Results

PHARMACOGNOSTICAL EVALUATIONS

PHARMACOGNOSTICAL EVALUATION OF *LEEA MACROPHYLLA*

1. Morphological Characteristics

The fresh root tubers are reddish brown in color with smooth surface, 5-30 cm in length and 4-6 cm in width, cylindrical tapering slightly at both ends with few root hairs, sweetish smell and astringent taste with tingling sensation (Figure 8).

2. Microscopical Characteristics

The fresh sections of the root tuber showed presence of orange red colour pigment without staining (Figure 9). The transverse section of the root tuber showed outer narrow cork consisting of 3 to 5 rows of thick walled, yellowish brown coloured lignified cells; cortex is wide, consisting of 10 to 15 rows of parenchymatous cells, spherical to oval in shape with varying sizes. They are embedded at places with rosette acicular and very few prismatic crystals of calcium oxalate. This is followed by 6 to 8 rows of parenchymatous pericyclic band lies underneath, embedded at places with isolated or groups of wide lumened sclerids, occasionally lying adjacent with idioblast embedded with cluster crystals of calcium oxalate. The stellar region shows radially arranged xylem vessels associated with a tracheid and few fibre and is capped with phloem alternating with parenchymatous multiseriate medullary rays with central pith. The inner tissue of the stellar region being parenchymatous embedded at places with groups of sclereids, isolated mucilage cells and plenty of acicular crystals, few rosettes and prismatic crystals of calcium oxalate (Figure 10).

Powder microscopy exhibit the presence of cork cell, parenchymatous cell, lignified rectangular elongated stone cells. Slender shaped lignified fibers ranging from

189–290 µm in length and 4–7µm in width are present either alone or in group. Plenty of acicular crystals, few rosettes and prismatic crystals of calcium oxalate are present. Annular vessels of 131–278 µm length and 17–22 µm in width along with border pitted tracheids ranging from 90-178 µm are also found (Figure 11). Histochemical analysis, when performed on section of root tuber with different reagents showed positive test for the presence of starch, tannin, oil, crystal, mucilage and lignin (Table 12).

Figure 8: Root tubers of *Leea macrophylla* (Leeaceae)

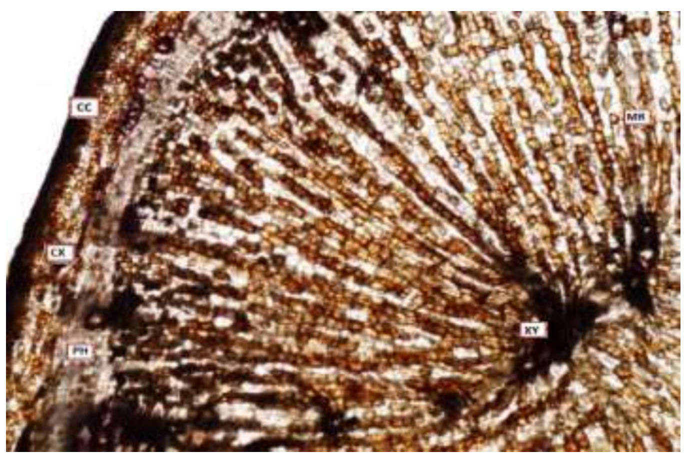


Figure 9: Transverse section of root tuber without staining.
In figure CC– Cork cell layer; CX– Cortex; PH– Phloem; MR– Medullary rays; XY– Xylem

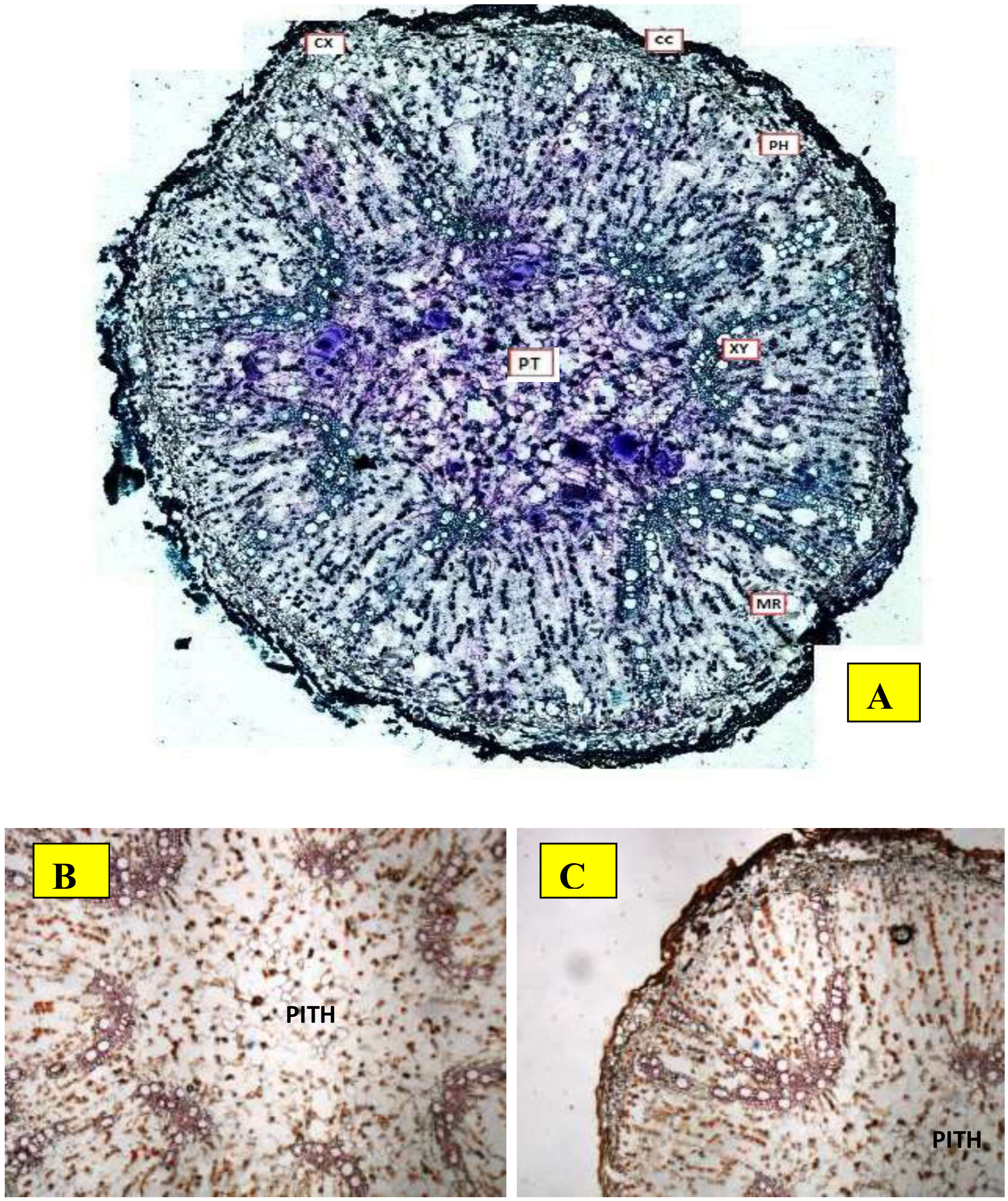


Figure 10: Microscopy of *Leea macrophylla* root tubers
[A]: Histology of root tuber [B] TS of root tuber showing xylem [C] TS of root tuber showing lignified cells stained pink [CC– Cork cell layer; CX– Cortex; PH– Phloem; PT– Pith; MR– Medullary rays]

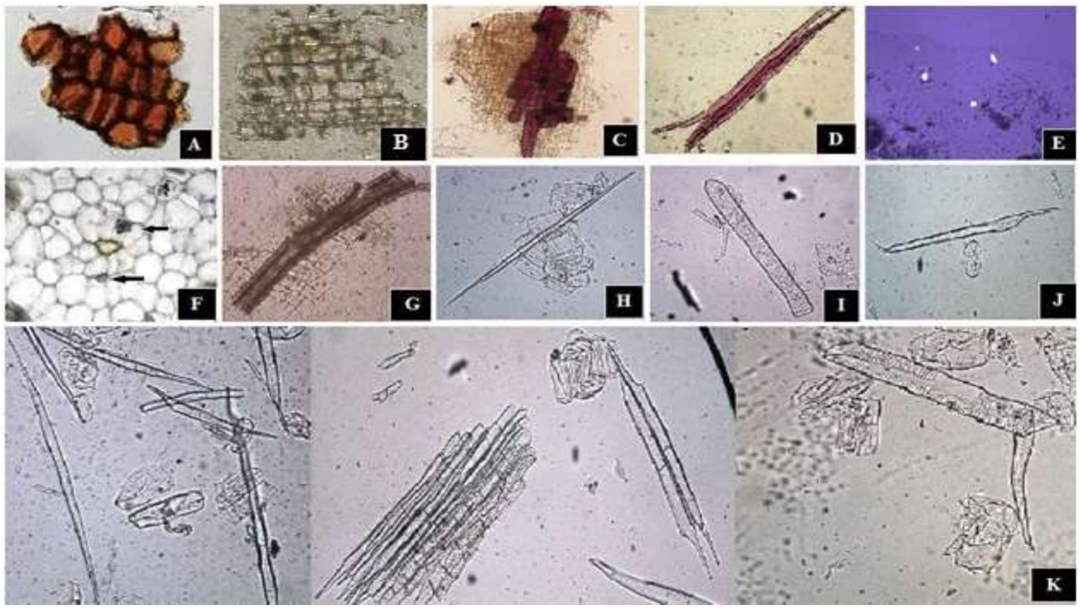


Figure 11: Powder characteristics (A-G) and maceration (H-K) study of *Leea macrophylla* root tuber. In figure [A]: Cork cells [B]: Parenchymatous cells [C]: Lignified stone cell [D]: Lignified Fibre in group [E]: Calcium oxalate crystals under polarized light [F]: Parenchyma with rosette crystals of calcium oxalate [G]: Lignified xylem vessel [H]: Fibre [I]: Vessels [J]: Trachied [K]: Macerated powder of *Leea macrophylla*

Table 12: Treatment of section of *Leea macrophylla* with different reagents

Drug	Reagent	Test for	Reaction	Result
Section	Iodine	Starch	Blue Colour	+
Section	Ferric Chloride	Tannin	Black Colour	+
Section	Sudan III	Oil	Pinkish to reddish Colour	+
Section	Dil. Hydrochloric acid	Calcium oxalate Crystal	Soluble	+
Section	Ruthenium Red	Mucilage	Pink Colour	+
Section	Phloroglucinol + dilute HCl	Lignin	Magenta Colour	+

(+) indicate presence.

