±0.1124^{**}$

Values are expressed as mean± SEM, (n=6). All the groups were compared with control group and standard group (Dunnett's t- test). Significant values are expressed as control (=p<0.01), standard (a=p<0.01, b=P<0.05).**

In vivo immunomodulatory activities

- **Haemagglutination titre test**
- **Delayed type hypersensitivity**
- **T- cell population**
- **Drug induced myelosuppression**

Haemagglutination titer of methanolic extract of *S. indicus*

- On 0 day animals were immunized with 0.1mL containing 1×10^8 cells of sheep red blood cells (SRBC) *i.p.*
- Animals were administered with different doses of methanolic extract of *S. indicus* (i.e., 100, 200 and 400 mg/kg bd.wt.) for 7 days.
- On 7^{th} day blood was withdrawn.
- Blood was centrifuged and serum was separated.
- 50µl + 50µl of SRBC and serum was added in titre plate and incubated at 30º C for 1h.
- Serial two fold dilutions were made in titer plate.
- Agglutination was observed[20].

Haemagglutination titer test for methanolic extract *Sphaeranthus indicus* whole plant

Groups	Treatment	Dose	Antibody titre
I	0.1 mL SRBC + Control	-	1.33±0.21
II	0.1 mL SRBC + Methanolic extract of *Sphaeranthus indicus*	100 mg/kg bd.wt.	6.166±0.307**, a
III	0.1 mL SRBC + Methanolic extract of *Sphaeranthus indicus*	200 mg/kg bd.wt.	4.16.±0.301 **, a
IV	0.1 mL SRBC + Methanolic extract of *Sphaeranthus indicus*	400 mg/kg bd.wt.	3.16±307**, a
V	0.1 mL SRBC + Standard (levamisole)	50 mg/kg bd.wt.	7.56±0.333**

Values are expressed as mean± SEM, (n=6). All the groups were compared with control group and standard group (Dunnett's t- test). Significant values are expressed as control (=p<0.01), standard (a=p<0.01).**

Delayed type hypersensitivity of methanolic extract of *S. indicus*

On 0 day animals were immunized with 0.1mL containing 1×10^8 cells of sheep red blood cells (SRBC) *i.p.*

The animals were administered with different doses of methanolic extract of *S. indicus* for 7 days (i.e., 100, 200 and 400 mg/kg bd.wt.)

On 7^{th} day prior to injection, right hind footpad thickness was measured with micrometer screw gauge (Mitutoyo digimatic).

Then the animals were challenged by injecting 1% SRBC (20 µL) into the right hind footpad.

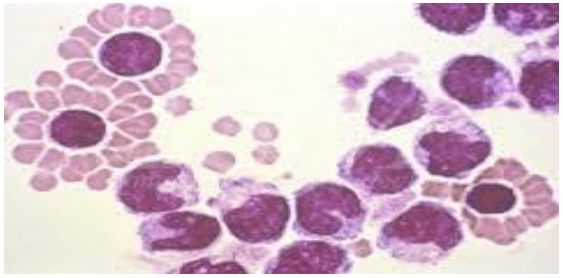
Difference between prior and post challenge footpad thickness was measured as DTH response[21].

Delayed type hypersensitivity of methanolic extract of *Sphaeranthus indicus* whole plant

Groups	Treatment	Dose	DTH response (mm) 24 h	DTH response (mm) 48 h
I	0.1 mL SRBC + Control	-	0.2453 ±0.0053	0.2413 ± 0.0053
II	0.1 mL SRBC + Methanolic extract of *Sphaeranthus indicus*	100 mg/kg bd. wt.	0.564 ±0.0317 **, a	0.507 ±0.0236 **, a
III	0.1 mL SRBC + Methanolic extract of *Sphaeranthus indicus*	200 mg/kg bd. wt.	0.730±0.0253 **, a	0.661 ±0.0335 **, a
IV	0.1 mL SRBC + Methanolic extract of *Sphaeranthus indicus*	400 mg/kg bd. wt.	1.302±0.0158 **, a	1.166±0.0592 **, a
V	0.1 mL SRBC + Standard (levamisole)	50 mg/kg bd. wt.	1.666 ±0.0435 **	1.533 ± 0.0269 **

Values are expressed as mean± SEM, (n=6). All the groups were compared with control group and standard group (Dunnett's t- test). Significant values are expressed as control (=p<0.01), standard (a=p<0.01).**

T- cell population of methanolic extract of *S. indicus*



The animals were administered with different doses of methanolic extract of *S. indicus* for 10 days (i.e., 100, 200 and 400 mg/kg bd.wt.)

On 11th day blood was withdrawn and anticoagulated with Alsever's solution in test tubes.

These test tubes should be kept in slant position such that RBC was settled down.

Supernatant was collected which contains lymphocytes,

50 µL of lymphocyte suspension + 50 µL of SRBC was incubated for an hour.

A drop of resultant suspension was taken on glass slide and observed for lymphocytes and rosettes under microscope[22].

