CHAPTER 4

ANTI-HAEMORRHOID ACTIVITY OF ETHANOLIC EXTRACT

4. Anti-haemorrhoid activity of ethanolic extract

4.1 Experimental work

4.1.1 Collection of plant material and authentication

Blumea lacera leaves (collected from local area of Varanasi in Uttar Pradesh, India) were authenticated by Prof. N. K. Dubey, Department of Botany, Banaras Hindu University. A voucher specimen No. Aster. 2016/3 was deposited in the herbarium of the Institute.

4.1.2 Extraction process optimization using BBD towards response surface

Shade-dried leaves of *B. lacera.* was coarsely powdered (150 g) and Soxhlet extraction process was used for extraction following optimization with the help of Box–Behnken design (BBD), where three astute factors; mesh size, extraction solvent and extraction time were evaluated which affect the extraction process. The powdered leaves were extracted using a Soxhlet extractor at 70 °C, as per the projected design. The resulting extract was then filtered and concentrated using Rota evaporator (IKA, Germany) to obtain the crude extract of *B. lacera* (EBL).

Design Expert (Version 12.0.0 Trial, Stat-Ease Ink.) was used to determine the boundary of the experiment, for evaluating response and for finally optimizing the process. The level of design factors has represented in **Table 4.1**.

Table 4. 1- Input factors with their ascribed values and coded levels, along with the expected outcomes of the response

Factor	**Levels used, Actual (coded)**		
	Low (-1)	**Medium (0)**	**High (+1)**
X_1=Mesh Size (sieve number)	10	20	40
X_2=Solvent Blend (ethanol:water)	80	90	100
X_3=Time (h)	6	15	24
Dependent Variables		**Constraints**	
Y_1=Extraction Efficiency (%)		Maximize	
Y_2=Phenolic content(mg/g)		Maximize	

The actual extractive experiments with values of independent variables and obtained results are shown in **Table 4.2**. A total of 20 extractive cycles were performed. The % yield of extraction is indicative of extraction efficiency while the phenolic yield of extract was estimated via standard methods. The extraction efficiency of 20 models was calculated after their extraction process. These extraction test models were put for time period obtained after input of independent variables. Repetition of models are recommended to obtain proper error of the model. These actual runs are compared with predicted values of the software. The values of mean, median, variance was calculated in the software which helps in integral analysis of the experiments. It also goes through the process of evaluating standard error, standard mean error which are important for knowing the spread ability of the model.

Table 4. 2- Actual extractive runs for obtainment of best results

Batch	**Independent variables**			**Dependent variables**	
	A: (Mesh Size)	B: Solvent Blend (Ethanol %)	C: (Time; h)	Y_1 (%)	Y_2 (mg/g)
1	40	80	15	16.47±4.72	113.826±1.38
2	20	80	6	31.09±2.35	121.994±6.54
3	10	80	15	12.43±7.04	125.493±3.92
4	20	90	15	39.85±5.23	129.986±4.63
5	20	100	6	15.63±3.13	112.115±4.38
6	40	90	24	21.54±4.62	117.474±5.46
7	10	100	15	15.36±6.27	117.539±3.78
8	20	90	15	37.66±2.55	131.307±2.93
9	40	90	6	19.67±5.64	110.923±3.24
10	20	90	15	39.24±3.82	134.221±5.63
11	20	80	24	37.58±3.47	117.501±1.42
12	10	90	6	16.9±2.49	112.327±3.84
13	20	90	15	38.01±6.25	129.457±4.59

14	20	90	15	27.22±2.98	131.787±6.32
15	10	90	24	19.26±3.56	115.963±1.49
16	20	90	15	32.6±4.83	129.347±3.74
17	20	90	6	32.47±7.32	121.346±4.27
18	20	90	15	37.92±5.66	128.418±1.93
19	20	100	24	30.98±4.37	112.684±2.53
20	40	100	15	23.95±3.48	120.718±2.66

4.1.3 Check point analysis (validation of method)

By creating a checkpoint batch (n = 3) of an optimised formula in ideal conditions as advised by Design-Expert software and analysing the corresponding CQAs, the predictability/validity of the rotatable BBD model was confirmed (saturation solubility). By calculating the percentage prediction error (percent Bias) using Eq. (4.1), a quantitative comparison between the theoretical predictions made using software and the actual experimental data was made [41, 43, 44].

$$\text{prediction error (\%)} = \frac{\text{experimental value} - \text{predicted value}}{\text{predicted value}} \times 100 \qquad \text{Eq(4.1)}$$

4.1.4 *In vitro* Anti-inflammatory activity

The inhibition in albumin denaturation method was adopted for determining the *in vitro* anti-inflammatory potential of EBL following the procedure earlier described with minor modification [103]. Freeze-dried EBL and aspirin, which was used as anti-inflammatory standard, were diluted serially in dimethyl sulfoxide (DMSO) in the concentration ranging from 50-600 µg/ml. Initially, 1 ml of a 1% aqueous solution of bovine albumin fraction was mixed with 1 ml of EBL or Aspirin. Further, the pH of the solution mixture was adjusted to 6.3 and then incubated at 37°C for 20 min followed by heating it for 30 min at 51°C. It was then allowed to cool to room temperature, and its absorbance was measured at 660 nm on a

UV-Visible spectrophotometer (Agilent Technologies, USA). The percentage inhibition of protein denaturation was computed using the following Eq (4.2). The findings were presented as IC_{50} values, where aspirin was referred to as positive control and DMSO as negative control.

$$\text{percentage inhibition}(\%) = \frac{\mathbf{A}\ \text{control} - \mathbf{A}\ \text{sample}}{\mathbf{A}\ \text{control}} \times 100 \qquad \text{Eq (4.2)}$$

Where A control: Absorbance of negative control i.e., DMSO and A sample: Absorbance of the EBL.

4.1.5 Experimental Animals

Healthy and adult Wistar rats (8–10 weeks old and weight between 220–250 g), were obtained from the Central Animal House of I.M.S (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).