3. Physicochemical evaluation

Air dried root tuber powder was used for the quantitative determination of different physicochemical constant which help in identification of the plants have been evaluated and are represented in the Table 13. The amount of foreign matter present was 2.0% w/w with 9.0% w/w loss on drying. Total ash, acid insoluble ash and water soluble ash values were found to be 13.6% w/w, 3.5% w/w and 1.5% w/w respectively. Both alcohol soluble extractive 29% w/w and water soluble extractive 25% w/w were found to be high with hot extraction method as compared to alcohol soluble extractive 22% w/w and water soluble extractive 18% w/w obtained with cold maceration. Foaming index was less than 100. Swelling index was found to be 6 ml/gm. The value of Hemolytic index was obtained as 48.27 units/gm.

Table 13: Physiochemical properties of *Leea macrophylla* root tuber

Sl. no	**Parameters**			**Results**
1	Foreign matter			2.00 % (w/w)
2	Loss on drying			4.57 % (w/w)
3	Total ash			13.60 % (w/w)
	Acid Insoluble ash			3.50 % (w/w)
	Water soluble ash			1.50 %(w/w)
4	***Cold maceration method***	Alcohol soluble extractive		22% (w/w)
		Water soluble extractive		18% (w/w)
	Hot Soxhlation/ reflux method	Alcohol soluble extractive		29% (w/w)
		Water soluble extractive		25% (w/w)
5	Foaming Index			Less than 100
6	Swelling Index			6 ml/gm
7	Hemolytic Index			units/gm
8	Crude fibre Content			5.36 %
9	**Chlorinated Pesticide residue**			
	TS1 (First elute)			0.009 mg/kg
	TS2 (Second elute)			0.008 mg/kg
	Phosphated Pesticide residue			
	TS1 (First elute)			0.002 mg/kg
	TS2 (Second elute)			0.004 mg/kg
	TS3 (Third elute)			Absent
10	***Heavy metals determination***	λ_{max}	***Fuel flow rate (L/min)***	***Concentrations (in ppm)***
	Arsenic (As)	193.7	2.4	0.1725 ppm
	Lead (Pb)	283.3	2.0	0.0159 ppm
	Cadmium (Cd)	228.8	1.8	0.00017 ppm
	Mercury (Hg)	253.7	1.8	0.0534 ppm

Fluorescence analysis

The fluorescence pattern given by the powdered drug with different reagents in day light, short UV and long UV is represented in table 14.The identification and comparison of the colours was done using the standard colour index chart.

Table 14: Fluorescence powdered drug analysis of *Leea macrophylla* root tuber

S.N.	Powder + Reagent	Fluorescence in daylight	Fluorescence (254nm)	Fluorescence (365nm)
1.	Powder as such	Brown	NF	NF
2.	Powder + 1N NaOH in methanol	Light Salmon	Medium Sea Green	NF
3.	Powder + 1N NaoH in water	Tan	Forest Green	Dark Green
4.	Powder + 1N HCl in methanol	Dark Salmon	Yellow Green	Dark Sea Green
5.	Powder + 1N HCl in water	Brown	Yellow Green	Yellow Green
6.	Powder + 1N HNO_3 in methanol	Light Corel	Pale Green	Pale Green
7.	Powder + 1N HNO_3 in water	Light Salmon	Light Green	Pale Green
8.	Powder + Iodine (5%)	Dark Red	Dark Green	NF
9.	Powder + $FeCl_3$ (5%)	Gold	Lime	NF
10.	Powder + KOH (50%)	Chocolate	Spring Green	NF
11.	Powder + Ammonia (25%)	Corel	Pale Green	Yellow Green
12.	Powder + Saturated Picric acid	Yellow	Yellow Green	Lawn Green
13.	Powder + Acetic acid	Corel	Lawn Green	Yellow Green

NF: No Fluorescence

Quantification of crude fibre content

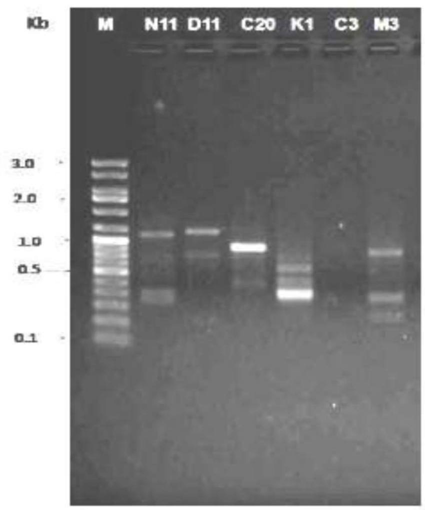
The crude fibre content in *Leea macrophylla* root tuber was found to be 5.36 % of plant material.

Quantification of pesticide residue and heavy metals content

Table 13 represents pesticide residue and heavy metal content quantified in the powdered drug. Both chlorinated and phosphate pesticide residues were found to be within the permissible range as described by WHO. The heavy metals like arsenic, lead, cadmium and mercury were within the limits specified by by WHO 1998 and FAO 1984.

4. DNA fingerprinting

Six RAPD decamer primers were used to amplify the DNA profile of the plant sample, out of which, Primer OPC-3 did not show any amplification. The primer OPN11 showed four DNA bands from 300 bp to 1000 bp. The primer OPD-11 produced three DNA fragment from 500 to 1000 bp. The primer OPC-20 and OPK-01 also produced three and four bands respectively. Primer OPM-03 showed three DNA bands from 200 – 800 bp (Figure 12).



Random Amplified Polymorphic DNA profile generated by OPN-11, OPD-11, OPC-20, OPK-01, OPC-03 and OPM-03, M- Molecular marker

Figure 12: DNA fingerprinting profile of the plant

5. Extraction, Qualitative and Quantitative Estimations & Standardization of Extract

5.1 Optimization of Extraction

Working with Box–Behnken design (BBD) towards response surface

Ethanolic extract of *Leea macrophylla* was obtained by using Soxhlet extraction of powdered root tuber. BBD was utilized to optimize the extractive process so as to maximize the extraction efficiency and the phenolic content obtained. 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 = $28.83 + 0.44X_1 + 0.99X_2 + 0.8X_3 - 0.2X_1X_2 + 1.15X_1X_3 - 0.25X_2X_3 - 8.45X_1^2 - 4.85X_2^2 - 5.23X_3^2$ Eq. 1

Phenolic Yield = $+28.83 + 4.11X_1 + 7.08X_2 + 0.3X_3 - 1.78X_1X_2 + 10.61X_1X_3 - 3.84X_2X_3 - 37.24X_1^2 - 20.91X_2^2 - 22.89X_3^2$ Eq. 2

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 by means of Table 15 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 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. Greater values of coefficient for X_1^2, X_2^2 and X_3^2 in both the equations in comparison to other terms showcase their greater significance on the outcome. The equations therefore essentially reduce to following form;

Extraction efficiency = $28.83 - 8.45X_1^2 - 4.85X_2^2 - 5.23X_3^2$ Eq. 3

Phenolic Yield = $+28.83 - 37.24X_1^2 - 20.91X_2^2 - 22.89X_3^2$ Eq. 4

A negative value in the regression equation for investigated response favors an antagonistic effect. Substituting squared values (lying in between -1 and +1) into the above equations 3 and 4 for X_1, X_2 and X_3, it was found that maximum extraction efficiency and phenolic content were obtained when the independent variables are in the middle of their utilized extremities or when X_1, X_2 and X_3 are equal to 0.

For instance when the coarsest sieve with (Sieve No 10 which offers large crude extractive feed) was used along with a relatively polar ethanol: water solvent blend (85:15) in Batch 1, the extraction efficiency was 14 % and phenolic yield was 113.24 mg/g. Whereas in Batch 15 with middle mesh openings (Sieve no 20), semi polar nature of solvent (90:10 ethanol water blend) and extraction time of 18 hrs, substantially greater extraction efficiency (29.0%) and phenolic yield

(181.54 mg/g) was obtained. The output from optimized batch predicted by Design Expert was also in close proximity as reflected by emboldened batch 16 in Table 6 were the extraction efficiency which was found to be 28.9% and as well as phenolic content was 184.9 mg/g. This batch was used for generating extract for further use in the study. 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 hrs 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.

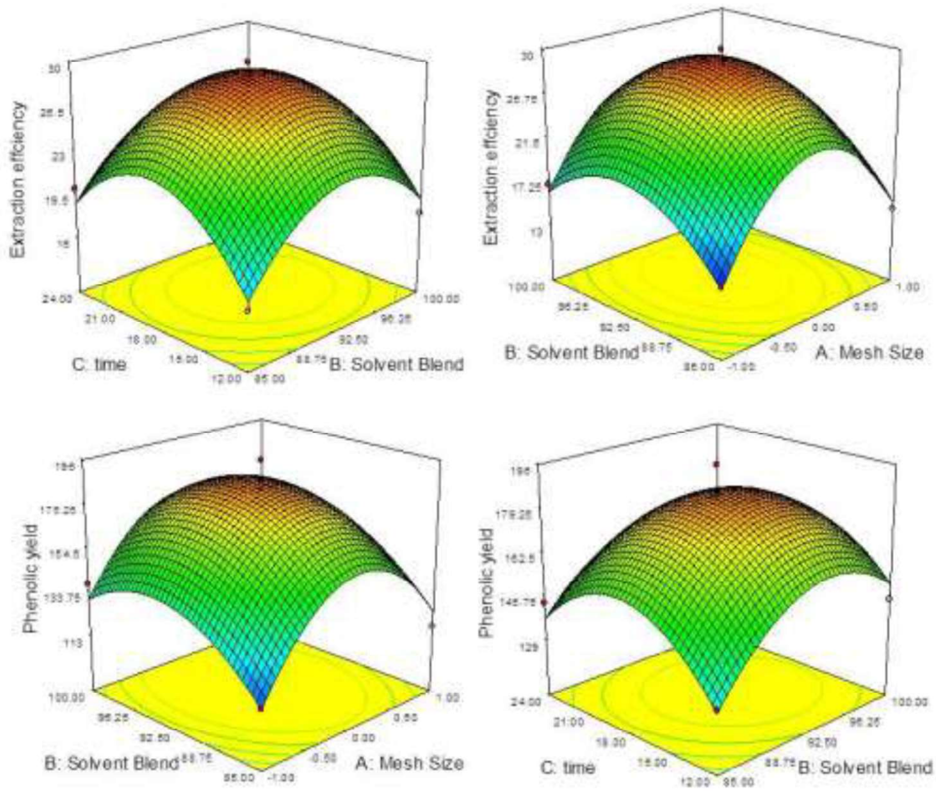


Figure 13 3D- response surface plots by Design Expert explaining the extractive process. (A) and (B) represent the effect of time, solvent blend and mesh size on extraction efficiency; (C) and (D) represent the effect of time, solvent blend and mesh size on phenolic yield.