Lymphocytes for methanolic extract of *Sphaeranthus* indicus whole plant

Groups	Treatment	Dose	Lymphocytes count	Rosettes count
I	0.1 mL SRBC + Control	-	131±1.932	10.00±0.365
II	0.1 mL SRBC + Methanolic extract of *Sphaeranthus indicus*	100 mg/kg bd.wt.	150.833±1.641$^{**, a}$	15.61±0.477$^{**, a}$
III	0.1 mL SRBC + Methanolic extract of *Sphaeranthus indicus*	200 mg/kg bd.wt.	159.166±0.0909$^{**, a}$	17.833±0.654$^{**, a}$
IV	0.1 mL SRBC + Methanolic extract of *Sphaeranthus indicus*	400 mg/kg bd.wt.	169±1.033$^{**, a}$	20±0.577$^{**, a}$
V	0.1 mL SRBC + Standard (levamisole)	50 mg/kg bd.wt.	171±1.713**	22±0.856**

Values are expressed as mean± SEM, (n=6). All the groups were compared with control group and standard group (Dunnett's t- test). Significant values are expressed as control (=p<0.01), standard (a=p<0.01).**

Drug induced myleosuppression of methanolic extract of *S. indicus*

On 0 day blood was withdrawn from animals and subjected to haematological parameters .

The animals were administered with different doses of methanolic extract of *S. indicus* for 14 days (i.e., 100, 200 and 400 mg/kg bd.wt.)

On 11^{th}, 12^{th} and 13^{th} day azathioprine was administered.

On 14^{th} day blood was again withdrawn and subjected to haematological parameters[14].

Drug induced myleosuppression - RBC count

Groups	Treatment	Dose	RBC count ($\times 10^6/mm^3$) 0 day	RBC count ($\times 10^6/mm^3$) 14th day
I	0.1 mL SRBC + Control	-	12.19±0.1816	12.03±0.2305
II	0.1 mL SRBC + Negative control (azathioprine)	2 mg/kg bd.wt.	11.22±0.248	5.61±0.3090**, a
III	0.1 mL SRBC + Methanolic extract of *Sphaeranthus indicus* + antagonist (azathioprine)	100 mg/kg bd.wt.	11.35±0.124	9.42±0.468**, a
IV	0.1 mL SRBC + Methanolic extract of *Sphaeranthus indicus* + antagonist (azathioprine)	200 mg/kg bd.wt.	12.80±0.124	9.60±0.318**, a
V	0.1 mL SRBC + Methanolic extract of *Sphaeranthus indicus* + antagonist (azathioprine)	400 mg/kg bd.wt.	14.10±0.255	12.69±0.525*,a
VI	0.1 mL SRBC + Standard (levamisole)	50 mg/kg bd.wt.	14.81±0.340	15.14±0.3090**

Values are expressed as mean±SEM, (n=6). All the groups were compared with control group and standard group (Dunnett's t- test). Significant values are expressed as control (=p<0.01, *=p<0.05), standard (a=p<0.01).**

Drug Induced Myleosuppression - WBC Count

Groups	Treatment	Dose	WBC count ($\times 10^3/mm^3$) 0 day	WBC count ($\times 10^3/mm^3$) 14th day
I	0.1 mL SRBC + Control	-	9.82±0.214	9.68±0.4282
II	0.1 mL SRBC + Negative control (azathioprine)	2 mg/kg bd.wt.	9.01±0.3117	4.75±0.396**, a
III	0.1 mL SRBC + Methanolic extract of *Sphaeranthus indicus* + antagonist (azathioprine)	100 mg/kg bd.wt.	9.80±0.301	7.59±0.360**, a
IV	0.1 mL SRBC + Methanolic extract of *Sphaeranthus indicus* + antagonist (azathioprine)	200 mg/kg bd.wt.	10.24±0.216	8.33±0.208**, a
V	0.1 mL SRBC + Methanolic extract of *Sphaeranthus indicus* + antagonist (azathioprine)	400 mg/kg bd.wt.	10.47±0.198	9.67±0.207*, a
VI	0.1 mL SRBC + Standard (levamisole)	50 mg/kg bd.wt.	11.285±0.22	11.97±0.287**

