CHAPTER 6

ANTI-HAEMORRHOID ACTIVITY OF SILVER NANOPARTICLES

6. Anti-haemorrhoid activity of silver nanoparticles

6.1 Experimental works

6.1.1 Experimental Animals

For assessing anti-hemorrhoid activity, healthy (weight between 220–250 g) and adult (8–10 weeks old) Wistar rats, whereas for acute and sub-acute oral toxicity study, female Charles foster rats were obtained from the central animal house of Institute of Medical Sciences, B.H.U. Rats were acclimatized in groups of six in polypropylene cages at 25 ± 1°C (ambient temperature) and at relative humidity of 45-55%, with a 12:12 hours of light/dark cycle Animals were given a regular pellet diet and free access to water *ad libitum* and were acclimatized for at least one week before commencement of the experiment. All experimental protocols were carried out in accordance with the IAEC (Institutional Animal Ethics Committee) guideline and clearance (letter No. Dean/2019/IAEC/1641).

6.1.2 Acute and sub-acute oral toxicity study

For acute oral toxicity study, PLSNPs (2000 mg/kg p.o.) was given to five female rats (fasted overnight) and observed (individually for 48 hours) to measure any behavioral and neurological changes viz. salivation, tremors, sezuires, loose bowel, sleep and lacrimation, in order to get indication of acute toxicity (OECD guidelines 425) [104].

Sub-acute oral toxicity study was performed as per the guidelines of OECD 407 [175]. The experimental animals were divided into three groups containing ten (five male and five female) Wistar rats in each group. First two group animals were treated with PLSNPs at a dose of 500 mg/kg and 1000 mg/kg; p.o. while the third (control) group was treated with vehicle (2% tween 80 in DW) once daily for 28 days. During the observation period, any sign of toxicity and mortality was closely monitered and reported. In addition, the animals were observed for

changes in body weight, food and water consumption on weekly basis. At the end of the experiment, final observations were made and the blood samples were collected through cardiac puncture. Furthermore, the vital organs were disected rinsed with normal saline and organ wieght was calculated followed by the histology. For the assessment of bichemical parameters, the blood samples were centrifuged at 3000 rpm for 15 min and serum was separated. Different diagnostic kits were used for the assessment of biochemical parameters. Reference varies for comparison and are dependent on the method of analysis, animal type including other experimental factors. Thus, in current study, the datas obtained for the control group were considered as the reference values; and statistical analysis was conducted against the control group [175-177].

6.1.3 Anti-haemorrhoid activity

6.1.3.1 Induction of haemorrhoids

For induction of haemorrhoids, a croton oil preparation (COP) containing 1:4:5:10 ratio of deionized water, pyridine, diethyl ether (S D Fine-Chem Limited, Mumbai, India) and 6% croton oil (Sigma Aldrich, St, Louis Mo. U.S.A.) in diethyl ether. Cotton swabs (Sterile) of 4 mm diameter soaked in 100 µl of COP was inserted into the anus of rats (fasted overnight) up to recto-anal portion (20 mm deep into the anal opening) and kept as it is for 10 s. After the COP application, all animals were observed up to 7–8 h for the development of edema. Haemorrhoids were tested in two independent groups of rats: in the first set, plasma exudation of Evans blue dye was measured, while in the second, hemorrhoidal severity, biochemical, and histological parameters were investigated [105, 106].

6.1.3.2 Grouping and treatment:

The rats were divided into seven groups i.e. Group I: Normal control (NC) group administered with 1% tween 80 (5 ml/kg, p.o.); Group II: Haemorrhoid control (DC) group administered with 1% tween 80 (5 ml/kg, p.o.); Group III: Haemorrhoid induced group treated with EAF suspension prepared using tween 80 at 100 mg/kg, p.o.; Group IV: Haemorrhoid induced group treated with EAF suspension prepared using tween 80 at 200 mg/kg, p.o.; Group V: Haemorrhoid induced group treated with PLSNPs in DW at 100 mg/kg, p.o.; Group VI: Haemorrhoid induced group treated with PLSNPs in DW at 200 mg/kg, p.o. and Group VII: Haemorrhoid induced group treated with Standard Pilex granules in DW (200 mg/kg, p.o., Himalaya Drug Company, Bengaluru, India). EAF, PLSNPs and standard Pilex granules were administered once daily for seven days.