Table 15: 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	6.15	.03	-.23	-.38	596.22		Linear	28.04	.05	-.19	-.38	12726.7	
2FI	7.16	.04	-.66	-1.19	945.64		2FI	31.87	.11	-.54	-1.06	18927.4	
Quadratic	1.56	.97	.92	.64	151.64	**Selected**	Quadratic	9.83	.94	.8528	.48	4686.67	**Selected**
Cubic	1.26	.99	.94		+	**Aliased**	Cubic	10.51	.97	.8316		+	**Aliased**
	S.S.	**df**	**M.S.**	**F-value**	**p-value**			**S.S.**	**df**	**M.S.**	**F-value**	**p-value**	
Model	418.18	9	46.6	19.04	0.0023	**Significant**	Model	8699.66	9	966.63	19.04	0.0023	**Significant**
X_1	*1.53*	1	1.53	0.63	.4641		X_1	135.05	1	135.05	0.63	.4641	
X_2	*7.80*	1	7.80	3.20	.1338		X_2	*401.01*	1	401.01	3.20	.1338	
X_3	*5.12*	1	5.12	2.10	.2071		X_3	*0.71*	1	0.71	2.10	.2071	
X_1X_2	*0.30*	1	0.30	0.12	.7391		X_1X_2	*12.71*	1	12.71	0.12	.7391	
X_1X_3	*5.29*	1	5.29	2.17	.2009		X_1X_3	*449.86*	1	449.86	2.17	.2009	
X_2X_3	0.25	1	0.25	0.10	.7618		X_2X_3	58.91	1	58.91	0.10	.7618	
X_1^2	263.90	1	263.90	108.16	0.0001	**Significant**	X_1^2	5120.9	1	5120.79	108.16	0.0001	**Significant**
X_2^2	87.00	1	87.00	35.66	0.0019	**Significant**	X_2^2	1614.9	1	1614.90	35.66	0.0019	**Significant**
X_3^2	100.96	1	100.96	41.38	0.0013	**Significant**	X_3^2	1934.73	1	1934.73	41.38	0.0013	**Significant**
Residual	12.20	5	2.44				Residual	482.80	5	96.56			
Lack of Fit	9.03	3	3.01	1.90	.3629	**Not significant**	*Lack of Fit*	261.65	3	87.28	0.79	0.6006	**Not significant**
Pure Error	3.17	2	1.58				*Pure Error*	220.95	2	110.48			
Cor Total	430.38	14					Cor Total	9182.46	14	9182.46			

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(X_1, X_2, $X_{3...}$ X_3^2) 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 valu.

5.2 Phytochemical evaluation of *Leea macrophylla*

Preparation of extract and successive fractions

The optimized extract of *Leea macrophylla* was then subjected to fractionation, phytochemical evaluation and quantification. The sub-fractions of ethanolic extract of *Leea macrophylla* obtained successively from fractionation showed the yield as follows -hexane (1.0% w/w), chloroform (2% w/w), ethyl acetate (7.5% w/w), n-butanol (25% w/w) and aqueous fraction (14.5% w/w/). The results for the preliminary phytochemical screening of ethanolic extract of *Leea macrophylla* and its subfraction are represented in Table 16.

Table 16: Preliminary phytochemical screening of ethanolic extract of *Leea macrophylla* and its successive fractions

Phytoconstituents	**Ethanolic extract (ELM)**	**Fractions**				
		Hexane fraction	**$CHCl_3$ fraction**	**Ethyl acetate fraction**	**Butanol fraction**	**Aqueous fraction**
Alkaloids	+	–	–	+	+	+
Glycosides	+	–	–	+	+	+
Flavonoids	+	–	+	+	+	+
Steroidal/triterpenes	+	+	+	+	+	+
Phenolic & tannins	+	–	–	–	+	+
Saponins	+	–	–	–	–	+
Mucilages	+	–	–	–	–	+
Proteins	+	–	–	–	+	+
Amino Acids	+	–	–	–	–	+
Sugars	+	–	–	–	+	+

(+) indicate presence, (–) indicate absence.

The major phytochemicals present in ethanolic extract of *Leea macrophylla* and its subfractions were found to be polyphenolics (tannins, phenolics and flavonoids), glycosides, alkaloids, saponins, steroids, mucilage, proteins, amino acids and carbohydrates. While coumarins were seemed to be absent. Alkaloids and steroids/triterpenes were present in ethanolic extract as well as its all subfractions i.e. in hexane, chloroform, ethyl acetate, butanol and aqueous fraction. Except hexane subfraction glycoside and flavonoid were present in ethanolic extract as well as its other subfractions. The ethanolic extract and its butanolic and aqueous subfractions showed the prescence of phenolics, tannins and protein. Saponin, mucilage and amino acid were present only in ethanolic extract and its aqueous subfraction.

The amount of alkaloids present in the plant material was 1.19±0.13 % w/w. Total phenolic content quantified in the root tubers was found to be 195.82±2.55 mg/g tannic acid equivalent, while total tannin reported was 97.21±1.07 mg/g tannic acid equivalent. The estimated value of total flavonoids and flavonols was observed to be 81.82±0.86 mg/g and 2.62±0.17 mg/g rutin equivalent, respectively. Total saponin content in the plant material was found to be 44.48±1.42 mg/g diosgenin equivalent, while total carbohydrate was found to be 58.88±0.81 mg/g d-fructose equivalent (Table 17).

Table 17: Quantification of phytoconstituents in ethanolic extract of *Leea macrophylla* extract and its fractions

Phytoconstituents	ELM extract	ELM fractions			
		LMC	LMEA	LMBU	LMAQ
Total alkaloids (% w/w)	1.19±0.13	-	0.21±0.10	0.52±0.12	0.38±0.10
Total phenolics (mg/g TAE)	195.82±2.55	-	-	96.78±4.94	76.12±1.61
Total tannins (mg/g TAE)	97.21±1.07	-	-	45.66±2.50	25.33 ± 1.9
Total flavonoids (mg/g RE)	81.82±0.86	3.39±1.26	17.18±4.46	49.72±2.02	40.05±3.78
Total flavonols (mg/g RE)	2.62±0.17	0.36±0.31	0.58±0.20	1.55±0.49	0.65±0.29
Total saponins (mg/g DE)	44.48±1.42	-	-	-	16.43±3.27
Total carbohydrates (mg/g FE)	58.88±0.81	-	-	21.99±1.21	35.38±3.38

TAE: Tannic acid equivalent; RE: Rutin equivalent; DE: Diosgenin equivalent; FE: fructose equivalent

5.3 Quantification of chlorogenic acid by HPLC

Figure 14 represents the HPLC chromatogram of standard chlorogenic acid and ethanolic extract of *Leea macrophylla*. From the standard plot of chlorogenic acid and the linear regression equation, the content of chlorogenic acid in the crude ethanol extracts of *Leea macrophylla* was found to be 9.01% w/w.

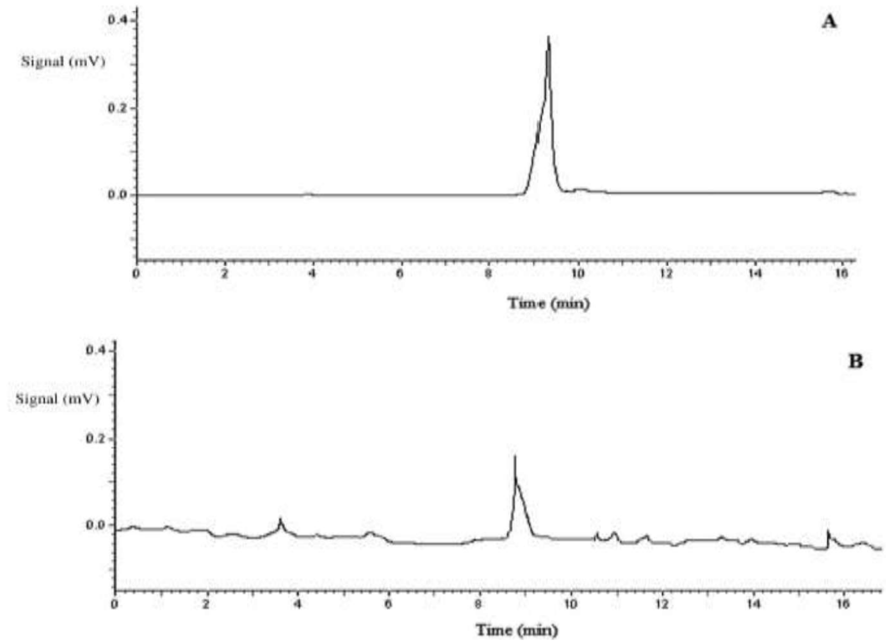


Figure 14: HPLC chromatogram of ethanolic extract of *Leea macrophylla* (A) standard chlorogenic acid and (B) ethanol extract of *Leea macrophylla*

Chlorogenic acid is the major representative of hydroxyl cinnamic acids. Chemically, chlorogenic acid is an ester formed between caffeic acid and quinic acid and is a natural antioxidant abundantly distributed among plant species.

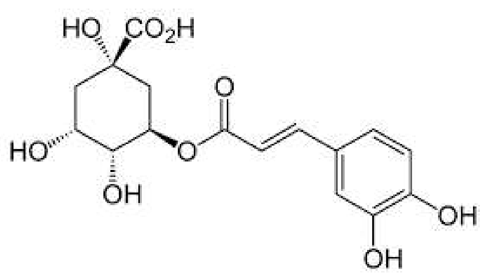


Figure 15: Chlorogenic Acid

6. Evaluation of *in vitro* antioxidant activities of *Leea macrophylla* extract (ELM) and its successive fractions

The results of the total antioxidant capacity, reducing power and scavenging activity of DPPH, hydrogen peroxide and hydroxyl radical for ethanolic extract of *Leea macrophylla* and its fractions i.e. (hexane, chloroform, ethyl acetate, butanol and aqueous) are represented respectively in Table 18, Figure 16-17. The total antioxidant capacity was determined by linear regression equation and was expressed as number of equivalent of ascorbic acid. Antioxidant capacity of ELM was found to be 365.67±1.08 μg/mL. Assay of reducing power is a concentration dependent reaction i.e. higher concentration indicates higher reducing power.

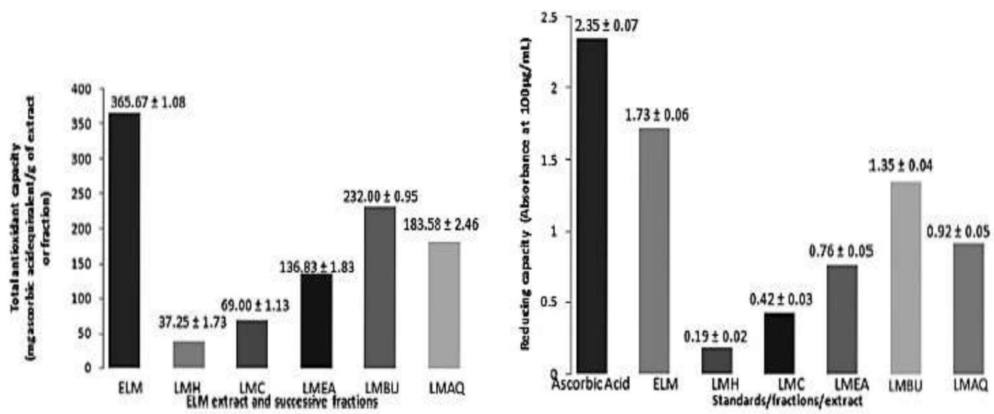


Figure 16: (A) Total antioxidant activity (B) Assay of Reducing Power of ethanolic extract of *Leea macrophylla* extract and successive fractions.

