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DEVELOPMENT, CHARACTERISATION AND PRECLINICAL EVALUATION OF SOME NOVEL ENZYMES WITH VITAMIN

Amol S.Rakte*[1], Dr. Basavaraj K.Nanjwade[2]

*[1]Department of Pharmaceutics, Faculty of Pharmacy, Pacific University, Udaipur-313024, Rajasthan, India.

[2]Department of Pharmaceutics, Faculty of Pharmacy, Omer Al-Mukhtar University, Tobruk, P. O. Box 919, Libya.

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***Correspondence for Author**
Amol S.Rakte
Department of Pharmaceutics, Faculty of Pharmacy, Pacific University, Udaipur-313024, Rajasthan, India.

ABSTRACT

Aim of the present research was to formulate and evaluate a multienzyme (Bromelain, Papain, Bacterial Protease, Rutin) with Vitamin C effervescent tablet with sufficient mechanical integrity, good palatability, robust formulation, good stability, inexpensive and shop floor friendly product produced in normal thermo-hygrometric conditions. In the preformulation study, identification of each enzymes were done with the help of MALDI-TOF instrument and compatibility evaluation was performed which implies that drug and excipients were compatible with each other. The formulations of tablets were done by using different methods like wet granulation, dry granulation and direct compression. Total ten trials for each method was prepared and evaluated for hardness, friability, effervescent time, weight variation, and pH. Finally one batch from each method having good physical parameters was kept for stability {DC-6 (direct compression) DG-7(dry granulation) and WG-8(wet granulation)}. From that techniques stability of tablets with wet granulation method was found to be satisfactory than other methods. The preclinical evaluation of this composition study has been undertaken to investigate the anti-inflammatory and toxicity studies in laboratory animals (Albino Wistar Rats).The main method used for Anti-inflammatory was Carrageenan induced paw edema and for toxicity studies OECD 407 (Repeated Dose 28-Day Oral Toxicity Study in Rodents) and OECD 425 (Acute Oral Toxicity – Up-and-Down Procedure).

KEYWORDS: Effervescence, Enzyme Premix, OECD, MALDI-TOF, Carrageenan induced paw edema.

INTRODUCTION

Effervescence is the reaction (in water) of acids and bases producing carbon dioxide. Typical acids used in this reaction are citric, malic, tartaric, adipic, and fumaric. Citric acid is the most commonly used, and it imparts a citrus-like taste to the product. Tartaric, adipic, and fumaric acids are used sparingly because of their low water solubility's. Typical bases used in the effervescent reaction are sodium bicarbonate, potassium bicarbonate, sodium carbonate, and potassium carbonate. Sodium bicarbonate is very common in effervescent formulas and produces a clear solution after tablet disintegration. When sodium levels are a concern, potassium bicarbonate is used. Both types of carbonates are used mainly as desiccants. Binders are normally necessary in effervescent tablets to bring the tablet hardness to a point where handling is possible. A binder should be used very cautiously because binders can carry free moisture into the tablet, which is undesirable and can increase disintegration times when used in large quantities. The ideal amount of binder is one that makes the tablet hard enough to handle, but soft enough to disintegrate (harder the tablet, slower the disintegration) and dry enough to be stable.[1] Effervescent system also available with powder form, the problem with this type of formulation is content of uniformity, but not associated with effervescent tablets. Generally any effervescent product requires strictly temperature (65^0F to 75^0F) and humidity (10 to 20 % RH) controlled area. So, we were trying to prepare such type of product in a normal thermo-hygrometric controlled area (24^0C/35% RH) with good palatability, robust formulation, good stability, inexpensive and shop floor friendly product. Following advantages with effervescent dosage form,

1) Ingredients are fully bio-available[2]
2) The Presence of carbon dioxide enhances absorption[3]
3) Creates a buffered solution[4]
4) Incorporation of more active nutrients[5]
5) Consistent and reliable dose[5]
6) Great taste
7) Gentle action, less stomach upsets
8) Effervescent vitamins are easier to swallow[6]

MATERIAL AND METHODS

Materials

Enzyme blend were obtained as gift sample from AE, Mumbai. Citric acid (anhydrous), Fumaric acid, Sodium carbonate, Sodium benzoate procured from Thomas Baker, Mumbai.