6.1.3.3 Evaluation of hemorrhoidal parameters

On the eighth day blood (1.5 ml) was withdrawn through retro-orbital plexus of the rats from the second set [108], for estimating biochemical parameters. Later, recto-anal tissue (20 mm) were dissected and weighed after sacrificing the animals after anesthizing them using thiopental sodium. After, placing a small portion of the tissue in 10% formaldehyde solution for histological examination and the rest was stored at -20 °C for estimation of biochemical parameter.

For determining the recto-anal coefficient (RAC) the recto-anal tissues of the rats were weighed and compared with the individual body weight of the rats and was calculated using the following formula: RAC = Weight of recto-anal tissue (mg) / Body weight (g).

The transverse section of recto-anal tissue was taken using microtome and was then examined for the presence of inflammatory cells, necrosis, congestion, haemorrhage and vasodilatation[105, 107]. Further, the macroscopic severity scoring of tissues was also carried out after mounting it on a white paper and inflammation score (from 0-2) was noted as described by Azeemuddin *et al.* (2014) [105].

6.1.3.4 Estimation of TNF- α and IL-6 in serum

The level of tumor necrosis factors (TNF-α) and interleukins (IL-6) present in serum seperated from the blood was determined using enzyme-linked immune sorbent assay kits following the manufacturers guidelines (Krishgen Biosystems, USA).

6.1.3.5 Histology and histomorphological scoring

For histological examination, previously fixed recto-anal tissue in 10% formalin was processed following a conventional procedure in order to prepare microscopic sections with a thickness of 5 mm, and subsequently stained with hematoxylin (H) and eosin (E). To ascertain any pathological changes occurred in the tissues, the prepared slides were examined under Nikon Eclipse E200 microscope. For describing the severity of lesions, the wound area was determined on an arbitrary scale i.e. – corresponds to 0-5%, + corresponds to 5-10%, ++ corresponds to 10-25%, +++ corresponds to 25-50% and ++++ corresponds to 50% & above [107]. Validation of a histomorphological scale was done to assess the severity of hemorrhoids in comparison to recto-anal tissue of a normal rat. Lesions in recto-anal tissue of rat in all groups were scored based on the histomorphological scale in a blinded fashion for evaluating the sevirity. The normal recto-anal tissue was considered as the best possible outcome and was scored as 28 (Maximum total score).

6.1.4 *In vivo* antioxidant studies

On the eighth day, at the end of the study, a portion of recto-anal tissue isolated after sacrificing the animals was rinsed with ice-cold phosphate buffered saline (0.1 M, pH 7.4) thoroughly the tissue was homogenised in 1.15% KCl and centrifuged at 16,000 × g for one hour at 0 °C to prepare a 10% w/v suspension., which was utilized for evaluation of *in vivo* antioxidant activity *viz*. lipid peroxidation (LPO), catalase (CAT) and superoxide dismutase (SOD) activity as per standard procedures [109-111].

6.1.5 Statistical analysis

The experimental results are represented as mean ± SEM (n=6) followed by one-way ANOVA (Tukey's test), whereas for analyzing score values Kruskal-Wallis (Dunn's) test was performed. GraphPad Prism 5.03 software was used for analyzing the data statistically and $p < 0.05$ was considered significant.

6.2 Results

6.2.1 Acute and subacute oral toxicity of PLSNPs

Upon administration of single dose of PLSNPs (2000 mg/kg) in acute toxicity test, there was no death, or any sign of toxicity observed and intake of water and food in all the animals remained unchanged. Thereafter, neither a sign of behavioural toxicity nor neurological toxicity was observed in experimental animals in the entire study period. Hence, the LD_{50} of the PLSNPs for Wistar female rats was higher than 2000 mg/kg body weight.