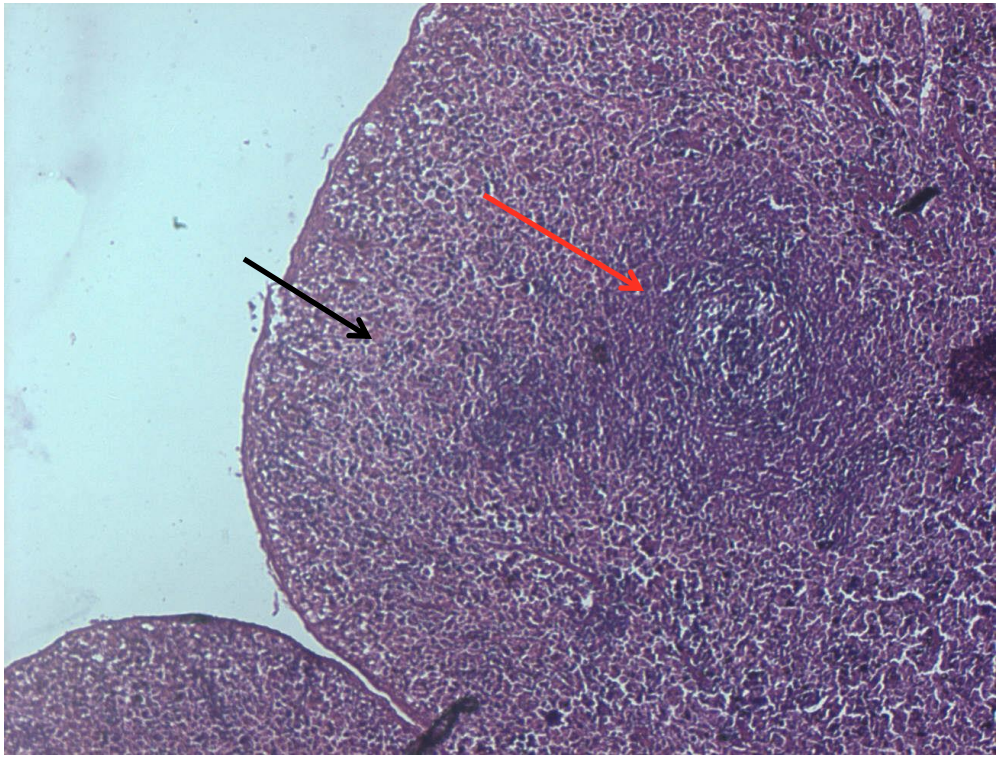
Values are expressed as mean±SEM, (n=6). All the groups were compared with control group and standard group (Dunnett's t- test). Significant values are expressed as control (=p<0.01, *=p<0.05), standard (a=p<0.01).**

Drug Induced Myleosuppression - Hb count(g/dL)

Groups	Treatment	Dose	Hb count (g/dL) 0 day	Hb count (g/dL) 14th day
I	0.1 mL SRBC + Control	-	10.23±0.274	10.08±0.239
II	0.1 mL SRBC + Negative control (azathioprine)	2 mg/kg bd.wt.	9.39±0.34	6.12±0.194**, a
III	0.1 mL SRBC + Methanolic extract of *Sphaeranthus indicus* + antagonist (azathioprine)	100 mg/kg bd.wt.	9.91±0.31	8.83±0.274**, a
IV	0.1 mL SRBC + Methanolic extract of *Sphaeranthus indicus* + antagonist (azathioprine)	200 mg/kg bd.wt.	10.51±0.343	8.93±0.189*, a
V	0.1 mL SRBC + Methanolic extract of *Sphaeranthus indicus* + antagonist (azathioprine)	400 mg/kg bd.wt.	11.23±0.231	9.65±0.327*, a
VI	0.1 mL SRBC + Standard (levamisole)	50 mg/kg bd.wt.	11.47±0.187	11.74±0.256**

Values are expressed as mean±SEM, (n=6). All the groups were compared with control group and standard group (Dunnett's t- test). Significant values are expressed as control (=p<0.01, *=p<0.05), standard (a=p<0.01).**