4.1.6 Acute oral toxicity study

Five female rats were used for the acute oral toxicity study and the study was conducted as per OECD guidelines 425 [104]. EBL (2000 mg/kg p.o.) was given to rats (fasted overnight) and observation of all the drug-treated rats (individually for 48 hours) was done to assess any changes in behavior and neurological activity *viz.* tremors, convulsions, salivation, diarrhea, sleep and lacrimation, as a sign of acute toxicity. Furthermore, the animals were observed for two weeks and finaly the animals were sacrifieced and vital organs were i.e., liver, kidney and

heart was isolated. Histological examination was done by observing the transverse sections (10 µm) to evaluate any damage to the vital organs; prepared with the help of cryomicrotome (SLEE MEV, Germany), stained using hematoxylin and eosin, observed under bright field microsope (Magnus MLX plus, India) at 10X resolution.

4.1.7 Anti-haemorrhoid activity

4.1.7.1 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 were 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. The edema lasting up to 12 h was considered haemorrhoids. 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, haemorrhoidal severity, biochemical, and histological parameters were investigated [105, 106].

4.1.7.2 Grouping and treatment

The EBL and Pilex was prepared as suspension using 1% tween 80. The rats were divided into six 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; Group III: Haemorrhoid induced group treated with EBL suspension at 100 mg/kg, p.o., Group IV: Haemorrhoid induced group treated with EBL suspension at 200 mg/kg, p.o., Group V:

Haemorrhoid induced group treated with EBL suspension at 400 mg/kg, p.o. and Group VI: Haemorrhoid induced group treated with standard Pilex granules (200 mg/kg, p.o., Himalaya Drug Company, Bengaluru, India). EBL and standard Pilex granules were administered once daily for seven days.

4.1.7.3 Assessment of Evans blue exudation

The method described by Dey *et al.* (2016)[107] and Azeemuddin *et al.* (2014)[105] was used to determine COP-induced Evans blue dye plasma exudation in recto-anal tissue. Evans Blue dye (30 mg/kg; i.v.) was injected into the tail veins of the rats 30 minutes before the administration of COP (set 1 as discussed earlier) and retained for 24 hours to induce haemorrhoids. The animals were then treated with different doses of EBL and Pilex granules for seven days. On the eighth day, the recto-anal tissue (20 mm) of animals was dissected and weighed and Evans blue dye was extracted from tissue using formamide. Quantification of Evans blue dye in the sample was done through a standard calibration curve, and λ_{max} was recorded at 620 nm using UV-Visible spectrophotometer. Evans blue concentration was expressed in µg/mg of recto-anal tissue.

4.1.7.4 Evaluation of haemorrhoidal 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 followed by cervical dislocation. After placing a small portion of the tissue in 10% formaldehyde solution for histological examination, the rest was stored at -20°C to estimate 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 prepared using microtome and then examined for inflammatory cells, necrosis, congestion, hemorrhage 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].

4.1.7.5 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).

4.1.7.6 Histology and histomorphological scoring

For histological examination, previously fixed recto-anal tissue in 10% formalin was processed following a conventional procedure 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].

A histomorphological scale was validated to assess the severity of haemorrhoids compared 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 the best possible outcome and scored as 28 (Maximum total score).

4.1.8 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 homogenized 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.

4.1.8.1 Estimation of lipid peroxidase (LPO)

The thiobarbituric acid reactive substances (TBARS) were measured using the colorimetric method as per previous reports [109] to estimate LPO level in recto-anal tissue. For this purpose, 2 mL of 0.37% TBA, 0.25 M HCl, and 15% TCA in a 1:1:1 ratio (TBA-trichloroacetic acid-HCl reagent) were added to 0.1 ml of tissue homogenate and was incubated in a water bath for 15 min and then cooled. It was then centrifuged for 10 min at 3500×g and the clear supernatant was collected and its absorbance was meassured at 535 nm and the final reading was expressed as mM/100 g-tissue.

4.1.8.2 Estimation of catalase (CAT)

For performing CAT activity, 0.1 ml of tissue homogenate was treated with 1.0 ml of 0.01 M phosphate buffer (pH 7.0) and 0.4 ml of H_2O_2. Further, a 5% dichromate-acetic acid reagent was prepared by using potassium dichromate and glacial acetic acid in a ratio of 1:3 and 2.0 ml of this reagent was added to the above reaction mixture to stop the reaction. Finally, the

absorbance was noted at 620 nm, and the catalase activity was reported as μM of H_2O_2 consumed/min/mg protein [110].

4.1.8.3 Estimation of superoxide dismutase (SOD)

In this method 0.5 ml of tissue homogenate was diluted with 1 ml of water, followed by addition of 2.5 ml of cold ethanol and 1.5 ml of chloroform and centrifugation at 4°C after 1 min vigorous shaking. For initiation of the reaction, NADH solution was added to the mixture, which contained 1.2 ml sodium pyrophosphate buffer (0.025 M, pH 8.3), 0.1 ml PMS (186 μM), 0.3 ml NBT (30 μM), enzyme preparation (diluted appropriately) and water (3 ml in a total volume). It was then incubated for 90 sec at 30°C, followed by addition of 1 ml glacial acetic acid to stop the reaction. Further, 4 ml of n-butanol was added to the mixture and was stirred and shaken vigorously for few minutes. Finally, the butanol layer was removed, and its absorbance was measured at 560 nm against n-butanol blank. A sample without enzyme was taken as a blank. The enzyme reaction that inhibited NBT reduction up to 50% in 1 min under the same experimental envirement was defined as one unit of the enzyme activity [111].

4.1.9 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 is performed. GraphPad Prism 5.03 software is used for analyzing the data statistically and $p < 0.05$ was considered significant.

4.2 Results

4.2.1 Extraction process and its optimization

Hydroalcoholic extract of *B lacera* was obtained through Soxhlet extraction involving the powdered leaves. The process was optimized using BBD to increase the extraction efficiency and phenolic content to maximum level. Best response model was obtained by varying three astute factors viz. time of extraction process, solvent blend and mesh size based on which optimized extraction cycle and condition was predicted.