In the entire duration of subacute toxicity study, all the animals were active with no change in behaviour and at the end of study (29^{th} day) all the animals were alive. Relative organ weight was unaffected by the treatment of PLSNPs as there was no significant difference observed in the weight of male and female vital organs treated with PLSNPs **(Table 6.1).**

Table 6. 1- Organ weights (g) of the experimental animals in the sub-acute toxicity study of PLSNPs

Organs	Female*			Male*		
	Control	PLSNP 500 mg/kg	PLSNP 1000 mg/kg	Control	PLSNP 500 mg/kg	PLSNP 1000 mg/kg
Lungs	1.466±0.13	1.59±0.10	1.506±0.13	1.516±0.08	1.614±0.07	1.542±0.11
Kidney	0.96±0.03	0.966±0.03	1.072±0.11	0.872±0.05	0.836±0.05	0.856±0.06
Liver	7.004±0.11	6.822±0.18	6.926±0.28	7.954±0.12	7.404±0.40	7.626±.22
Heart	0.83±0.04	0.778±0.06	0.786±0.09	0.81±0.04	0.832±0.06	0.774±0.08

*The results are average of three determinations ± SEM (n= 5)

Furthermore, the results of haematological and biochemical evaluation revealed no significant toxic effect in the treatment groups which is evident from **Table 6.2 and 6.3**. Histology results of selected vital organs (heart, kidney, liver) and recto-anal portion were in accordance with the haematological and biochemical results, confirming PLSNPs to be non-toxic **(Figure 6.1).** Therefore, PLSNPs were found to be safe at the maximum dose of 1000 mg/kg throughout the treatment period of 28 days**.**

Table 6. 2- Serum hematological values of experimental animals orally treated with PLSNPs

Hematological parameters	Female*			Male*		
	Control	PLSNP 500 mg/kg	PLSNP 1000 mg/kg	Control	PLSNP 500 mg/kg	PLSNP 1000 mg/kg
Hb (g/L)	152.792± 2.6	151.264±2.76	154.666±2.29	152.372±2.31	53.14±2.58	152.936±1.99
RBC (*10^6/mm^3)	7.354±0.25	8.028±0.26	8.052±0.22	7.692±0.22	8.16±0.19	8.246±0.15
PCV (%)	47.556±0.71	46.406±1.17	47.042±0.28	47.754±0.73	48.204±0.55	47.826±0.38
WBC (*10^3/mm^3	9.942±0.33	10.34±0.39	10.708±0.19	10.434±0.26	11.172±0.30	11.834±0.12
Platelets (*10^3/mL)	319.45±13.03	348.78±24.24	390.62±12.22	327.01±13.53	29.03±14.34	330.37±13.60

Neutrophils ($*10^3/mm^3$)	2.21±0.26	2.482±0.18	2.576±0.21	3.272±0.18	3.506±0.14	3.29±0.19
Lymphocytes ($*10^3/mm^3$)	5.942±0.15	5.544±0.29	5.998±0.18	5.208±0.17	5.706±0.27	5.232±0.17
Eosinophils ($*10^3/mm^3$)	0.0616±0.01	0.0646±0.01	0.073±0.01	0.0822±0.01	0.0684±0.01	0.0798±0.01
Monocytes ($*10^3/mm^3$)	0.0368±0.01	0.0268±0.01	0.0262±0.01	0.0222±0.01	0.0218±0.01	0.0256±0.01
Basophils ($*10^3/mm^3$)	0.008±0.03	0.0076±0.02	0.005±0.02	0.0082±0.03	0.0058±0.02	0.0046±0.01

*The results are average of three determinations ± SEM (n= 5)
The values were non-significant ($p<0.05$) when compared to control group.

Table 6. 3- Biochemical parameters in the serum of experimental rats treated orally with PLSNPs.