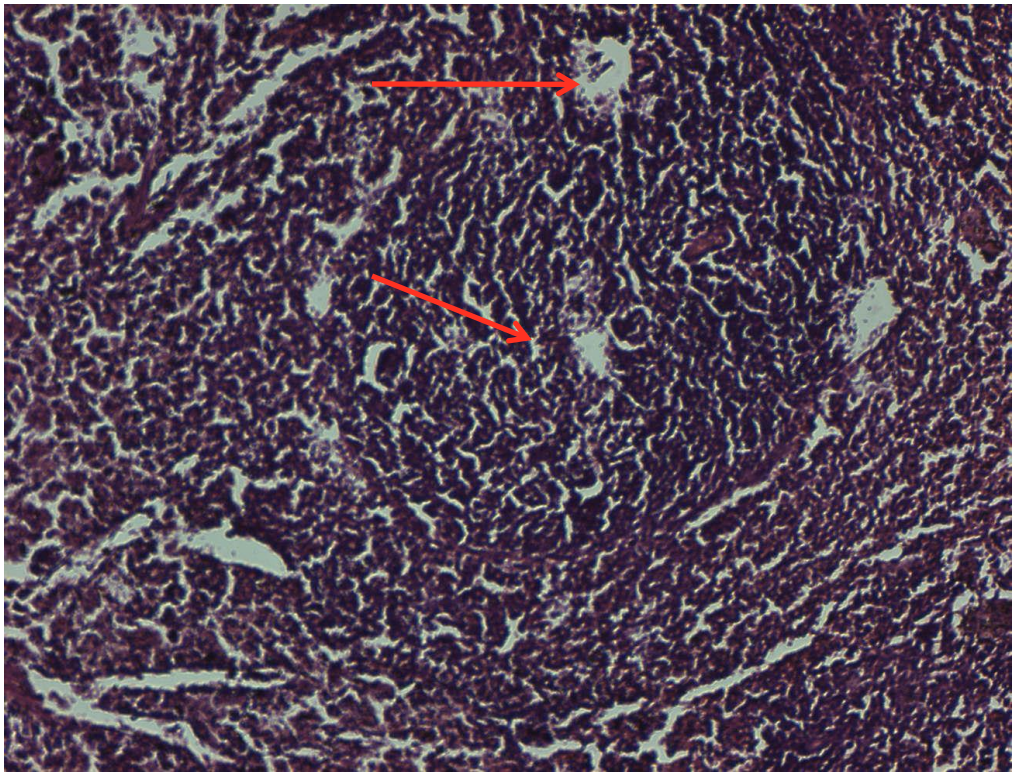
Histopathology studies



Histopathology of Control Rat Spleen

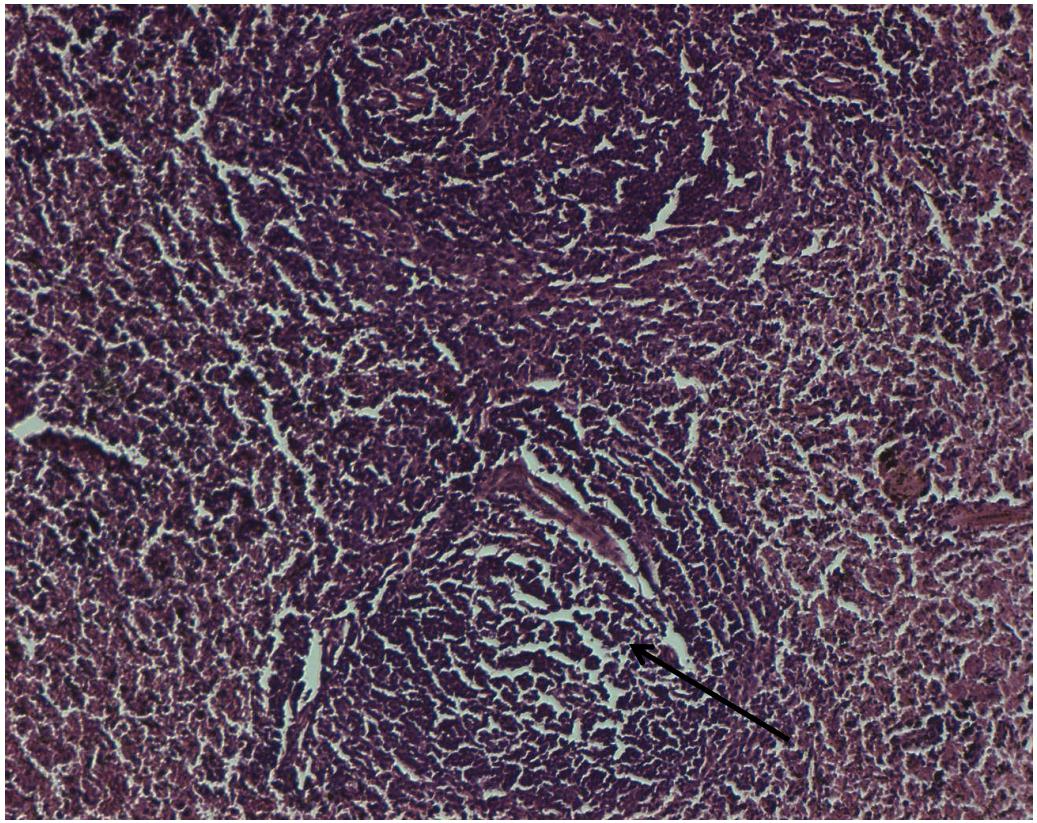
Spleenic cortex appeared normal – black arrow

Lymphatic follicles located in the medullary region appeared normal – red arrow



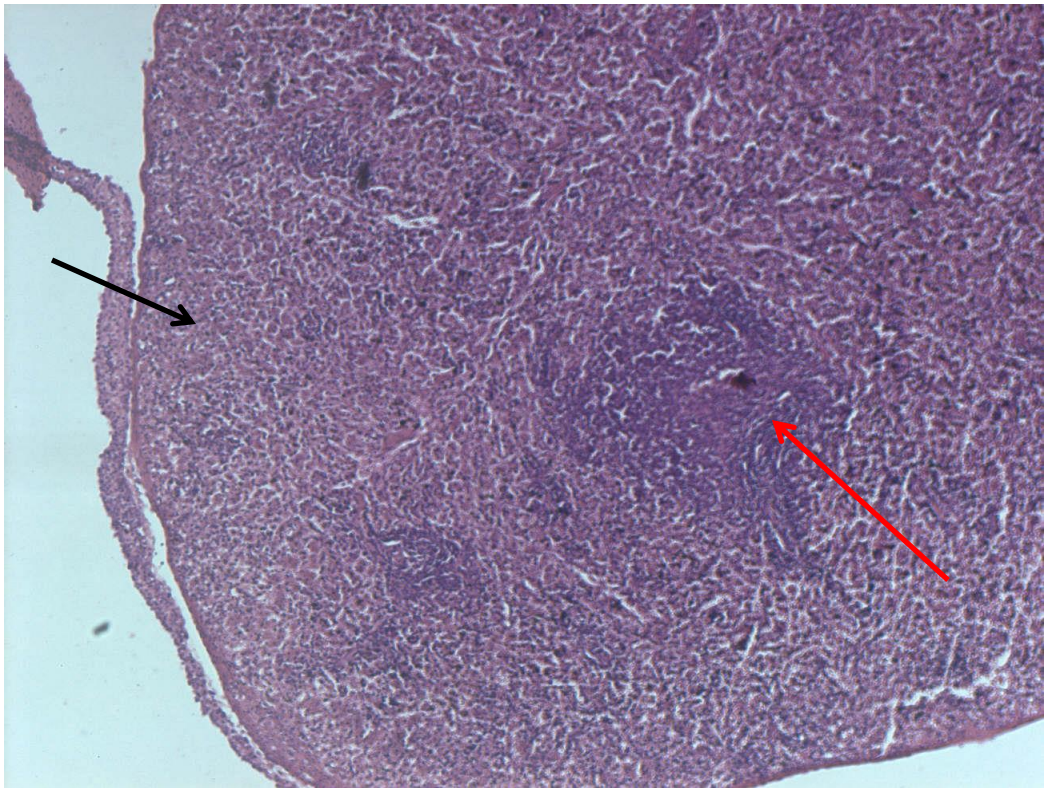
Histopathology of rat spleen treated with 100 mg/kg bd.wt. methanolic extract of *S. indicus*.