The results demonstrated a potent reducing potential of ELM (1.73±0.05 μg/mL). The capability to reduce DPPH by donating an electron or hydrogen to DPPH is indicative of free radical scavenging activity of the extract. ELM showed an IC_{50} value of 39.80±2.05 μg/mL as compared to ascorbic acid (IC_{50}: 23.67±1.67 μg/mL). Griess reagent was used to determine the nitric oxide scavenging activity, which illustrated a moderate scavenging activity of ELM (IC_{50}: 101.11±2.37 µg/mL) in comparison to rutin (IC_{50}: 37.81±3.57 µg/mL). A considerably moderate scavenging potential of hydrogen peroxide by ELM was observed with an IC_{50} value of 74.15±2.84 µg/mL compared to standard rutin IC_{50} 30.63±3.21 µg/mL. Fenton reaction was used to assess the potential of ELM in inhibiting the hydroxyl radical production through iron (II)–dependent deoxyribose damage assay. The results showed low scavenging activity with an IC_{50} value of 52.22±0.97 µg/mL compared to positive control BHA (IC_{50} 17.59±1.00 µg/mL).

Table 18: *In vitro* antioxidant activity of ethanolic extract of *Leea macrophylla* and its fractions

Drug	IC_{50} concentration in µg/mL required for scavenging the free radical			
	DPPH radical	**Nitric Oxide Scavenging**	**H_2O_2 radical**	**Hydroxyl radical**
Standard	Ascorbic acid 23.67±1.67	Rutin 37.81 ± 3.57	Rutin 30.63 ± 3.21	BHA 17.59 ± 1.00
Extract				
ELM extract	39.80 ± 2.05	101.11 ± 2.37	74.15 ± 2.84	52.22 ± 0.97
ELM Fractions				
LMH fraction	248.74 ± 7.29	320.18 ± 1.36	409.48 ± 5.27	227.37 ± 5.55
LMC fraction	233.53 ± 5.00	290.77 ± 3.78	264.57 ± 4.10	203.07 ± 1.32
LMEA fraction	90.33 ± 0.36	149.05 ± 0.95	121.90 ± 1.97	123.45 ± 2.82
LMBU fraction	65.21 ± 1.12	114.15 ± 2.01	90.30 ± 4.01	86.52 ± 1.98
LMAQ fraction	71.49 ± 1.07	129.66± 3.88	118.20 ± 2.70	107.07 ± 0.68

Among the tested fractions, LMBU depicted the highest antioxidant capacity (232.00±0.95 µg/mL ascorbic acid equivalent) than the other fractions i.e. LMAQ: 183.58±2.46, LMEA: 136.83±1.83, LMC 69.00±1.13 and LMH: 37.25±1.73 µg/mL ascorbic acid equivalent respectively. In assay of reducing power LMBU exhibited potent antioxidant potential followed by LMAQ, LMEA, LMC and LMH, while in free radical scavenging activity using DPPH method, all the fractions tested, demonstrated a considerable free radical scavenging activity as indicated by the obtained IC_{50} values.

Standard ascorbic acid was found to have lowest IC_{50} value of 23.67±1.67 µg/mL followed by LMBU (IC_{50}: 65.21±1.12 µg/mL), LMAQ (IC_{50}: 71.49±1.07 µg/mL), LMEA (IC_{50}: 90.33±0.36 µg/mL), LMC (IC_{50}: 233.53±5.00 µg/mL) and LMH (IC_{50}: 248.74±7.29 µg/mL). Further, LMBU exhibited a highly potent scavenging activity (IC_{50} value 114.15±2.01 µg/mL) of all the fractions, followed by LMAQ, LMEA and LMC while hexane fraction showed the least scavenging activity in the assay of nitric oxide scavenging activity.

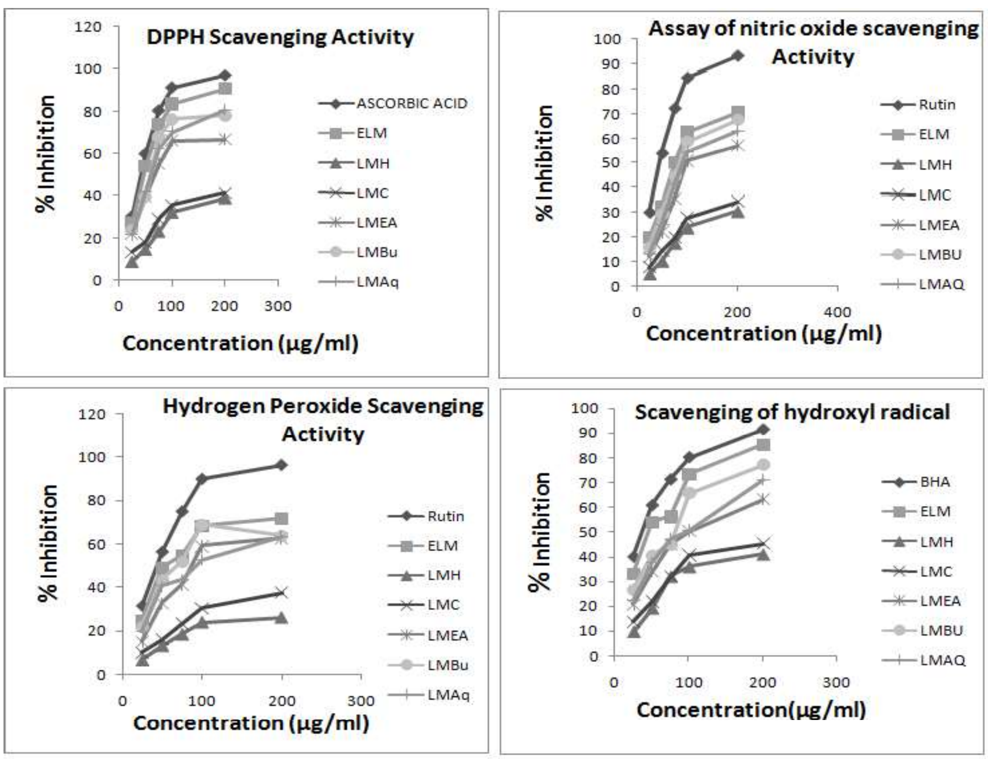


Figure 17: Radical scavenging activity of ethanolic extract of *Leea macrophylla* and its successive fractions

In scavenging of hydrogen peroxide method rutin, used as standard demonstrated the highest scavenging activity with an IC_{50} value of 30.63±3.21 µg/mL followed by LMBU, LMAQ, LMEA, LMC and LMH in descending order. The results revealed a potent hydroxyl radical scavenging activity of all fractions. As seen through the obtained IC_{50} values, standard BHA (IC_{50}: 17.59±1.00 µg/mL) showed maximum activity which was followed by LMBU (IC_{50}: 86.52±1.98 µg/mL), whereas LMH depicted the least scavenging activity.

7. Antibacterial activity

The assessment of antibacterial activity of *Leea macrophylla* extract and its successive fractions from root tubers against bacterial strains was found to be more pronounced in case of Gram positive bacteria compared to Gram negative bacteria (Table 26). Among the tested extract and fractions, ethanolic extract of *Leea macrophylla* demonstrated the most potent activity which was followed by LMBU, LMAQ, LMEA, LMC and LMH at their respective higher concentrations as observed by measuring the diameter of zone of inhibition. Among the tested strains, ethanolic extract of *Leea macrophylla* was highly effective against *S. aureus*, *S. flexneri* and *S. boydii,* whereas was found to be less effective against *S. typhi* and *K. pneumoniae*. MIC depicted a wide range of antibacterial activity with values ranging from 0.195 to 3.125 mg/mL (Table 19).

Table 19: Effect of different fractions of *Leea macrophylla* on zone of inhibition (in mm) and MIC (mg/ml) against different bacterial strains

Strains	SA	EF	SF	ST	EC	KP	PA	SB
Drug/Extract/Fraction (mg/ml)	**ZONE OF INHIBITION (in mm)**							
ELM 50	12.41±0.31	10.21±0.47	9.54±0.30	7.18±0.51	10.03±0.29	8.63 ±0.32	11.63±0.54	10.53±0.53
ELM 100	18.54±0.34	14.52±0.26	12.54±0.30	11.45±0.34	13.57±0.38	10.60±0.53	16.72±0.43	14.80±0.25
LMH50	7.81±0.34	–	7.46±0.23	5.81±0.46	7.28±0.20	–	6.51±0.43	7.17±0.51
LMH100	10.38±0.55	–	8.91±0.47	7.08±0.44	10.5±0.37	–	9.80±0.42	10.53±0.30
LMC50	9.85±0.43	6.85±0.34	7.61±0.21	6.02±0.48	7.81±0.34	–	6.84±0.36	8.14±0.52
LMC100	12.9±0.35	10.42±0.33	9.34±0.22	7.65±0.36	11.46±0.28	–	11±0.15	11.30±0.32
LMEA50	10.81±0.25	7.23±0.47	8.06±0.35	6.58±0.38	8.43±0.41	6.03±0.41	8.50±0.44	8.93±0.39
LMEA100	14.33±0.33	10.90±0.45	10.15±0.51	8.55±0.60	12.34±0.47	7.90±0.24	12.87±0.40	12.53±0.54
LMBU50	11.28±0.51	7.93±0.43	8.37±0.31	6.88±0.46	8.97±0.53	7.46±0.38	9.31±0.45	9.93±0.48
LMBU100	16.80±0.30	12.44±0.32	11.95±0.18	9.68±0.40	12.63±0.32	9.50±0.51	14.13±0.46	14.22±0.55
LMAQ50	11.03±0.45	7.78±0.39	8.24±0.46	6.82±0.12	8.54±0.32	7.08±0.61	8.84±0.46	9.27±0.28
LMAQ100	15.26±0.66	11.67±0.44	10.89±0.29	9.08±0.58	12.4±0.35	8.60±0.37	12.93±0.36	13.13±0.28
Cipro 0.5	27.85±0.25	30.64±0.34	21.33±0.45	25.80±0.54	28.59±0.32	28.80±0.30	26.34±0.25	25.81±0.60
				MIC(mg/ml)				
ELM	0.195	0.781	0.390	3.125	0.781	1.562	0.390	0.781
LMH	0.781	–	0.781	3.125	6.25	–	3.125	6.25
LMC	0.390	0.781	1.562	0.781	0.390	–	6.25	3.125
LMEA	0.781	0.781	1.562	0.390	1.562	0.781	1.562	6.25
LMBU	0.390	0.781	0.390	1.562	0.781	0.390	0.781	1.562
LMAQ	0.781	1.562	0.781	0.390	1.562	0.781	0.390	6.26