Biochemical	**Female***			**Male***		
Parameters	**Control**	**PLSNP 500 mg/kg**	**PLSNP 1000 mg/kg**	**Control**	**PLSNP 500 mg/kg**	**PLSNP 1000 mg/kg**
ALP (IU/L)	146.6±12.91	159±9.81	150.6±12.96	151.6±7.55	161.4±7.09	154.2±9.82
Bilirubin (mg	2.952±0.21	2.966±0.03	3.072±0.11	2.872±0.05	3.02±0.07	3.016±0.08
AST(IU/L)	209.2±6.06	215.6±5.56	210.926±4.12	204.954±5.31	209.404±5.67	207.826±4.76
ALT (IU/L)	53.43±2.32	49.378±1.73	55.786±2.75	50.21±2.66	51.832±3.40	50.374±1.88

*The results are average of three determinations ± SEM (n= 5)

The values were non-significant ($p<0.05$) when compared to control group.

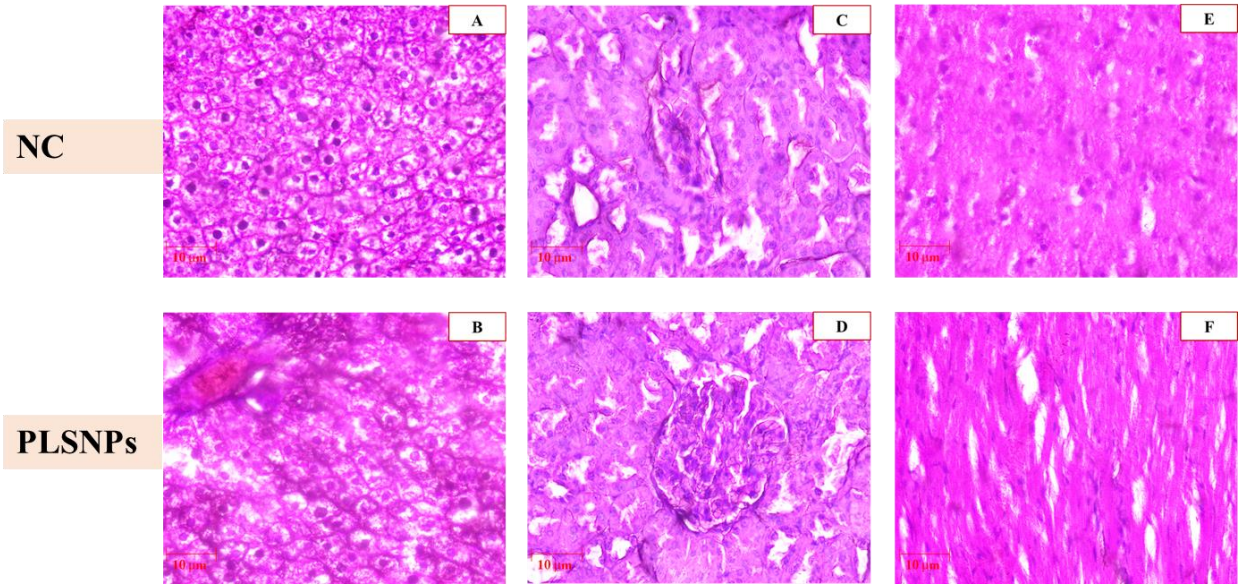

Figure 6. 1: Histological sections of liver (A & B), kidney (C & D), and heart (E & F) of NC and PLSNPs (1000 mg/kg; p.o.) treated groups

6.2.2 Anti-haemorrhoid activity

The recto-anal coefficient and macroscopic severity score was significantly increased upon administration of COP as evident in NC group rats, which on treatment with EAF and PLSNPs decreased in significant manner (p<0.05). The effect of PLSNPs at 200 mg/kg; p.o. was comparable to standard Pilex granules (**Figure 6.2A-B**).

High level of pro-inflammatory markers i.e., TNF-α and IL-6 in the recto-anal tissue was noted in DC group indicating severe inflammation. However, treatment with EAF (200 mg/kg; p.o.) and PLSNPs (200 mg/kg; p.o.) significantly reduced the levels of these cytokines (**Figure 6.2C & 6.2D**). Treatment with PLSNPs (200 mg/kg; p.o.) exhibited higher total histomorphological score when compared with DC group (p<0.05) (**Figure 6.2E**).