Mild to moderate lymphoid depletion noticed in the lymphatic follicles located in the medullary region of spleen – red arrow.



Histopathology of rat spleen treated with 200 mg/kg bd.wt. methanolic extract of *S. indicus*.

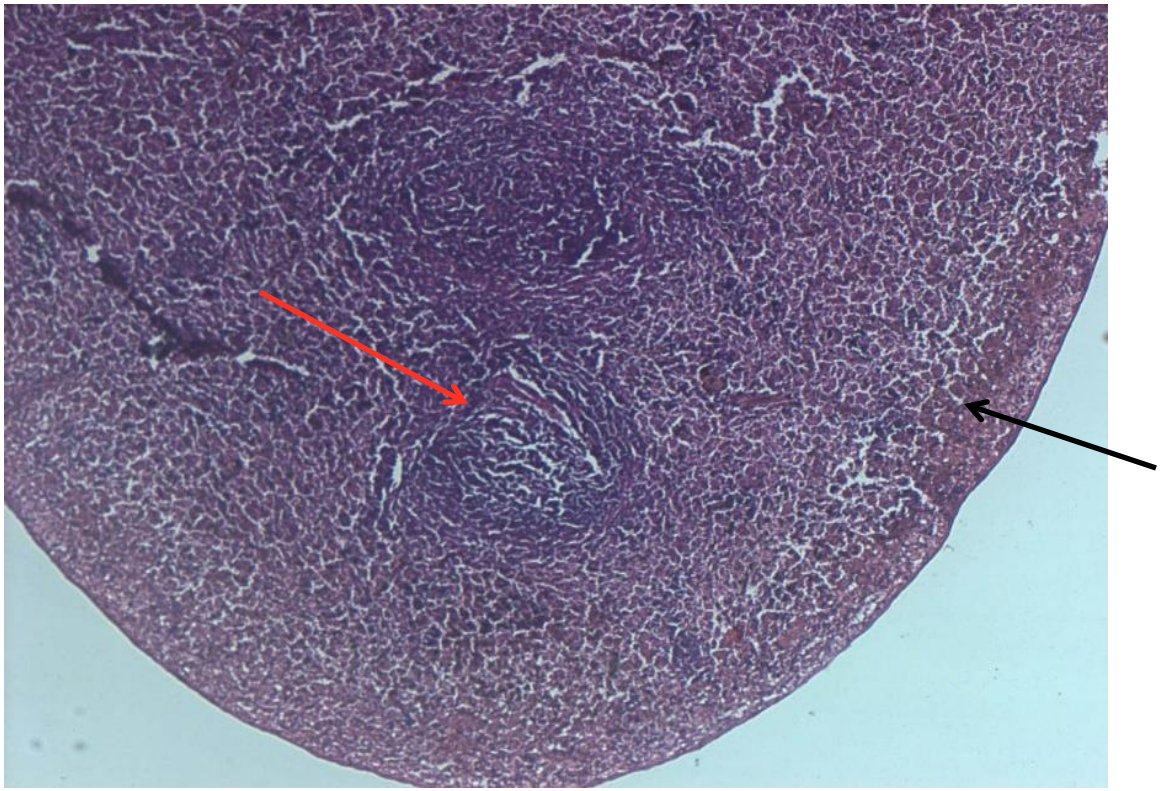
Mild to moderate lymphoid depletion noticed in the lymphatic follicles located in the medullary region of spleen- black arrow.



Histopathology of rat spleen treated with 400 mg/kg bd.wt. methanolic extract of *S. indicus.*