Three input factors, solvent blend, mesh size and duration of extractive processing were varied over three levels to arrive at the best response model which was used to predict the conditions for optimized extraction cycle. The final quadratic equations produced by the software with coded variables were as follows:

Extraction efficiency = 38.8217 + 2.21 * A + -1.06303 * B + 2.92485 * C + 2.35934 * AB + -0.744951 * AC + 2.215 * BC + -17.0434 * A^2 + -4.93551 * B^2 + -2.22603 * C^2 Eq. (4.3)

Phenolic Yield = 131.148 + -1.04757 * A + -1.294 * B + 0.888279 * C + 4.05437 * AB + 1.28161 * AC + 1.26548 * BC + -7.10199 * A^2 + -4.75992 * B^2 + -9.76613 * C^2 Eq. (4.4)

Selection of response model from amongst linear, 2FI, quadratic and cubic models to delineate obtained and expected outcomes, fitting of the data for observed responses to selected models and determination of significant factors are explained using **Table 1.3** and would not be expanded textually for the sake of preventing digression. Very briefly, though, quadratic model was selected to extract relationship amongst independent and dependent variables. The values of the coefficient for mesh size, solvent blend ratio and time of extraction, as given in equations relates to effects of these factors and their comparative significance on the extraction efficiency and phenolic yield of Soxhlet extraction.

For instance, when the finest sieve with (Sieve no. 40 which offers fine crude extractive feed) was used along with a relatively polar ethanol: water solvent blend (80:20) in Batch 1, the extraction efficiency was 16.47 % and the phenolic yield was 113.86 mg/g. Whereas in Batch 10 with middle mesh openings (Sieve no. 20), semi polar nature of solvent (90:10) ethanol water blend) and extraction time of 15 h, substantially greater extraction efficiency (39.24%) and phenolic yield (134.221 mg/g) was obtained. The 3D response surface plots **(Figure 4.1)** of DoE for extraction efficiency and phenolic yield suggested the following outcomes. Drug particles screened through sieve 20 (median sized openings) resulted in maximum extraction efficiency and phenolic yield while a decrease was observed when particles were screened with either sieve 10 (coarse particles) and 40 (very fine). Grinding of plant material into small particles enhances the surface area for efficient mixing with solvent, which in turn should hasten the mass transfer of active principle from plant material to the solvent, however, this principle holds true up to a certain extent only. Particles sized small (those produced by sieve no. 40) create difficulty during extraction due to attainment of slimy character, clogging the sample plug and slowing down the extraction process. During extraction, attainment of equilibration time is essential. The equilibration time is the time after which the amount of analyte extracted remains constant. Intuitively prolongation of extraction periods should give maximum yields. However, this study showed that extraction efficiency and phenolic yield was maximum at 15 h while increase in time upto 24 h reduces the yield. Unnecessary continuous extraction for prolonged time may deteriorate the phytoconstituents due to excessive effect of heat which may reduce the yield [112].

The study indicated that when absolute ethanol (100%) or highly polar solvent blend (ethanol: water 80:20) was used the extraction efficiency and phenolic yield was lower as

compared to the solvent blend of ethanol: water (90:10). The choice of solvent selected for extraction is vital as it is intended to isolate phytoconstituents. Solvents vary in their ability to extract phytoconstituents from plant tissue due to variable degree of affinity to them [113]. Efficiency of extraction can also be enhanced by the addition of co-solvent to liquid phase as it may alter the polarity of final solvent blend [114]. Selection of solvent is based upon the polarity of targeted phytoconstituent and, in turn, depends on the molecular affinity of solvent towards solute, mass transfer, use of co-solvent, environmental safety, human toxicity and financial feasibility [115].

Therefore, the optimum condition for highest extraction efficiency (39.24%) and total phenolic content (134.221 mg/g) was observed when powder was screened through sieve no. 20 and extracted with hydroalcoholic solvent (90:10) for 15 hours.

Table 4. 3- Model summary statistics and selection of significant factors

Extraction Efficiency							Phenolic Content						
Model Selection	**Std. Dev.**	**R^2**	**Adj. R^2**	**Pred. R^2**	**PRESS**	**Remark**	**Model Selection**	**Std. Dev.**	**R^2**	**Adj. R^2**	**Pred. R^2**	**PRESS**	**Remark**
Linear	10.14	0.04	-0.14	-0.52	2602.57		Linear	8.03	0.08	-0.09	-0.39	1564.16	
2FI	11.10	0.07	-0.36	-1.66	4570.08		2FI	8.55	0.15	-0.24	-1.12	2378.67	
Quadratic	5.34	0.83	0.68	-0.38	2376.89	**Selected**	Quadratic	2.50	0.94	0.8941	0.48	575.86	**Selected**
Cubic	4.55	0.93	0.77		+	**Aliased**	Cubic	1.96	0.98	0.9351		+	**Aliased**
	S.S.	**df**	**M.S.**	**F-value**	**p-value**			**S.S.**	**df**	**M.S.**	**F-value**	**p-value**	
Model	1430.95	9	158.99	5.58	0.0064	**Significant**	Model	1061.03	9	117.89	18.82	<0.0001	**Significant**
A	39.07	1	39.07	1.37	0.2687		A	8.78	1	8.78	1.40	0.2639	
B	8.59	1	8.59	0.3014	0.5950		B	12.73	1	12.73	2.03	0.1845	
C	70.57	1	70.57	2.48	0.1466		C	6.51	1	6.51	1.04	0.3321	
AB	23.50	1	23.50	0.8249	0.3851		AB	69.40	1	69.40	11.08	0.0076	
AC	2.35	1	2.35	0.0826	0.7796		AC	6.97	1	6.97	1.11	0.3164	
BC	19.62	1	19.62	0.6888	0.4259		BC	6.41	1	6.41	1.02	0.3358	
A^2	997.95	1	997.95	35.03	0.0001	**Significant**	A^2	173.28	1	173.28	27.66	0.0004	**Significant**
B^2	112.73	1	112.73	3.96	0.0747	**Significant**	B^2	104.85	1	104.85	16.74	0.0022	**Significant**
C^2	24.03	1	24.03	0.8434	0.3800	**Significant**	C^2	462.53	1	462.53	73.83	<0.0001	**Significant**
Residual	284.92	10	28.49				Residual	62.65	10	6.26			
Lack of Fit	160.50	4	40.13	1.94	0.2240	**Not significant**	*Lack of Fit*	39.62	4	9.91	2.58	0.1438	**Not significant**
Pure Error	124.41	6	20.74				*Pure Error*	23.02	6	3.84			
Cor Total	1715.87	19					Cor Total	1123.68	19				