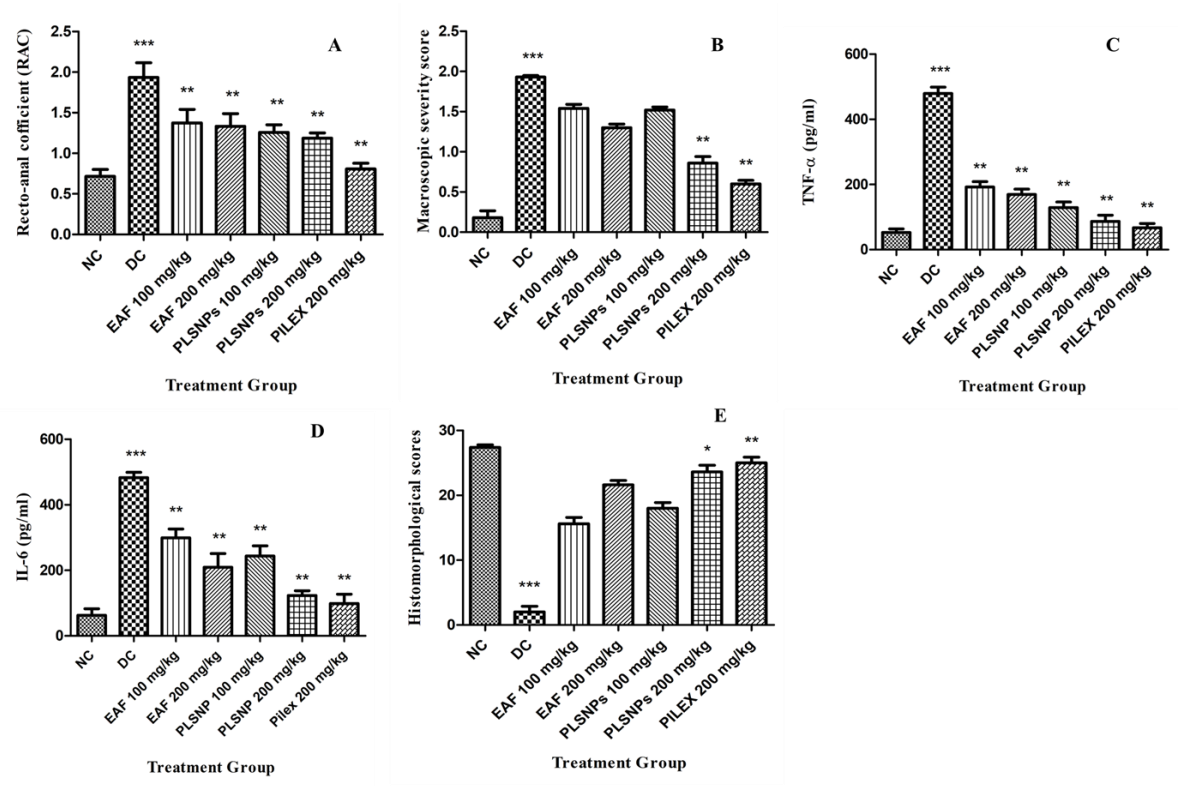

Figure 6. 2: Effect of PLSNPs on A) recto-anal coefficient B) macroscopic severity scores; levels of C) TNF-α & D) IL-6 and E) histomorphological scores in croton oil induced haemorrhoid rat model showing PLSNPs at dose level 200 mg/kg p.o. are most effective against haemorrhoids. Results expressed as mean ± SEM (n=5) where ***: $p < 0.05$ when compared to normal control (NC) group and **: $p < 0.05$ when compared to disease control (DC) group.