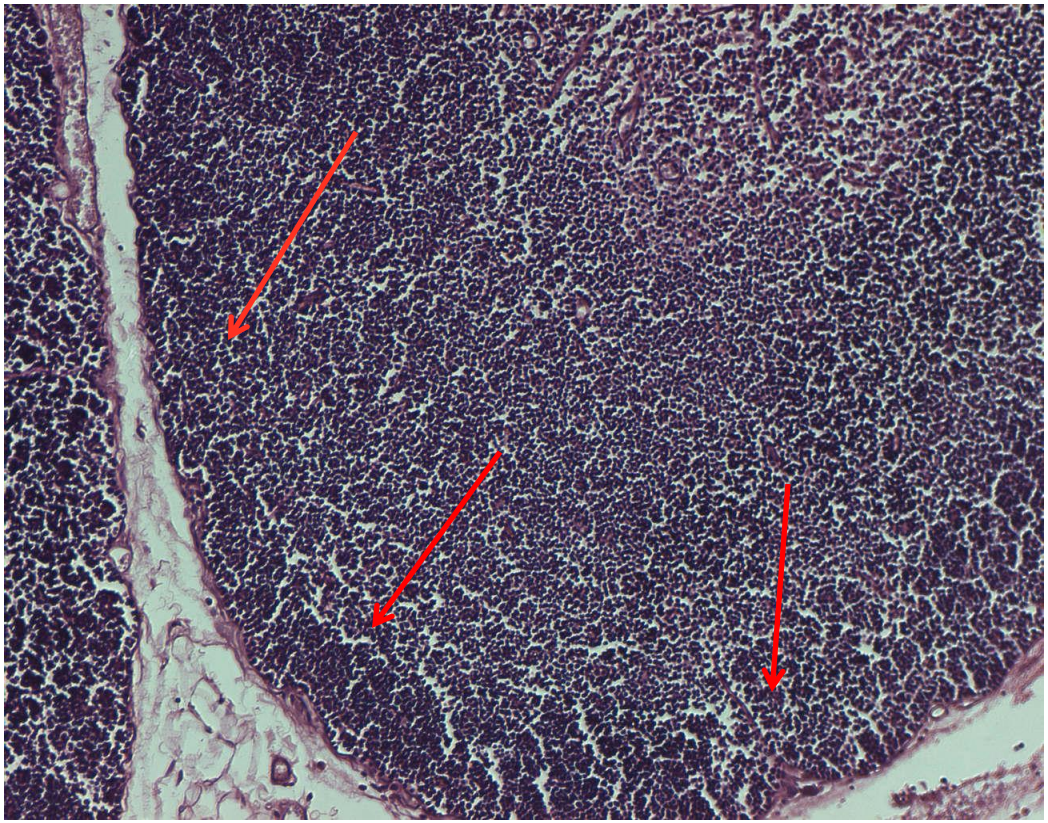
Spleenic cortex appeared normal – black arrow

Mild to moderate lymphoid follicular atrophy noticed in few follicles – Red arrow.



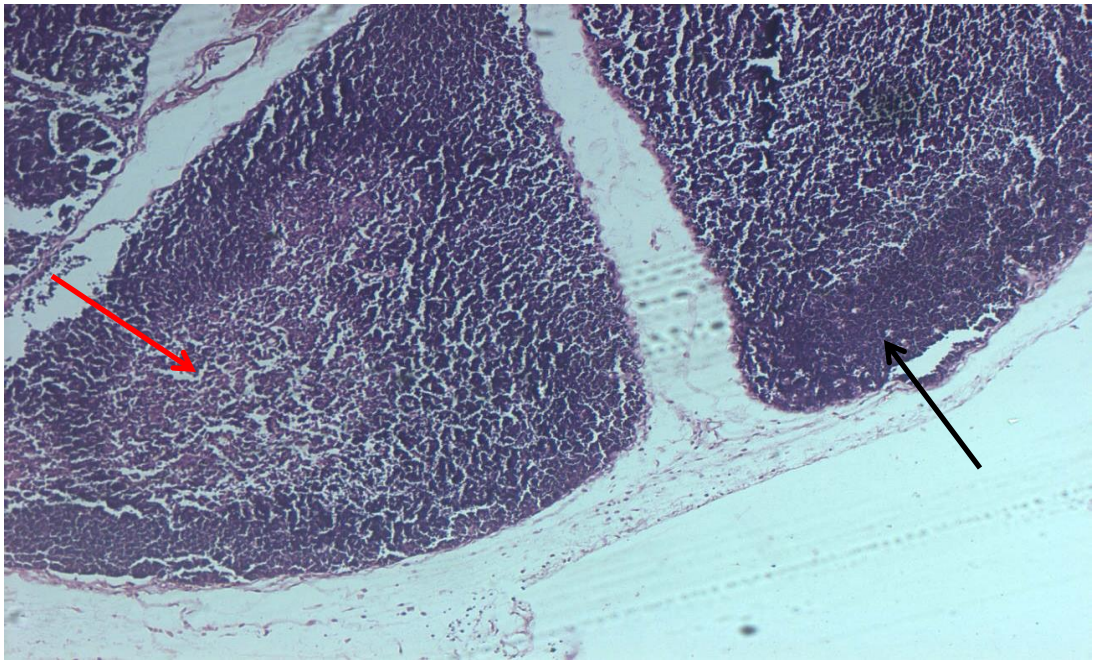
Histopathology of Rat Spleen treated with standard (levamisole 50 mg/kg bd.wt)

Spleenic cortex appeared normal and it contain large amount of RBCs- black arrow. Medullary region containing lymphatic follicles showed mild atrophy and lymphoid depletion- red arrow.