Footnote: Std. Dev.: Standard deviation of obtained values over 15 runs; R^2: Regression coefficient (the model maximizing R^2 is selected); Adj. R^2: Adjusted R^2; Pred. R^2: Predicted R^2 (The selected model should maximize summation of Adj. R^2 and Pred. R^2); PRESS: Predicted residual sum of squares (PRESS statistic with the lowest value indicates the best model); 2FI:2 Factor Interaction Model;+: PRESS statistic can't be estimated; Aliased(Implies the number of experimental runs is not adequate to fit cubic model in the response curve); S.S.: Sum of Squares; M.S.: Mean Square; df :Degree(s) of freedom. After adapting quadratic model, influence of each factor (A, B, C...C^2) was verified by computing F values. Factor with large F value explains the variance more. Probability of obtaining this F value, if the investigated independent variable did not have any effect on the response is measured by p values. Small probabilities, $p<0.05$, indicate a significant factor. The insignificant lack of fit for the quadratic model counter intuitively proves that the model is a good fit and can be pursued for optimization of soxhlet extraction.

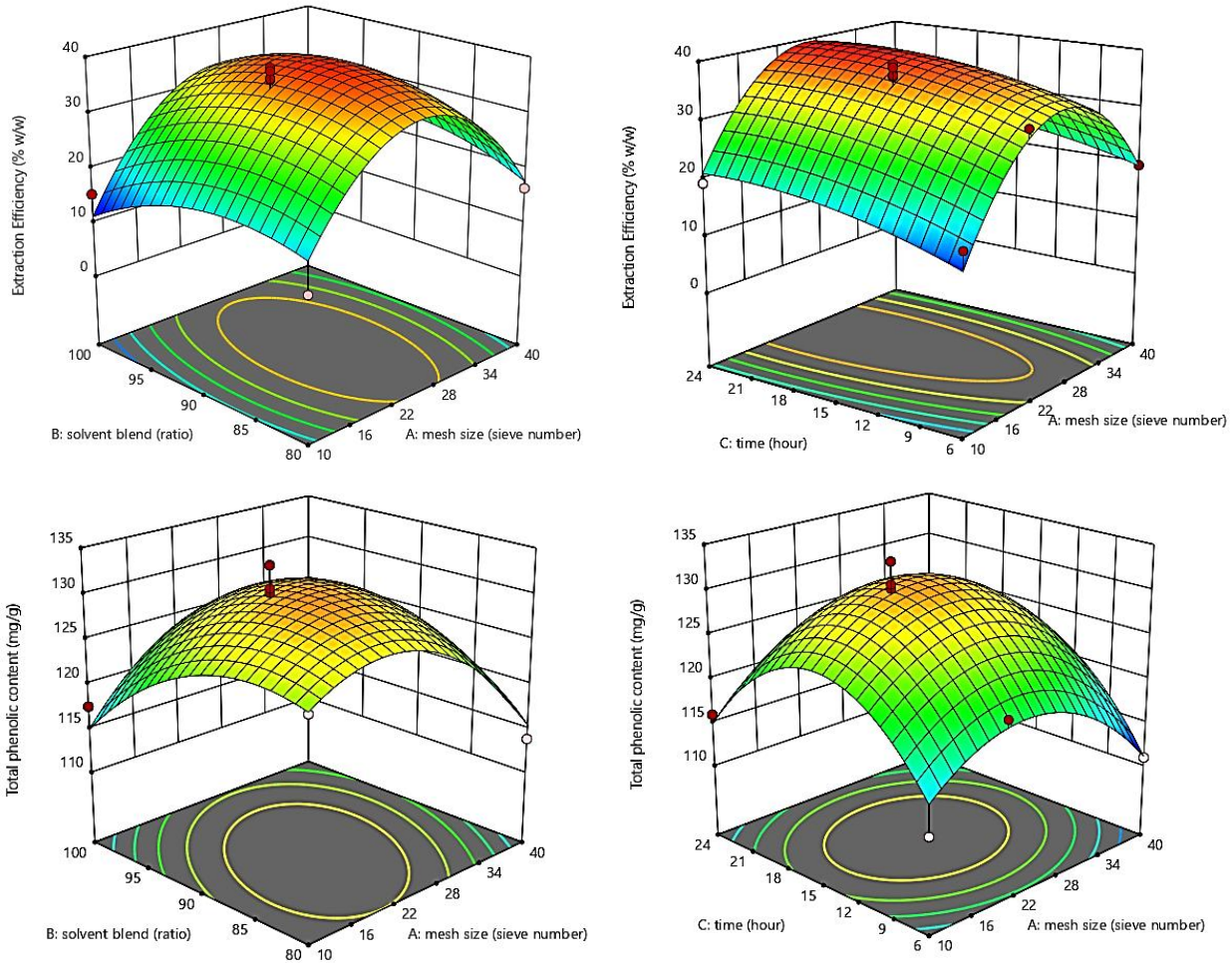


Figure 4. 1: 3D- response surface plots by Design Expert explaining the extractive process.