In a histological investigation, the muscular, mucosal, and submucosal layers of the recto-anal tissue were intact and showed normal structure in the NC group (**Figure 6.3A**). Contrarily, recto-anal tissue from the DC group displayed acute inflammatory reactions **(Figure 6.3B)**, necrosis, long-term oedema, and significant degradation of the mucosal deposit. In comparison to the NC group, expanded blood vessels and focal areas of congestion and haemorrhages were also seen in DC group. The inflammation-affected area also showed modest to moderate fibroblast acceleration and mucosal layer widening. Lesions in the recto-anal tissues were less

severe in treatment groups III, IV, and V compared to the DC group, with a minimal to mild score (**Figure 6.3C-E**). PLSNPs (200 mg/kg; p.o.) and the pilex granules-treated group both showed a marked to moderate relief against histological damage (**Figure 6.3F & 6.3G**).

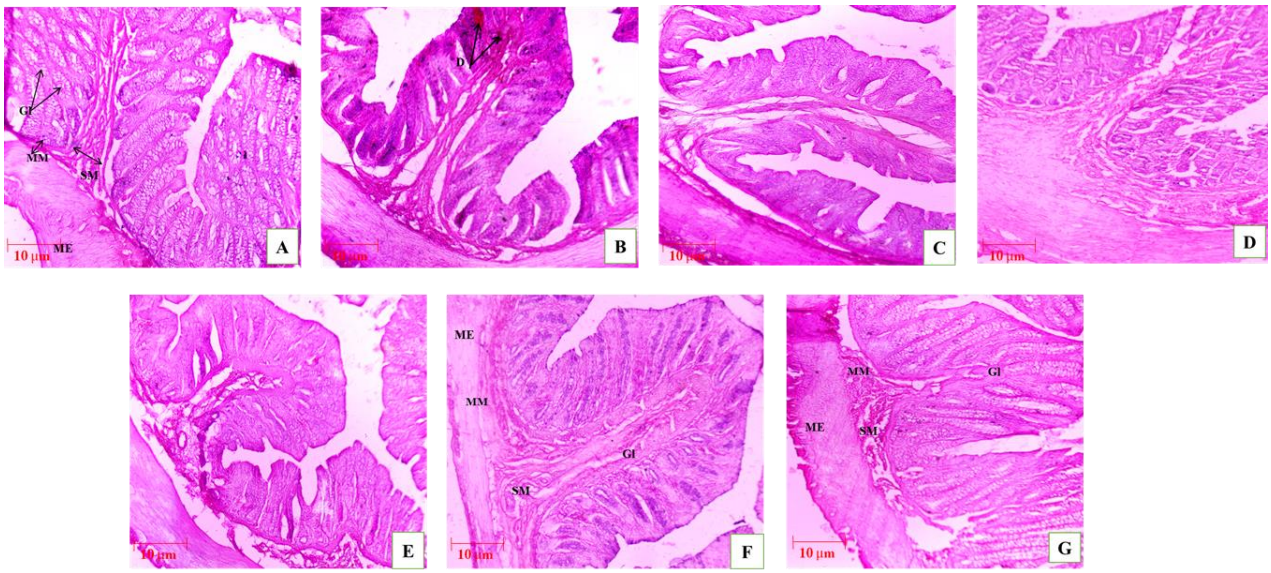

Figure 6. 3: Histopathological sections (at 40X magnification) of recto-anal tissue in rat model of croton oil-induced haemorrhoids. (A) showing the intact architecture of rectum in normal control (NC) group. (B) showing severe inflammation and dilatation of blood vessels in disease control (DC) group. (C and D) showing moderately intense inflammation and dilatation of blood vessels in rats treated with EAF 100 and 200 mg/kg; p.o. respectively (E and F) Showing lesions of minimal intensity inflammation with minimal blood vessel dilation in rats treated with PLSNPs 100 and 200 mg/kg; p.o. respectively (G) showing very little inflammation and slight dilatation of blood vessels in rats treated with Pilex (200 mg/kg; p.o.). Gl- glands, SM- submucosal layers with veins, MM- muscularis mucosa, ME- muscularis externa, D- dialated blood vessels with lesions.