Histopathology of Control Rat thymus

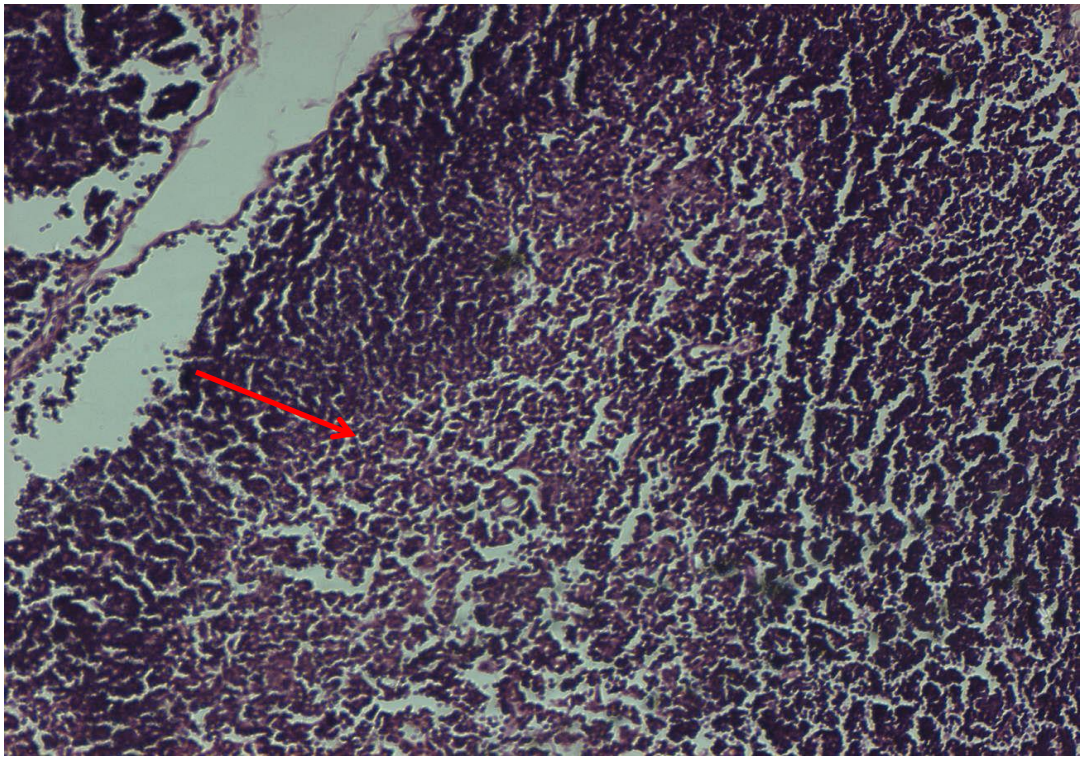
Cortex region of thymus appeared normal [red arrow] it contain pockets/ spread of lymphocytes appeared normal.



Histopathology of rat thymus treated with 100 mg/kg bd.wt. methanolic extract of *S. indicus*.

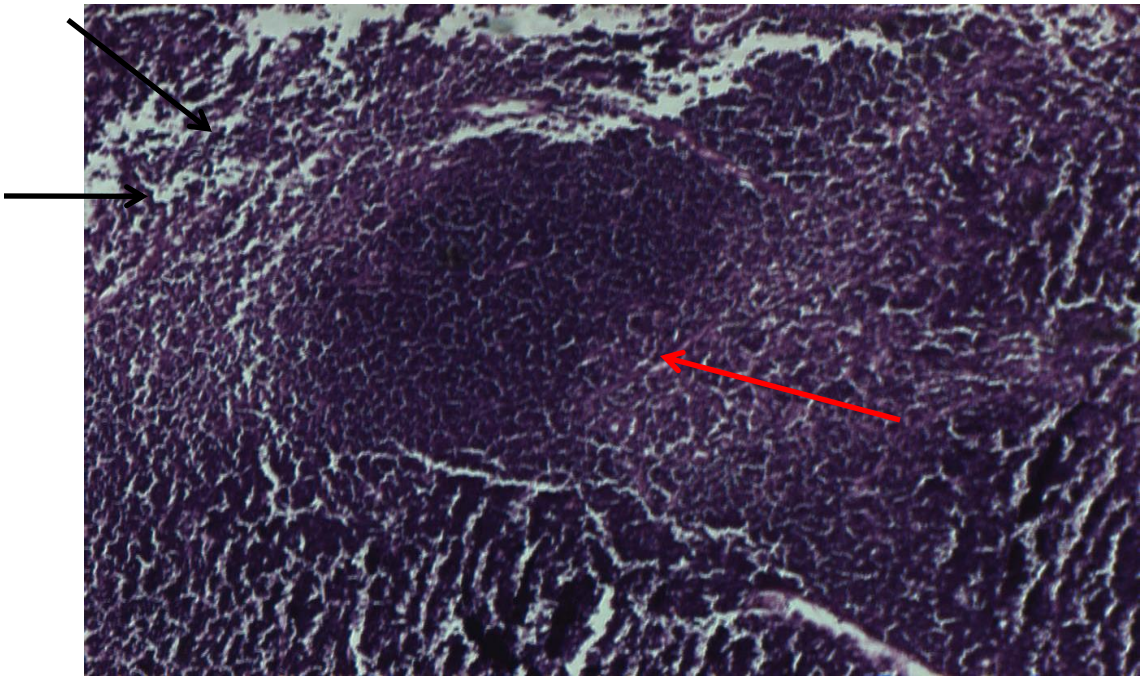
Thymic atrophy/fibrosis noticed in the medullary region of thymus - Red arrow

Cortex region containing lymphoid pockets with follicles appeared normal- black arrow.