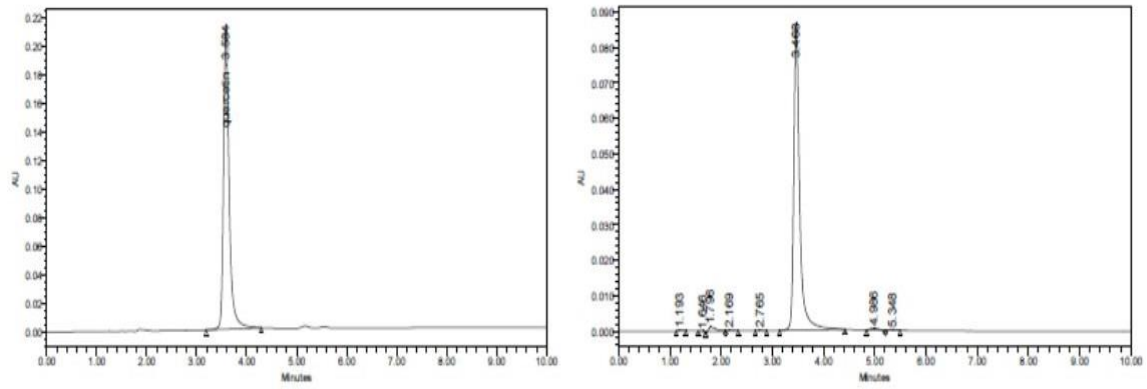
Where (A) and (B) represent the effect of time, solvent blend and mesh size on extraction efficiency; (C) and (D) represent the effect of time, solvent blend and mesh size on phenolic yield. The study shows that the particle size of powder for extraction should be optimum for higher extraction efficiency and phenolic yield. Too fine or too coarse sizes have an adverse effect. Usually, the amount of analyte extracted plateaus after particular time period however as is evident from curves in our case extraction efficiency and phenolic yield diminished at 24 h which might be due to degradation of phytoconstituents upon sustained application of heat. Extraction efficiency and phenolic content is also affected by solvent used for extraction as affinity of phytoconstituents towards extractive media varies with polarity of solvent.

4.2.2 Checkpoint analysis (validation of method)

The predicted values of Y1 and Y2 were 39.157 ($n = 3$) and 131.189 mg/mL, respectively and the actual experimental values of Y1 and Y2 were 37.905± 3.81 and 131.289±1.79. The percentage prediction error (% Bias) for the checkpoint batch was found to be − 3.197% and 0.076% respectively, which was < 5% validating the authenticity of predictive capacity and accuracy of the design model [116].

4.2.3 Standardization of EBL using HPLC

The chromatogram of standard quercetin and confirmation of quercetin in EBL has been represented in **Figure 4.2**. Under optimum operational conditions, the HPLC method was applied for the identification of quercetin in EBL, and the presence of quercetin was confirmed by comparing the HPLC chromatogram of EBL with that of standard quercetin. A peak with retention time at 3.468 minutes was confirmed as quercetin in EBL and the quantity of quercetin in EBL was found to be 31.603±1.141 mg/g.



(A) **(B)**

Figure 4. 2: HPLC chromatogram of quercetin. In ethanolic extract of *B. lacera*. Peak of standard quercetin is shown in figure A while peaks of quercetin in ethanolic extract of *B. lacera* is shown in figure B.

4.2.4 *In vitro* Anti-inflammatory activity

The *in vitro* anti-inflammatory activity of EBL was evaluated against protein denaturation, where EBL showed IC_{50} value of 94.79 ±4.32 µg/ml which was quite comparable to the protein protection capacity of standard aspirin (IC_{50} value of 77.10 ±5.26 µg/ml).

4.2.5 Acute oral toxicity of EBL:

During the entire period of acute oral toxicity study of EBL, there were neither sign of behavioral toxicity nor neurological toxicity in rats which also showed zero mortality rate up to 2 g/kg. Histological examination of vital organs indicated no significant histological damage in liver, heart, and kidney at a dose of 2 g/kg as repreesented in **Figure 4.3**.

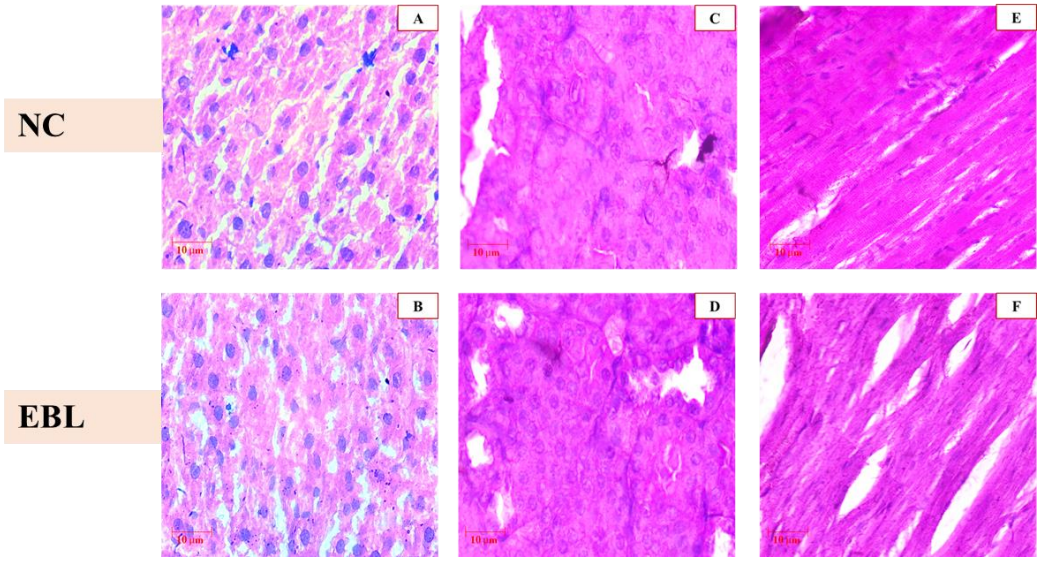


Figure 4. 3: Histological sections of liver (A & B), kidney (C & D), and heart (E & F) of NC and PLSNPs (2000 mg/kg; p.o.) treated groups.

4.2.6 Anti-haemorrhoid activity

From the observations, COP caused a significantly (p<0.05) increased extravasation of Evans blue dye as observed from the recto-anal tissues of the negative control as compared to the normal control. However, on treatment with EBL (100, 200, 400 mg/kg; p.o.), and pilex

granules (200 mg/kg; p.o.) a significant (p<0.05) reduction in Evans blue concentration was noted as compared to the negative control **(Figure 4.4)**. From the overall observation, EBL at 400 mg/kg; p.o. was found to be most effective which was quite comparable with the standard pilex granules.