In table, ELM: Ethanolic extract of root tubers of *Leea macrophylla*, LMH: Hexane fraction of Ethanolic extract of root tubers of *Leea macrophylla,* LMC: Chloroform fraction of Ethanolic extract of root tubers of *Leea macrophylla,* LMEA: Ethyl acetate fraction of Ethanolic extract of root tubers of *Leea macrophylla* LMBU: Butanol fraction of Ethanolic extract of root tubers of *Leea macrophylla,* LMAQ: Aqueous fraction of Ethanolic extract of root tubers of *Leea macrophylla* and Cpr: Ciprofloxacin; SA: S. aureus, EF: E. faecalis, SF: S. flexneri, ST: S.typhi, EC: E. coli, KP: K. pneumonia, PA: P .aeruginosa and SB: S. boydii

PHARMACOLOGICAL EVALUATIONS

1. Topical Formulation and its Texture Profile Analysis (TPA)

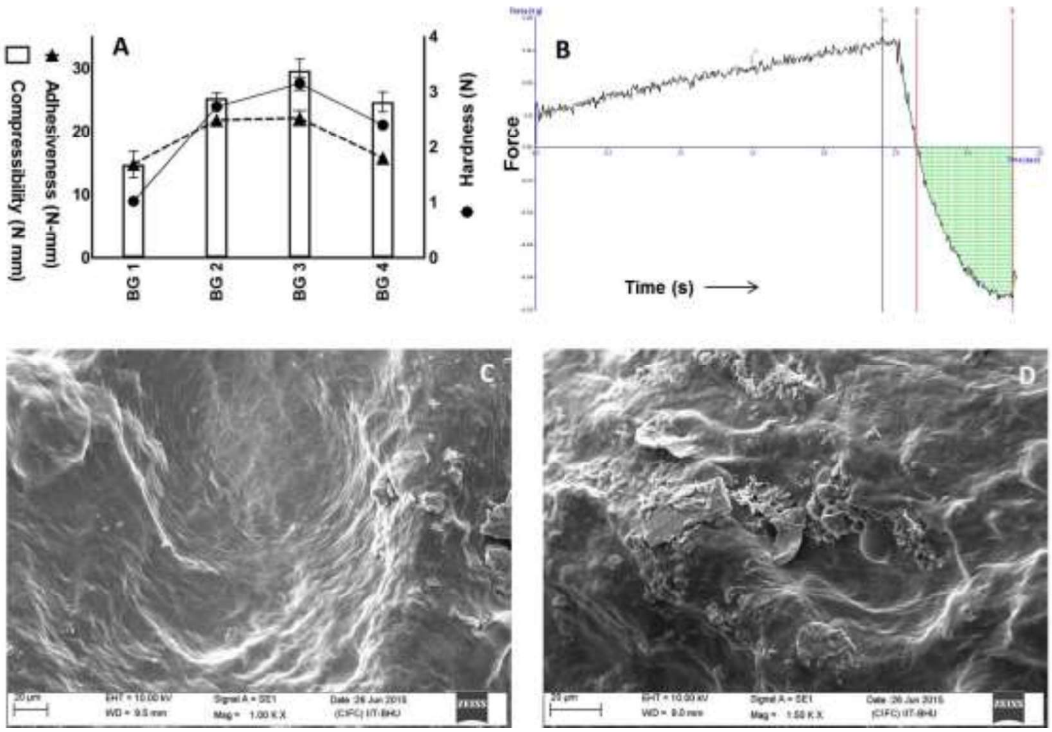


Figure 18: Physicochemical characteristics of bioadhesive gel of ethanolic extract of *Leea macrophylla*. (A) Hardness, compressibility and adhesiveness of the batches of bioadhesive gel formulated. Considering the highest adhesiveness, moderate hardness and compressibility BG2 was selected as the best topical formulation (B) Force time plot of best selected topical formulation BG2. Surface morphology of (C) blank gel and (D) bioadhesive gel BG2 captured by scanning electron microscope.

The mechanical properties of the extract containing topical bioadhesive gel were obtained using texture profile analysis, as presented in Figure 18 A. Figure 18 B is a force time plot of BG2.The hardness of gels was: BG1 (1.022±0.03N), BG2 (2.730±0.04N), BG3 (3.150±0.12N), BG4 (2.400±0.08N). The compressibility was: BG1 (14.78±2.10N-mm), BG2 (25.26±0.83 N-mm), BG3 (29.66±1.80 N-mm), BG4 (24.65±1.60 N-mm). The adhesiveness was: BG1 (14.79±0.31), BG2 (21.80±0.67 N-mm), BG3 (22.09±1.19 N-mm), BG4 (15.80±0.54 N-mm). As it may be discernible from the figure, increasing concentration of SCMC from 2.5to 4% (w/v) and PVP 2.5

to 4% (w/v) in BG1 to BG3 significantly increased formulation hardness, compressibility and adhesiveness. Considering the highest adhesiveness, moderate hardness and compressibility of BG2 was selected as the best topical formulation and used for further studies. BG2 and an equivalent blank gel were lyophilized and observed under a SEM.

SEM micrographs for blank gel (Figure 18 C) revealed a smooth undulating texture. The presence of distorted irregular entities in case of lyophillized BG2 (Figure 18 D) is probably evidence of precipitated microparticles of *Leea macrophylla* extract upon drying.

2. Acute Toxicity Studies

2.1. Acute oral toxicity study

The results of acute oral toxicity study found the safety limit of extract to be 5 g/kg, when administered via p.o. route. The extract did not exhibit any behavioral modulation, or symptoms of toxicity or morbidity on the experimental animals.

2.2. Acute skin irritation test

Acute dermal toxicity study showed that formulated bioadhesive gel containing ethanolic extract of *Leea macrophylla* did not induce any noticeable swelling, inflammation, irritation or any others abnormalities on the skin of the treated animals.

3. Wound healing activity

3.1. Incision wound model

Figure 19 represents the effect of orally administered and topically applied ethanolic extract of *Leea macrophylla* on tensile strength of wounds. The wound breaking strength (WBS) after oral administration and topical application was increased significantly when incision study was performed. However the increase in results was more pronounced when the extract was applied topically. The blank gel alone didn't produce any significant rise in wound breaking effect. Highly significant increment in WBS was obtained in treated animals, with percentage increase of 23.41%, 27.66% and 27.13% when treated with ELM orally at 500, 750 mg/kg and topically at 2.5% w/v respectively which were comparable with oral standard Vitamin E (30.85%).

Table 20- Effect of Oral administration and Topical application of ethanolic extract of *Leea macrophylla* on wound breaking strength in rats

Treatment Oral* (mg/kg p.o.)/ Topical# (%), 10 days	**Wound breaking strength (g)**	**% Increase**
Control*	313.33 ± 8.82	–
Gel Base#	321.67 ± 7.03	2.66
ELM 250*	346.67 ± 7.15	10.64
ELM 500*	386.67 ± 6.15	23.41
ELM 750*	400.00 ± 8.56	27.66
ELM 2.5#	398.33 ± 10.14	27.13
ELM 5#	453.33 ± 9.19	44.68
ELM 7.5#	461.67 ± 10.78	47.34
VTE*	410.00 ± 10.65	30.85
ALOE#	481.67 ± 9.80	53.73

Value are mean ± SEM (n=6). Statistical comparison determined by one way ANOVA followed by the Newman–Keuls multiple comparison test, where $^{a}p < 0.05$ vs. control, $^{b}p < 0.05$ vs. gel base, $^{c}p < 0.05$ vs. ELM 250, $^{d}p < 0.05$ vs. ELM 500, $^{e}p < 0.05$ vs. ELM 750, $^{f}p < 0.05$ vs. ELM 2.5, $^{g}p < 0.05$ vs. ELM 5, $^{h}p < 0.05$ vs. ELM 7.5, $^{i}p < 0.05$ vs. VTE

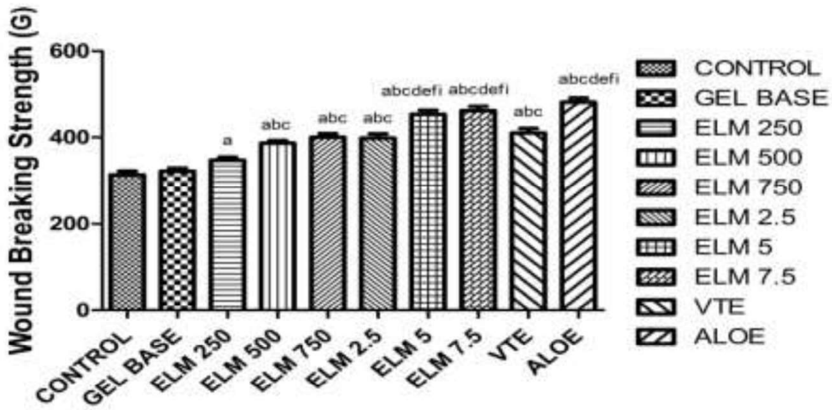


Figure 19: Effect of oral and topical treatment of ethanolic extract of *Leea macrophylla* on breaking strength in rats.

A percentage increase of 44.68% and 47.34% were observed with topical treatment at 5% w/v and 7.5% w/v ELM bioadhesive gel respectively which were comparable to topical standard Aloe vera cream (53.73%). However, the WBS of rats plateaued at doses exceeding 500 mg/kg p.o. Interestingly 5% w/v and 7.5% w/v bioadhesive gel of *Leea macrophylla* extract exerted almost parallel effects on the tensile strength of wounded tissue. Therefore, 500 mg/kg orally was fixed as effective dose of ELM and 5% w/v for topical application for further investigation (Table 20).

3.2. Excision wound model

In excision wound model the untreated control rats exhibited chronologically retracting wound contraction, with complete wound contraction attained on 24th day. Healing rate of excised wound was faster in rats treated orally or topically with ethanolic extract compared to the control group. A statistically significant difference, within the treatment modules (oral and topical) was also observed. Topical treatment with ELM showed optimum wound contraction on 16th day (96.8%), which was faster as compared to oral treatment (91.7%) , also evident with complete wound contraction which was found to be on 20th day with topical treatment while that with oral treatment was observed on 22nd day (Figure 20, 21, Table 21).

The results also depicted that topically treated animals showed faster healing as compared to oral treatment and the results were comparable to standard Vitamin E as well as Aloe vera cream. Complete wound contraction in animals treated topically with ELM gel 5% w/v were achieved in 20 days while oral treatment with ELM 500 mg/kg exhibited complete wound contraction in 22 days. Control animals showed delayed wound healing with complete wound contraction in 24 days.