Histopathology of rat thymus treated with 200 mg/kg bd.wt. methanolic extract of *S. indicus*.

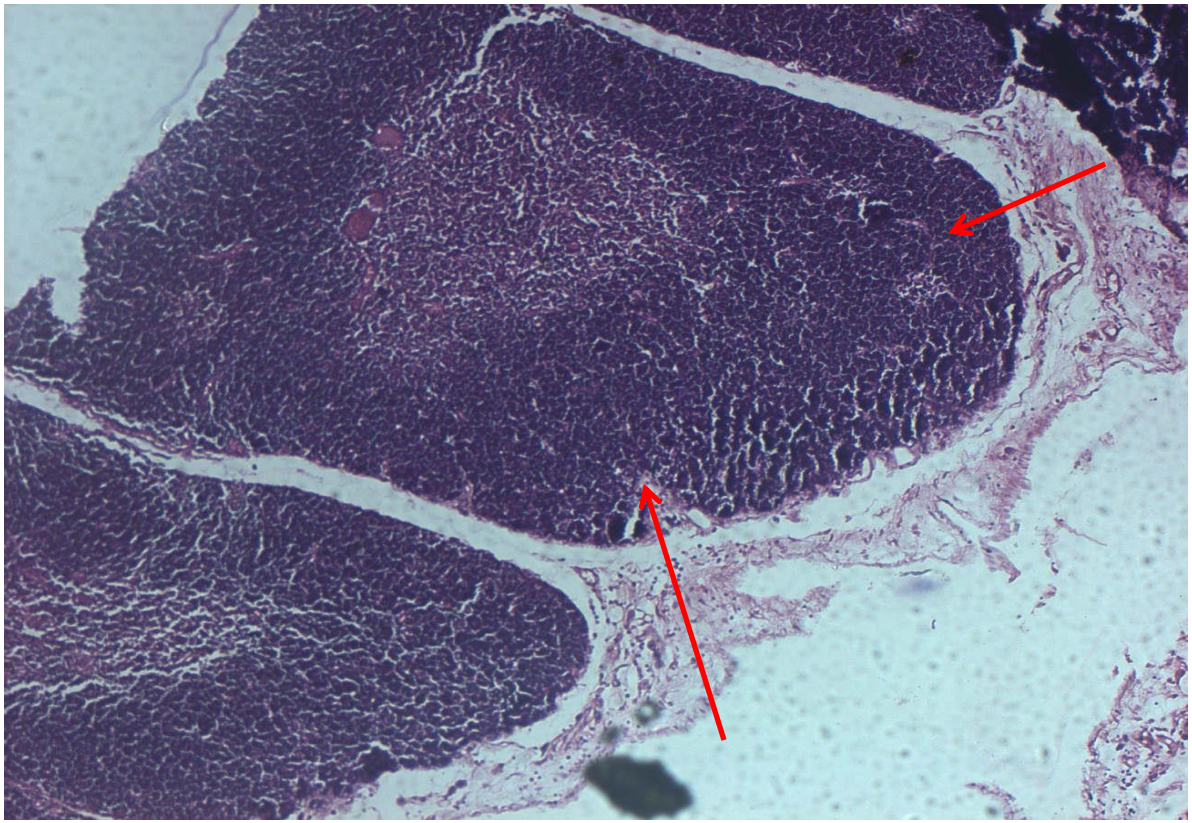
Thymic atrophy / fibrosis noticed in the medullary region of thymus– red arrow



Histopathology of rat thymus treated with 400 mg/kg bd.wt. methanolic extract of *S. indicus*.

Medullary region appeared normal – red arrow

Mild to moderate lymphoid cells proliferation or follicular hypertrophy noticed the cortex region of thymus – black arrow.



Histopathology of rat thymus treated with standard (levamisole 50 mg/kg bd.wt.)

Cortex region appeared normal it contain large amount of lymphocytes appeared as follicles- red arrow.

Histopathology Studies

The histopathology studies of spleen and thymus gland further conformed the immunostimulatory activity of the methanolic extract of *Sphaeranthus indicus*.

The methanolic extract had shown the significant protective effect from SRBC indicated by mild atrophy and lymphoid depletion in the spleen and thymus gland.

Even there was moderate follicular atrophy in the cortical region of the thymus gland.

Its effect was comparable to that of standard levamisole. Earlier studies reported that saponins, flavonoids and glycosides are most likely candidates eliciting the immunostimulatory activity.

Hence the immunostimulant activity of the methanolic extract of *Sphaeranthus indicus* can be attributed to the phenols, glycosides and saponins present in it.

Conclusions

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Certificate

This is certify that the project title "Screening of immunomodulatory activity of Sphaeranthus indicus Linn: whole plant" has been approved by the IAEC.

Name of Chairman/ Member Secretary IAEC: Dr. U. Ganji

Name of CPCSEA nominee:

Signature with date 17/02/2015

Chairman/ Member Secretary of IAEC:

CPCSEA nominee:

(Kindly make sure that minutes of the meeting duly signed by all the participants are maintained by Office)

THANK YOU