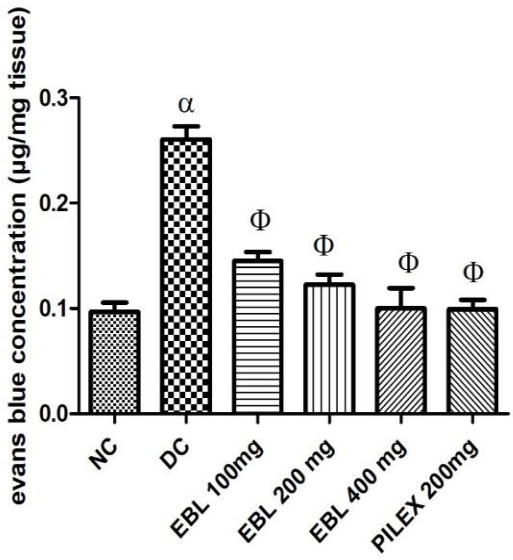


Figure 4. 4: Effect of EBL on Evans blue concentration in croton oil induced hemorrhoid rat model which shows that EBL at dose of 400 mg/kg p.o. is most effective against hemorrhoids. Results expressed as mean ± SEM (n=6) where α: $p < 0.05$ when compared to normal control (NC) group and Φ: $p < 0.05$ when compared to disease control (DC) group.

The results revealed a significant increase in the recto-anal coefficient on administration of COP as evident through the negative control group rats and was found to significantly (p<0.05) reduce on treatment with EBL in a dose-dependent manner, where EBL at 400 mg/kg; p.o. was found to be most effective and showed similar effect as that of standard Pilex granules **(Table 4.4).**

The macroscopic severity score was also observed to increase in negative control group after COP administration when compared to normal control group, which also significantly (p<0.05)

reduced on treatment with EBL and standard thus, confirming minimal recto-anal damage as compared to croton oil control (negative) rats **(Table 4.4)** and is well supported with the results of the histopathological examination.

Table 4. 4- Effect of EBL on macroscopic severity score and recto-anal coefficient

Hemorrhoidal parameters	*NC*	*DC*$^{\alpha}$	*EBL100*$^{\phi}$	*EBL200*$^{\phi}$	*EBL400*$^{\phi}$	*Pilex200*$^{\phi}$
Macroscopic severity score	0.183±0.070	1.875±0.077	1.47±0.049	1.35±0.043	1.1±0.072	0.975±0.054
Recto-anal coefficient	1.183±0.064	3.61±0.312	2.32±0.11	1.61±0.12	1.19±0.11	1.19±0.12

Values are expressed as mean ± SEM.

α: $p < 0.05$ when compared to NC. Φ: $p < 0.05$ when compared to DC.

The results also suggested the increased level of pro-inflammatory markers i.e., TNF-α and IL-6 imparting inflammation on the recto-anal tissues. The increased level of these cytokines was in correlation with high severity score in negative control. However, a significant decrease in the levels of these cytokines after the treatment with EBL established the anti-inflammatory nature of the EBL (100, 200 and 400 mg/kg; p.o.) **(Figure 4.5A and 4.5B)**.

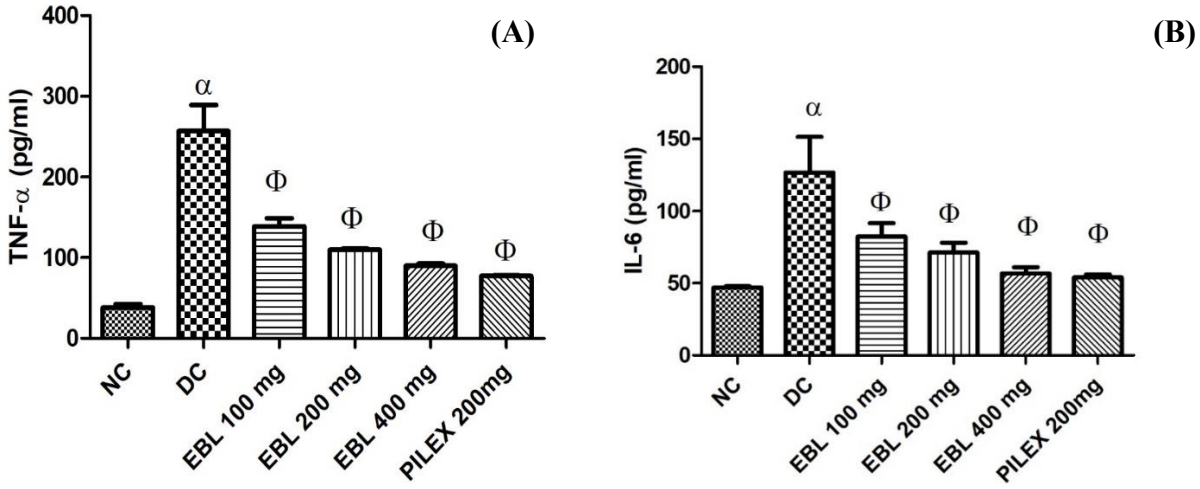


Figure 4. 5: Effect of EBL on A) level of cytokines-TNF-α B) level of cytokines-IL-6 in croton oil induced haemorrhoid rat model. Graph shows that EBL at dose of 400 mg/kg p.o. is most effective against haemorrhoids. Results expressed as mean ± SEM (n=6) where α: $p < 0.05$ when compared to normal control (NC) group and Φ: $p < 0.05$ when compared to disease control (DC) group.