6.2.3 *In vivo* antioxidant studies

PLSNPs at 200 mg/kg; p.o. demonstrated an overall robust antioxidant capacity that was relatively comparable to that of commercial Pilex granules, according to the findings from the

in vivo antioxidant tests. This was amply supported by elevated levels of SOD and CAT and a concurrent decline in LPO following PLSNP therapy (**Table 6.4**).

Table 6. 4- *In vivo* antioxidant studies of PLSNPs.

P.O. treatment (mg/kg, once daily x 7 days)	**Protein**	**SOD**	**LPO**	**CAT**
	mg/ml	**IU/mg protein**	**nmol/mg protein**	**nmol/min/mg protein**
NC	10.41±0.47	1.38±0.47	0.34±0.04	0.70±0.09
DC	24.80±4.25	0.58±0.20	0.91±0.04	0.25±0.01
EAF100	18.46±1.43	0.77±0.26	0.72±0.10	0.29±0.01
EAF200	15.69±0.61**	0.91±0.31**	0.62±0.01**	0.38±0.01**
PLSNP100	15.53±0.54**	0.92±0.32**	0.66±0.01**	0.33±0.03**
PLSNP200	13.91±0.22**	1.03±0.35**	0.52±0.04**	0.46±0.01**
Pilex 200	11.86±0.45**	1.21±0.41**	0.49±0.02**	0.50±0.02**

*The results are average of three determinations ± SEM

**: $p < 0.05$ when compared to disease control (DC) group.

6.3 Discussion

Scientists from all over the world have shown interest in nanoscale-based formulations due to its versatility, low toxicity, and enhanced palatability [150]. Synthesis of biogenic nanoparticles by “one step-one pot process” is comparatively cheaper, less perilous as well as easy. In previous few decades, several attempts have been made to adapt nanotechnology in diagnostics, mitigation, cure and purport of any disease or medical condition [178].

According to earlier accounts, the plant *Blumea lacera* reportedly has antioxidant, antibacterial, anti-inflammatory, and anti-haemorrhoid action but lacks clear evidence that how it acts; also requires higher dose due to crude extract [30]. Therefore, developing the bio-fabricated silver nanoparticles involving *Blumea lacera* could result as better alternative with

significantly reduced dose as compared to treatment with crude extract. Consequently, in this study, the author planned to evaluate the of PLSNPs from EAF as a potential anti-haemorrhoid agent. As a high concentration of flavonoids and sterols could produce more PLSNPs, polyphenol-enriched plants are typically favoured for synthesizing biogenic nanoparticles [151-153].

In order to assess the clinical signs and symptoms as well as the toxic effects of drugs, like decreased body weight and other clinical signs and symptoms that are the main observations among numerous toxicity indicators, it is required to conduct an acute oral toxicity research [86]. In the current investigation, rats given PLSNPs at doses of 2000 mg/kg showed no significant change in body weight and organ weight. Experimental animals with different doses (100 and 200 mg/kg body weight) of PLSNPs did not exhibit any harmful behavioural effects.

Toxicology studies are crucial in modern medicine to ensure the safety of extracts or medications used in clinical medicine because interactions with toxic compounds or their metabolites can cause significant changes in haematological parameters and quick or gradual changes in the structure and function of the affected tissues. In order to assess the toxicity of compounds, examinations of haematological indices offer information on the negative effects of foreign compounds on the components of blood. In this investigation, PLSNPs were found to safe upto 1000 mg/kg body weight of the experimental animals as there was no significant change was observed in the biochemical and haematological parameter after administration of the selected dose for a period of 28 days.

There were no reported fatalities at the text's conclusion on subacute toxicity. Throughout the 28-day observation period, the animals remained active and showed no behavioral changes in

response to the Hippocratic screening stimulus. Additionally, during the 14-day satellite surveillance period, no delayed development of toxic effects was seen, and there was no statistically significant change in the amount of water consumed or gained during this time. However, compared to the control group, the male rats in the satellite group consumed less food. Although there was a statistical difference in the mean consumption, no biological significance was given to this because the body weight increase values were the same across groups. Organ weight is a crucial sign of an animal's pathological and physiological state. The PLSNPs did not induce any harmful effect on the relative organ weights of female and male treated rats. There were no changes in renal function-related biochemical measures or histological changes indicating the presence of disease.