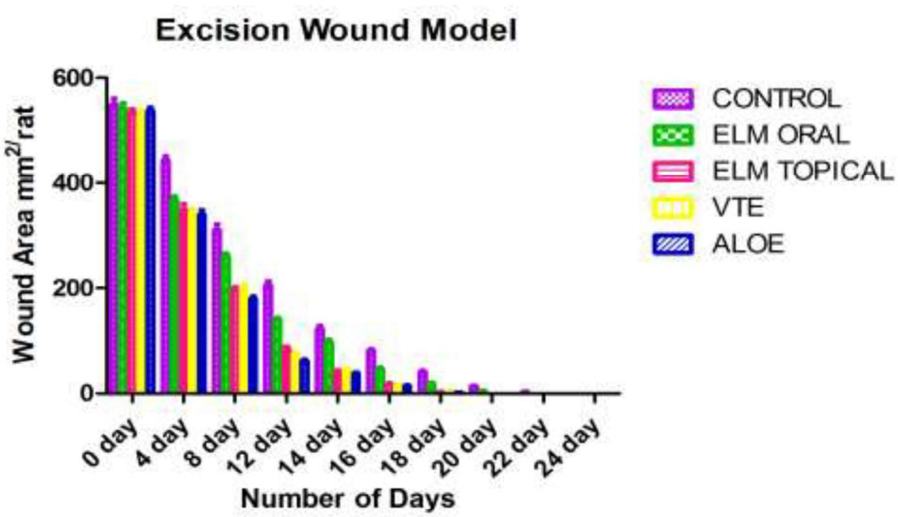


Figure 20: Effect of oral and topical treatment of ethanolic extract of *Leea macrophylla* on wound area

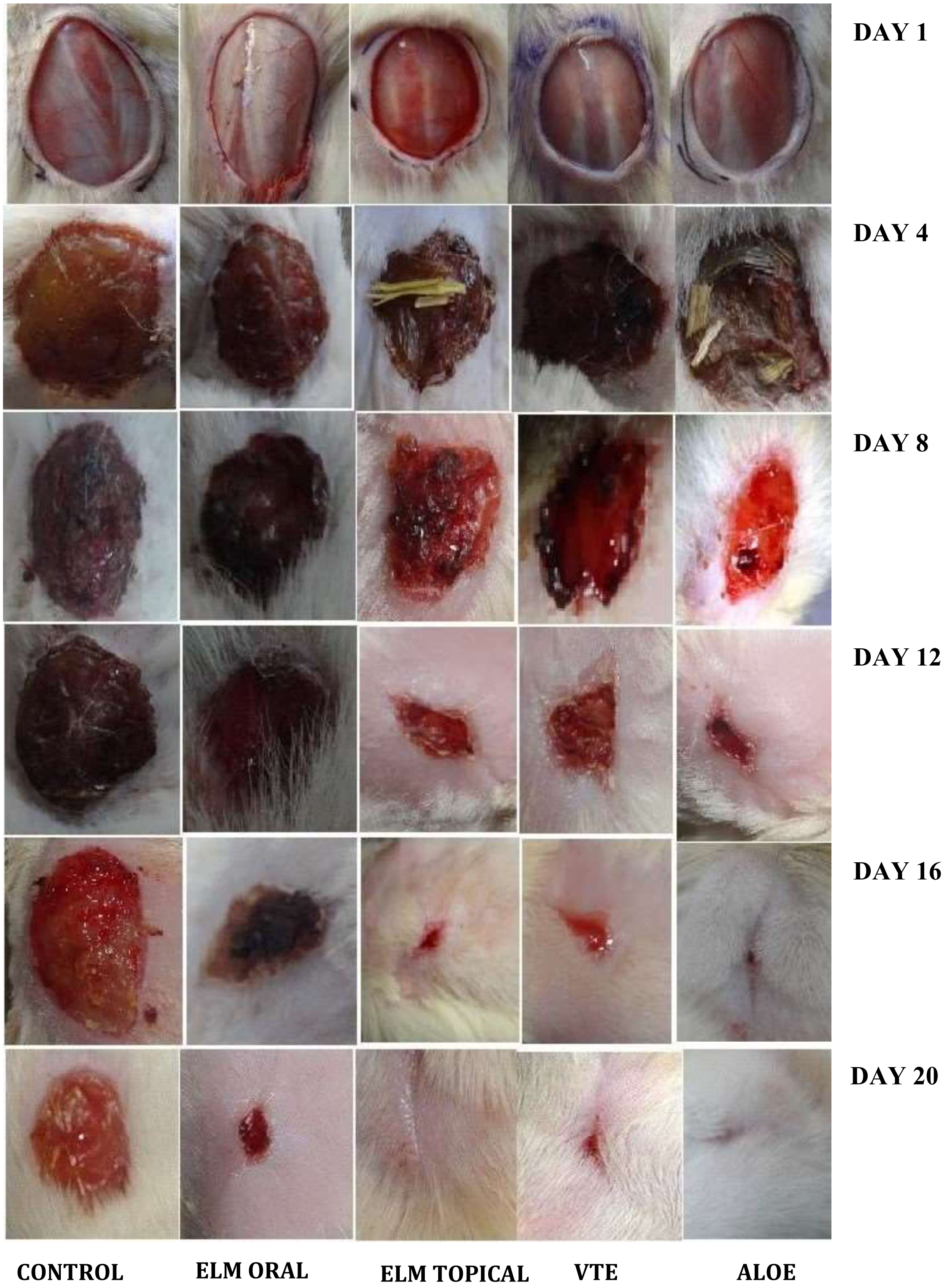


Figure 21: Images of excision wound with oral and topical treatment of ethanolic extract of *Leea macrophylla*.

EXCISION WOUND STUDY IN RATS

Table 21- Effect of Oral administration and Topical application of ethanolic extract of *Leea macrophylla* on wound contraction

Treatment (mg/kg p.o.)	Wound area in mm^2/rat (%)								
	0 day	4^{th} day	8^{th} day	12^{th} day	14^{th} day	16^{th} day	18^{th} day	20^{th} day	22^{nd} day
Control	549.00±11.50 (0.0)	443.17±8.03 (19.3)	310.67±10.94 (43.4)	205.83±7.33 (62.5)	121.83±6.24 (77.8)	81.50±2.17 (85.2)	40.67±2.09 (92.6)	13.50±1.18 (97.5)	3.17±0.48 (99.4)
ELMO	541.17±10.80 (0.0)	367.33±7.56 (32.1)	259.83±6.07 (52.0)	140.50±3.43 (74.0)	97.67±4.18 (82.0)	45.17±3.25 (91.7)	18.83±1.25 (96.5)	4.33±0.71 (99.2)	0.00±0.00 (100.0)
ELMT	532.33±8.09 (0.0)	349.83±10.53 (34.3)	195.67±6.50 (63.2)	85.17±4.07 (84.0)	40.50±2.75 (92.4)	17.00±2.28 (96.8)	2.67±0.49 (99.5)	0.00±0.00 (100.0)	0.00±0.00 (100.0)
VTE	527.00±11.72 (0.0)	344.17±7.85 (34.7)	197.67±8.92 (62.5)	73.50±5.48 (86.1)	44.33±3.40 (91.6)	15.83±1.80 (97.0)	4.67±0.76 (99.1)	0.00±0.00 (100.0)	0.00±0.00 (100.0)
ALOE	536.50±6.74 (0.0)	339.67±9.18 (36.7)	179.33±4.32 (66.6)	62.17±2.44 (88.4)	37.00±2.03 (93.1)	14.33±1.28 (97.3)	1.17±0.54 (99.8)	0.00±0.00 (100.0)	0.00±0.00 (100.0)

Value are mean ± SEM (n=6). Statistical comparison was performed by two way ANOVA followed by Bonferroni post hoc test.

Mean epithelization period and scar area justified significant healing effect of topically treated animals in comparison to oral treatment (Figure 22, Table 22). The topical treatment with ELM showed higher reduction in mean epithelisation period and scar area in comparison to oral treatment.

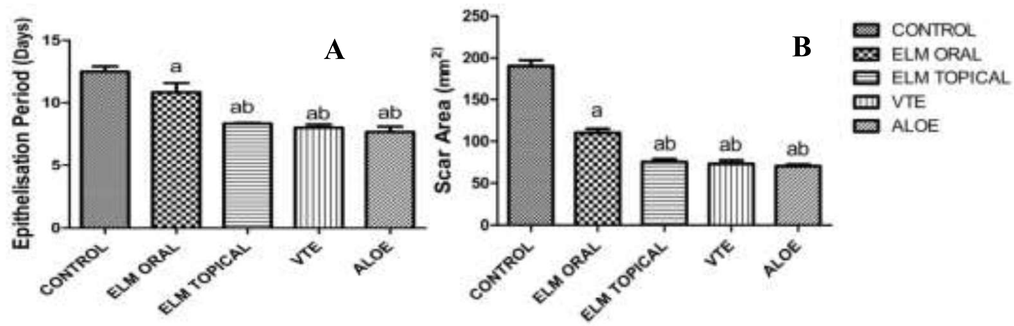


Figure 22: Effect of oral and topical treatment of ethanolic extract of *Leea macrophylla* on (A) Epithelisation period and (B) Scar area

Table 22- Effect of Oral administration and Topical application of ethanolic extract of *Leea macrophylla* on epithelization period and scar area

Oral Treatment (mg/kg,)	Epithelization period (Days)	Scar area (mm^2)
Control	12.50 ± 0.43	189.67 ± 7.82
ELMO	10.83 ± 0.75	110.17 ± 4.44
ELMT	8.33 ± 0.21	75.50 ± 3.33
VTE	8.00 ± 0.26	73.17 ± 4.28
ALOE	7.67 ± 0.42	70.50 ± 1.84

Value are mean ± SEM (n=6). Statistical comparison was determined by one way ANOVA followed by the Newman–Keuls multiple comparison test where $^{a}p< 0.05$ vs. control, $^{b}p< 0.05$ vs. ELM oral, $^{c}p< 0.05$ vs. ELM topical, $^{d}p< 0.05$ vs. VTE.

3.3. Biochemical estimations

Animals which underwent topical treatment showed greater increase in weight of granulation tissue and protein in comparison to those receiving oral treatment (Figure 23, Table 23). (22.04%) however the increase in dry tissue protein was not significant.

Table 23- Effect of Oral administration and Topical application of ethanolic extract of *Leea macrophylla* on granulation tissue protein content

Oral treatment (mg/kg, once daily x10 days)	Wet Tissue		Dry Tissue	
	Weight (mg/100 g bw)	**Protein (mg/g wet tissue)**	**Dry tissue (mg/ 100 g bw)**	**Protein (mg/g dry tissue)**
Control	254.20±7.36	61.56±1.03	61.38 ± 1.67	203.23 ± 4.31
ELM Oral	299.13±9.25	69.42±1.22	74.91 ± 2.66	218.44 ± 5.07
ELM Topical	350.64±10.08	78.85±1.16	84.03±2.77	278.44±9.59
VITAMIN E	322.50±6.76	72.21±1.31	81.27±3.42	257.08±6.86
ALOE	366.72±5.65	79.06±1.00	89.62± 3.64	296.98±7.18

Value are mean ± SEM (n=6). Statistical comparison was determined by one way ANOVA followed by Newman–Keuls multiple comparison test where [a] $p < 0.05$ vs. control, [b] $p < 0.05$ vs. ELM oral, [c] $p < 0.05$ vs. ELM topical, [d] $p < 0.05$ vs. VTE.