In a histopathological study, normal architecture of recto-anal tissue was observed in normal control with all the three layers intact *viz*. muscular, mucosal, and submucosal layers **(Figure 4.6A)**. Acute inflammatory reactions **(Figure 4.6B)** were seen in recto-anal tissue from COP treated rats along with necrosis, severe edema, and other degeneration of the mucosal layer. Dilated blood vessels and focal area of congestion and hemorrhages were also seen as compared to normal control rats. Thickening of the mucosal layer along with fibroblast escalation in mild to moderate form was also observed in the inflamed portion. In treatment groups, less severe lesions in the recto-anal tissues as compared to COP treated negative control group was noted with minimal to mild score **(Figure 4.6C & 4.6D)**. EBL (400 mg/kg; p.o.) and pilex granules treated group exhibited a marked to moderate protection respectively against histological damage **(Figure 4.6E & 4.6F).**

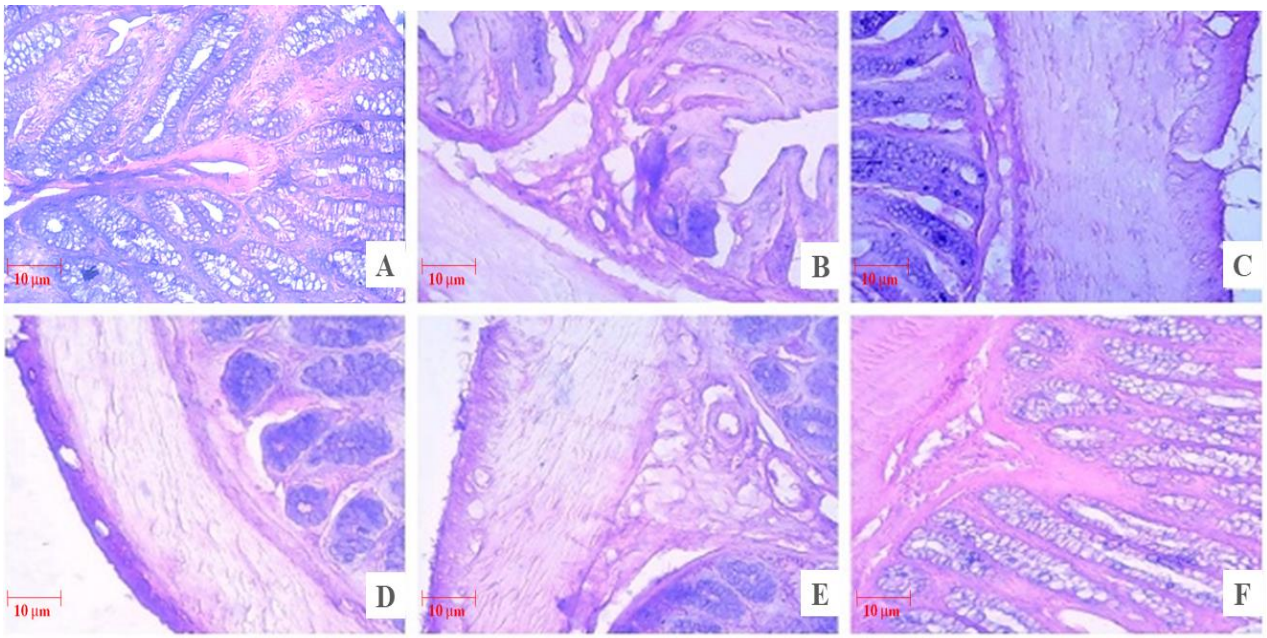


Figure 4. 6: Effect of EBL on histology of recto-anal tissue: Microscopic images (at 40X magnification) of recto-anal sections, demonstrating the effect of EBL on histology in rat model of croton oil-induced haemorrhoids. (A) showing the intact architecture of rectum in normal control (NC) group. (B) showing severe inflammation and dilatation of blood vessels in disease control (DC) rat. (C and D) showing moderately intense inflammation and dilatation of blood vessels in rats treated with EBL 100 and 200 mg/kg; p.o. respectively (E) Showing lesions of minimal intensity inflammation with minimal blood vessel dilation in rats treated with EBL 400 mg/kg; p.o. (F) showing very little inflammation and slight dilatation of blood vessels in rats treated with Pilex (200 mg/kg; p.o.).

Treatment with EBL (400 mg/kg; p.o.) and as well as pilex (200 mg/kg; p.o.) exhibited higher total histomorphological score when compared with croton oil control group (p<0.05). EBL at 100 and 200 mg/kg; p.o. did not show any significant recovery of lesions **(Figure 4.7).**

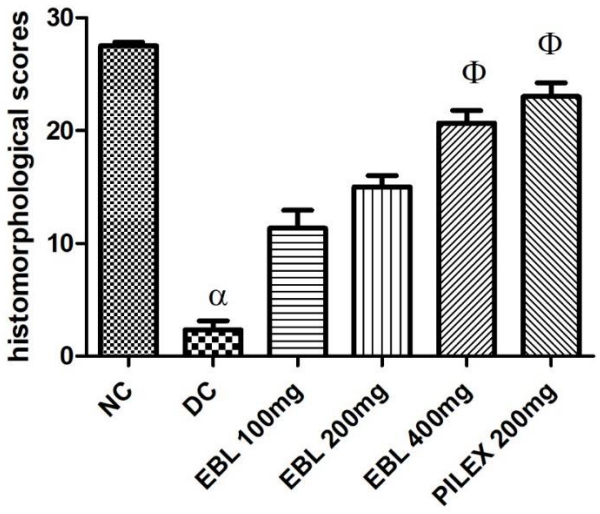


Figure 4. 7: Effect of EBL on histomorphological scores of recto-anal tissue. The graph shows that EBL at dose level 400 mg/kg p.o. is most effective against haemorrhoids. Results are expressed as mean ± SEM (n=6) where α: $p < 0.05$ when compared to normal control (NC) group and Φ: $p < 0.05$ when compared to disease control (DC) group.