Furthermore, the anti-haemorrhoid potential of these biosynthesised PLSNPs was evaluated by COP induced haemorrhoid rat model. As COP causes inflammatory reactions, polymorphonuclear leukocyte infiltration, and vasodilatation, which leads to the development of oedema in tissue, it has been utilized as an inducing agent for haemorrhoids in experimental animals. The croton oil facilitates the release of inflammatory mediators that speed up inflammation, such as prostaglandins, leukotrienes, bradykinins, nitric oxide, chemokines, and cytokines. Together, these factors cause the regulation of inflammatory cells including eosinophils, neutrophils, lymphocytes, and monocytes as well as newly assigned inflammatory cells like endothelial cells, mast cells, fibroblasts, and macrophages that cause severe inflammation [105, 117]. Application of COP in our study resulted in severe inflammation, considerably higher macroscopic severity scores, and a higher recto-anal coefficient in the DC group compared to the NC group, confirming the production of haemorrhoids and supporting past research findings [105, 107]. After seven days of therapy with EAF (200 mg/kg), PLSNPs

(100 and 200 mg/kg body weight), and Pilex (200 mg/kg body weight) there was a significant improvement in hemorrhoidal metrics, which is suggestive of the therapeutic benefits. The protective role of PLSNPs in hemorrhoids was also made clear by the decreased histomorphological examination scores. Based on the findings of anti-haemorrhoid activity, it can be stated that the PLSNPs are more effective in treating haemorrhoids as compared to EAF at comparatively half dose.

As was previously mentioned, the application of COP speeds up the migration and subsequent release of several inflammatory cells and mediators [118, 119]. The current investigation also shown considerably increased serum concentrations of cytokines including TNF-α and IL-6. [105, 120]; and severity score analysis corroborated this. After receiving therapy with PLSNPs, the levels of these cytokines in the serum decreased, confirming its ability to reduce inflammation, which is essential for any candidate with the potential to treat haemorrhoids [121].

The histopathological findings supporting the anti-inflammatory function of PLSNPs showed that animals treated with PLSNPs showed fewer inflammatory cells, decreased degenerative changes, hypertrophy, necrosis, and vasodilatation with a few haemorrhagic spots in comparison to recto-anal tissue of the negative control group. After using Pilex granules and PLSNPs, there was a marked improvement in the histological damage.

Croton oil has been shown to enhance the expression of reactive oxygen species, free radical scavengers, and leukocytes, involving a variety of neurotransmitters, which impairs the antioxidant state. This was also verified by observations in the DC group [106, 122, 123]. Treatment with PLSNPs resulted in a significant drop in LPO and an increase in SOD and CAT levels, indicating a potential prevention of peroxidative tissue damage due to the PLSNPs'

antioxidant properties. The above findings were indicative of strong anti-inflammatory and antioxidant properties of the PLSNPs as a potential agent for treating hemorrhoids, treatment with drug pilex granules exhibited the similar results [105, 107].

6.4 Conclusion

In thrust of developing a potential anti-haemorrhoid agent, the current study demonstrated the biosynthesis of PLSNPs using EAF fraction derived EBL and evaluation of its anti-haemorrhoid effect. The antihemorrhoid potential of PLSNPs was established through the results obtained by evaluating the hemorrhoidal and biochemical parameters after treatment in COP induced hemorrhoids in the experimental animals. Therefore, PLSNPs produced involving phytosterol enriched fraction might be promising agent in the treatment of haemorrhoids attributed to its significant anti-inflammatory and antioxidant properties. However, more thorough *in-vivo* research in the future may allow the researchers to gain a thorough understanding of the real mechanism underlying these pharmacological effects.