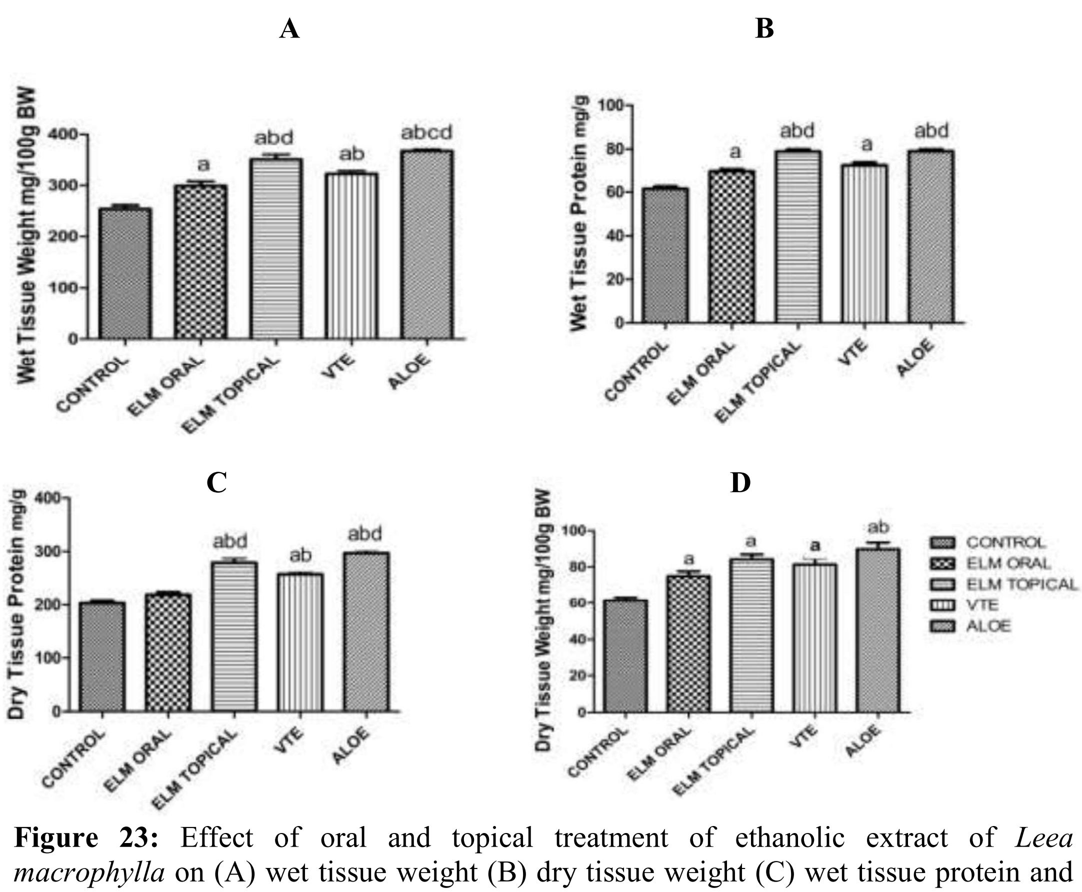


Figure 23: Effect of oral and topical treatment of ethanolic extract of *Leea macrophylla* on (A) wet tissue weight (B) dry tissue weight (C) wet tissue protein and (D) dry tissue protein

The increment in dry and wet weight of granulation tissue and protein content was highly significant in the topically treated group when compared to control group.

Increase in wet tissue and protein was found to be 37.94% and 28.09% respectively and dry tissue and protein was found to be 36.90% and 37.01% respectively when treated topically with ELM bio gel. Oral treatment also afforded significant wet granulation tissue (17.68%), protein content (12.77 %) and dry granulation tissue weight

ELM extract exhibited significant increase in value of antioxidants GSH, SOD and CAT whereas level of free radical generating enzymes LPO and NO, and inflammatory markers, MPO was reduced.

Table 24- Effect of Oral administration and Topical application of ethanolic extract of *Leea macrophylla* on granulation tissue protein, superoxide dismutase (SOD), reduced glutathione (GSH) and catalase (CAT) contents: Study on antioxidant status

Oral treatment (mg/kg, once daily x10 days)	**Protein**	**SOD**	**GSH**	**CAT**
	mg/g wet tissue	**IU/mg protein**	**ng/mg protein**	
Control	61.56±1.03	0.27±0.01	11.28±0.28	8.74±0.34
ELM Oral	69.42±1.22	0.36±0.01	13.57±0.80	16.44±0.40
ELM Topical	78.85±1.16	0.48±0.01	19.29±0.63	19.99±0.33
Vitamin E	72.21±1.31	0.45±0.01	17.64±0.78	19.33±0.53
ALOE	79.06±1.00	0.50±0.02	20.13±0.60	22.60±0.35

Value are mean ± SEM (n=6). Statistical comparison was determined by one way ANOVA followed by Newman–Keuls multiple comparison test where ^{a} $p< 0.05$ vs. control, ^{b} $p< 0.05$ vs. ELM oral, ^{c} $p< 0.05$ vs. ELM topical, ^{d} $p< 0.05$ vs. VTE.

.

Table 25- Effect of Oral administration and Topical application of ethanolic extract of *Leea macrophylla* on lipid peroxidase (LPO), nitric oxide (NO) and MPO contents: Study on free radicals status and anti-inflammatory marker

Oral treatment (mg/kg, once daily x10 days)	Protein	LPO	NO	MPO
	mg/g wet tissue	nM/mg protein	nM/mg protein	
Control	61.56±1.03	4.75±0.30	19.70±1.00	25.40±0.42
ELM Oral	69.42±1.22	2.55±0.09	9.81±0.29	20.59±0.44
ELM Topical	78.85±1.16	1.99±0.13	6.11±0.41	12.88±0.26
Vitamin E	72.21±1.31	2.40±0.12	8.07±0.37	16.58±0.35
ALOE	79.06±1.00	1.72±0.10	5.72±0.39	12.03±0.24

Value are mean ± SEM (n=6). Statistical comparison was performed by one way ANOVA followed by Newman–Keuls multiple comparison test where [a] $p < 0.05$ vs. control, [b] $p < 0.05$ vs. ELM oral, [c] $p < 0.05$ vs. ELM topical, [d] $p < 0.05$ vs. VTE.

Further advantageous effects on wound healing was reflected by significantly increased levels of hydroxyproline, hexosamine and hexuronic acid levels per unit of dried granulation tissue (g) as well as protein (mg). However, the effect was more significant with topical application (Table 24, 25, 26).

Table 26- Effect of Oral administration and Topical application of ethanolic extract of *Leea macrophylla* on granulation tissue weight, protein, hydroxyproline (HP), hexosamine (HA) and hexuronic acid (HUA) in granulation tissue in rats

Oral treatment (mg/kg, od x 10 days)	Dry tissue (mg/100g bw)	Protein (mg/g dry tissue)	Connective tissue parameters µg/mg protein		
			HP	HA	HUA
Control	61.38± 1.67	203.23± 4.31	140.57±7.17	103.67±7.37	30.08±1.27
ELM Oral	74.91± 2.66	218.44± 5.07	177.10±11.58	117.34±6.69	37.76±1.50
ELM Topical	84.03±2.77	278.44±9.59	211.12 ±13.70	144.98±6.21	51.79±2.58
Vitamin E	81.27±3.42	257.08±6.86	203.23±8.24	140.16±5.91	43.68±1.99
ALOE	89.62± 3.64	296.98±7.18	221.26±13.70	150.18± 5.55	53.22± 3.70

Value are mean ± SEM (n=6). Statistical comparison was determined by one way ANOVA followed by Newman–Keuls multiple comparison test where [a] $p< 0.05$ vs. control, [b] $p< 0.05$ vs. ELM oral, [c] $p< 0.05$ vs. ELM topical, [d] $p< 0.05$ vs. VTE.

Figure 24 represents the effect of extract on antioxidants, free radicals, inflammatory markers and collagen determinants.

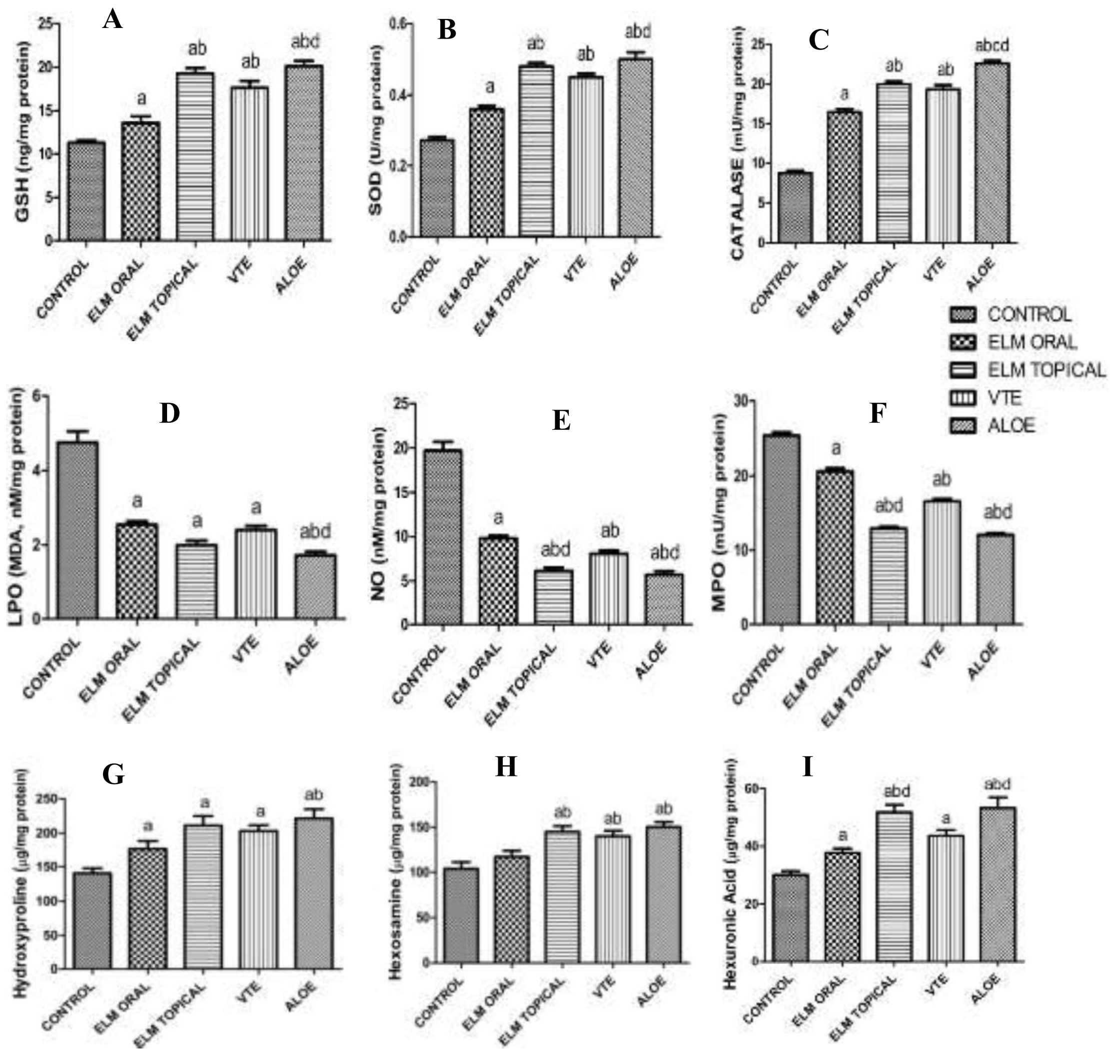


Figure 24: Effect of oral and topical treatment of ethanolic extract of *Leea macrophylla* on antioxidants, free radicals and collagen determinants (A) Reduced glutathione (B) Superoxide dismutase (C) Catalase (D) Lipid peroxidation (E) Nitric oxide (F) Myeloperoxidase (G) Hydroxyproline (H) Hexosamine and (I) Hexuronic Acid