4.2.7 *In vivo* antioxidant studies

Results of the *in vivo* antioxidant evaluations revealed that the antioxidant capacity of EBL at 400 mg /kg; p.o. was significant which was quite comparable with that of standard Pilex granules. This was significantly justified by increased levels of SOD and CAT with a concomitant decrease in the level of LPO on treatment with EBL **(Table 4.5).**

Table 4. 5- *In vivo* antioxidant studies of EBL

Oral treatment (mg/kg, once daily x7 days)	**Protein**	**SOD**	**LPO**	**CAT**
	mg/ml	**IU/mg protein**	**nmol/mg protein**	**nmol/min/mg protein**
NC	14.55±0.55	0.58±0.20	0.43±0.04	0.25±0.005
DC $^{\alpha}$	24.80±4.25	0.99±0.34	0.81±0.17	0.50±0.06
EBL100	21.33±1.94	0.94±0.32	0.68±0.05	0.38±0.05
EBL200	18.67±1.43	0.93±0.32	0.63±0.04	0.33±0.03
EBL400 $^{\Phi}$	15.39±0.92	0.77±0.26	0.56±0.06	0.27±0.004
Pilex 200 $^{\Phi}$	15.45±0.47	0.68±0.23	0.50±0.09	0.26±0.011

Values are expressed as mean ± SEM (n=6) where α: $p < 0.05$ when compared to normal control (NC) group and Φ: $p < 0.05$ when compared to disease control (DC) group. (In Table, p.o: per oral, EBL: Ethanol extract of *B. lacera*, LPO: Lipid peroxidation, SOD: Superoxide dismutase and CAT: catalase).

4.3 Discussion

Process optimization helps in getting the improved results in both ways i.e., qualitatively, and quantitatively. The practical yield of extract (60 g in 150 g crude leaves) was in accordance with the extraction efficiency (39.24%) calculated using BBD. The results of *in vitro* antioxidant and anti-inflammatory activity revealed that the EBL could be an effective agent in treating the diseases involving oxidative stress and inflammation such as haemorrhoids. To

assess the safety of EBL upon oral administration, acute oral toxicity study was performed which showed that EBL is safe up to 2g/kg.

The present study involves croton oil as an inducing agent for the induction of haemorrhoid in rats as it contains an active phorbol esters, 12-*O*-tetradecanoylphorbol-13-acetate, which exerts inflammatory reactions followed by polymorphonuclear leukocyte infiltration, vasodilatation that results in development of edema in tissue. This active constituent from croton oil enables the release of inflammatory mediators like prostaglandins, leukotrienes, bradykinins, nitric oxide, chemokines, and cytokines which accelerate inflammation. These factors together trigger the regulation of endothelial cells, mast cells, fibroblasts, and macrophages along with newly assigned inflammatory cells like eosinophils, neutrophils, lymphocytes, and monocytes that contribute to severe inflammation [105, 117]. In our study also, application of COP cased sever inflammation and significantly increased macroscopic severity scores and the recto-anal coefficient in negative control as compared to normal control, which confirmed the induction of haemorrhoids, complying with the earlier research findings [105, 107]. Significant amelioration ($p < 0.05$) of haemorrhoidal parameters after seven days of treatment with EBL and pilex is suggestive of the curative effects of EBL. Application of COP in the recto-anal portion causes extreme vasodilatation and subsequent inflammation, associated with bleeding is a diagnostic feature for haemorrhoids [3, 118]. Our study also revealed a significant increase ($p < 0.05$) in Evans blue concentration in recto-anal tissue of negative control affirming severe vasodilatation by COP when compared with normal control in Evans blue dye extravasation test, which was further justified by observations of recto-anal tissue histology (observed for bleeding, inflammation and marked edema). However, treatment with EBL caused a significant reduction ($p < 0.05$)

in the level of Evans blue dye confirming a marked reduction in the inflammation caused to recto-anal tissues. The lower scores of the histomorphological examinations and *in vitro* anti-inflammatory study also justified the protective role of EBL in haemorrhoidal conditions.

As discussed earlier, migration and subsequent release of many inflammatory cells and mediators are accelerated by COP application [118, 119]. In the same manner, the present study also showed a significantly higher levels ($p < 0.05$) of cytokines like TNF-α and IL-6 in serum on COP application which compliment with the earlier research outcomes [105, 120]; and was also confirmed by severity score analysis. The decrease in the levels of these cytokines in serum after on treatment with EBL affirmed the anti-inflammatory role of EBL, which is very essential for any candidate to have anti-haemorrhoid potential [121].

The anti-inflammatory role of EBL was also supported through the histopathological observations in which animals treated with EBL demonstrated less number of inflammatory cells and lowered degenerative changes, hypertrophy, necrosis as well as vasodilatation with a few hemorrhagic spots as compared to recto-anal tissue of negative control group. A moderate reduction in histological damage after treating with Pilex granules was also seen, however treatment with EBL showed marked reduction in the histological damage.

Studies have revealed that, croton oil causes increase in expression of reactive oxygen species, free radical scavengers and leukocytes infiltration, involving various neurotransmitters, which hampers the antioxidant status and was also confirmed through the observations of our negative control group rats [106, 122, 123]. Treatment with EBL significantly decreased LPO and increased SOD and CAT levels suggesting inhibition in peroxidative tissue damage, possibly because of antioxidant property of EBL. This suggests that anti-inflammatory, as well as

antioxidant properties of the EBL played a crucial role in exerting the anti-haemorrhoid effect, the same has been exhibited by the standard drug pilex granules [105, 107].

The results of preliminary phytochemical screening confirmed the availability of phenols, alkaloids, tannins, and flavonoids as major components in EBL. These phytoconstituents have well been reported to have anti-inflammatory and antioxidant activity [117, 124, 125], which directly or indirectly contributed in the anti-haemorrhoidal potential of EBL. Further, EBL was standardized with the help of HPLC using quercetin as marker.

4.4 Conclusion

From the overall observation, we have successfully justified the traditional claims of the plant *B. lacera* in treatment of haemorrhoid, which may be attributed to its potent anti-inflammatory and antioxidant effect. The phytoconstituents present in EBL could be the main contributor for the above therapeutic efficacy. However, for elucidation of the mechanism of action further studies like the isolation of lead molecule is needed. Furthermore, clinical and formulation studies are also required for properly validating the therapeutic potential of *B. lacera* for human use.