3.4. Estimation of IL-1β, IL-6, TNF-α and VEGF

Figure 25 represents the effect of ethanolic extract of *Leea macrphylla* on proinflammatory cytokines IL-1β, IL-6, TNF-α and growth factor VEGF. On 10th post-wounding day in excision model level of IL- 1β were significantly reduced (1232.89±9.26 pg/ml) in orally treated animals whereas the effect were more pronounced in topically treated animals (646.67±10.73 pg/ml) and were comparable to topical standard drug Aloe (601.38±8.18) while untreated control animals showed higher levels (2061.24±7.01 pg/ml). The results also indicated a significant decrease in IL-6 when treated with ethanolic extract of *Leea macrophylla.* Control animals demonstrated higher level of IL-6 (3328.13±3.84 pg/ml) and the decrease was more in topically treated animals (1468.67±8.67 pg/ml) than orally treated (2932.44±4.75 pg/ml). The data from ELISA analysis further showed higher reduction in TNF-α levels when animals were treated topically (2040.06±13.25 pg/ml) as compared to orally administered (4563±8.61 pg/ml) while levels were higher in control animals (6528.44±10.20 pg/ml). The results depicted significant augmentation in VEGF production in treated groups as compared to control group which showed low level of VEGF (418.35±4.71 pg/ml). Topical application (907.21±7.48 pg/ml) illustrated effect comparable to topical standard Aloe (1112.31±9.48 pg/ml) in comparison to oral treatment (714.35±5.41pg/ml).

Levels of IL-1β, IL- 6, TNF α and VEGF were determined using ELISA kits on granulation tissue homogenate obtained on 10th post-wounding day from excision wound model. ELM treatment lowered the level of pro-inflammatory cytokines while level of VEGF was augmented. The results were more pronounced with topical treatment.

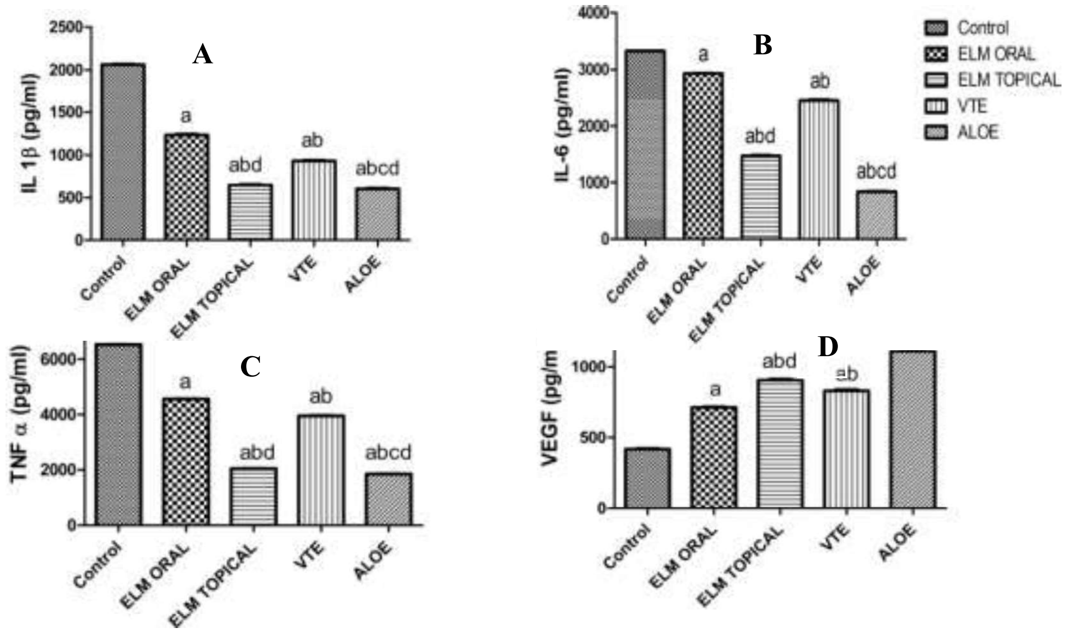
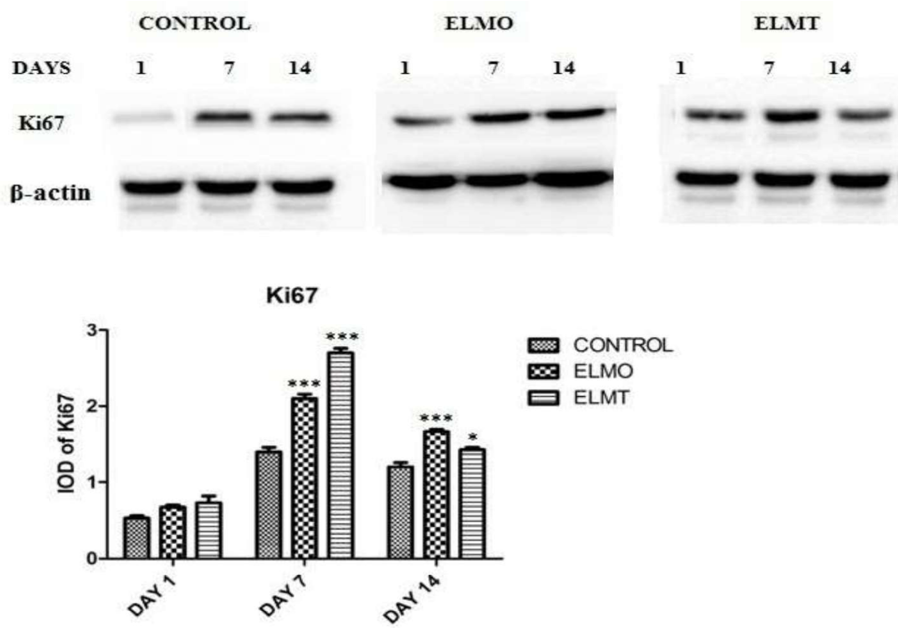


Figure 25: Effect of oral and topical treatment of ethanolic extract of *Leea macrophylla* on (A) IL-1β (B) IL- 6 (C) TNF α and (D) VEGF.

Value are mean ± SEM (n=6). Statistical comparison was determined by one way ANOVA followed by the Newman–Keuls multiple comparison test where ^{a} p< 0.05 vs. control, ^{b} p< 0.05 vs. ELM oral, ^{c} p< 0.05 vs. ELM topical, ^{d} p< 0.05 vs. VTE

3.5. Western Blot analysis for proliferation marker Ki-67

Cell proliferation in wounded skin investigated by western blot analysis of proliferation marker Ki67 indicated that the expression of Ki67 was evident from day 1 post-wounding in control and treated groups (orally and topically treated with ethanolic extract of *Leea macrophylla*) however there was no significant difference in its level between the groups. On 7th day rise in level of Ki67 was highly significantly in orally and topically treated animals as compared to control however the increase in Ki67 level was greater in topically treated. On 14th day decline in Ki67 was evident in all groups however the decline rate was considerably higher in topically treated groups followed by orally treated and control groups. The study illustrated that ethanolic extract of *Leea macrophylla* treatment triggered cell proliferation during wound healing while the effect was more prominent with topical treatment as compared to oral administration (Figure 26).



Value are mean ± SEM (n=3). Statistical comparison was performed by two way ANOVA followed by Bonferroni post hoc test. $^{*}p < 0.05$ and $^{***}p < 0.001$ compared to control group. Treated groups showed a significant increase in expression of proliferation marker Ki-67 this suggested treatment with ethanolic extract of *Leea macrophylla* promotes cellular proliferation and hence wound healing.

Figure 26: Effect of oral and topical treatment of ethanolic extract of *Leea macrophylla* on cellular proliferation. Ki67 in rat wound was determined by Western blot at indicated days. The IOD (integration optical density) of Ki67 protein band was calibrated using β-actin.

3.6. Histological studies

To gain a mechanistic insight into the stages of wound healing (initial inflammation, proliferation and skin remodeling) which results in re-epithelization; histology of excised skin-wound from experimental animals was observed on 'day 10' after treatment initiation. Accelerated healing was observed in topically treated group with greater collagen content, angiogenesis, keratinization, fibroblast proliferation and epithelialisation compared to orally treated group. The wound healing rate was protracted in the control group (Figure 27).

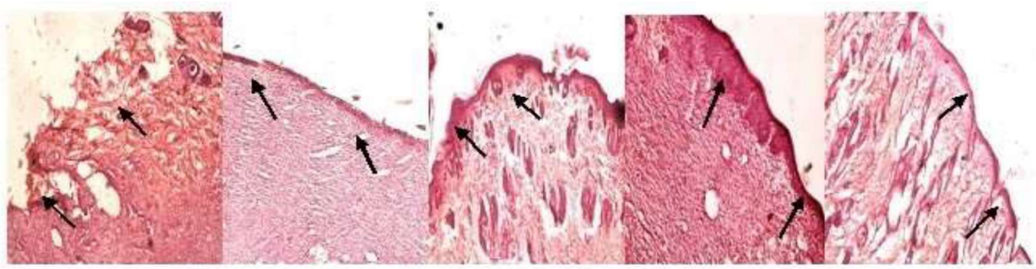


Figure 27: Histopathological view of excision wound on 10th post-operative day with oral and topical treatment of ethanolic extract of *Leea macrophylla*

Arrow indicates the epithelization zone which is more prominent in case of groups treated with ELM topical as compared to those treated with ELM oral. The histologic rearrangement suggests greater extent of wound healing in topically treated animals.