Sodium bicarbonate, Tartaric acid purchased from Lar Chemicals. Simethicone (30%) gift sample from Meyer Mumbai. Aerosil-200 gift sample from Evonik, Mumbai. Orange flavor gift sample from Quest fragrances, Mumbai. Povidone, Isopropyl alcohol, Methylenedichloride gift sample from AE. Mannitol, Lactose, Microcrystalline Cellulose, Crospovidone, Hypromellose, Tween-80 gift sample from Signet Chemical Corporation, Mumbai.

Methods

In this research three methods were used.

A) Direct compression

B) Dry granulation (Slug)

C) Wet granulation

A) Direct compression

Enzyme blend, citric acid (anhydrous), tartaric acid, sodium bicarbonate, sodium carbonate, sodium citrate, sodium glycine carbonate, hypromellose, povidone, sodium lauryl sulphate, sodium benzoate were separately weighed and passed through sieve no. #40. Orange flavor and acesulfame potassium weighed and passed through sieve no.#80.Purifed talc and colloidal silicon dioxide were weighed and passed through sieve no.#60.Then the ingredients mixed in geometric order and compressed it using 24.8 mm circular plain punches on single rotary 12 stations tablet compression machine. Following were the trial formulations prepared with this method shown in Table 1.

B) Dry Granulation (Slug)

Typically, the process involves compressing a powder mixture into a rough tablet or 'slug' on a heavy duty rotary tablet press. The slugs are then broken up into granular particles by a grinding operation, usually by passage through an oscillating granulator the individual steps include mixing of the powders, compression (slugging) and grinding.[7]

1) Slug preparation

Enzyme blend, povidone, half quantity of mannitol, lactose, crospovidone, sodium benzoate were weighed and passed through sieve no.#40 and compressed it using 16 mm FBE (Flat beveled edges) circular plain punches on single rotary tablet compression machine with hardness 6 – 7 Kg/cm^2.Obtained tablets were milled in 3.0 mm screen and then passed through sieve no.#16.

2) Granulation

Half quantity of citric acid (anhydrous), tartaric acid, sodium bicarbonate were weighed and passed through sieve no.#40.Taken the 1 % of water for granulation with total granulating material. Obtained wet mass were sift through sieve no. #8 and kept for drying at 50^0 C ± 5^0C for 30 minutes. Semidried mass were passed through sieve no. #20 and then dried at 50^0 C ± 5^0C until L.O.D. obtained below 1.0 %.

3) Prelubrication

Remaining half quantity of mannitol, citric acid (anhydrous), tartaric acid, and sodium bicarbonate were taken with microcrystalline cellulose and passed through sieve no. #40.Weighed the acesulfame potassium, orange flavor and passed through sieve no. #80

4) Lubrication

Colloidal silicon dioxide, purified talc weighed and passed through sieve no.#60 along with remaining half quantity of sodium benzoate already passed through sieve no. #40.The all steps were mixed and taken the trials using 24.8 mm circular plain punches on single rotary 12 stations tablet compression machine. Following were the trial formulations prepared with this method shown in Table 2.

Table.1. Direct compression formulation

API & Excipients	DC-1	DC- 2	DC- 3	DC- 4	DC- 5	DC- 6	DC- 7	DC- 8	DC- 9	DC-10
Enzyme Premix	400*	400*	400*	400*	400*	400*	400*	400*	400*	400*
Citric Acid (Anhydrous)	400	400	520	400	500	460	490	480	500	460
Tartaric Acid	800	450	-	350	300	915	980	960	1000	920
Sodium Bicarbonate	1200	600	1520	1245	1065	1350	1425	-	1500	-
Sodium Carbonate	45	600	-	-	200	-	-	1480	-	1350
Sodium Citrate	150	200	-	-	-	215	200	-	220	-
Sodium Glycine Carbonate	-	-	163	165	200	-	-	170	-	-
Sodium Lauryl Sulphate	30	20	-	20	15	25	20	20	15	30
Povidone (PVP-K-30)	120	100	120	110	115	110	90	110	100	90
Acesulfame Potassium	20	-	-	20	20	25	20	25	30	30
Sodium Benzoate	20	20	25	15	25	30	25	25	25	25
Purified Talc	5	12.5	12.5	10	5	10	10	10	10	5
Colloidal Silicon Dioxide	10	7.5	7.5	5	10	5	10	5	10	10
Orange Flavor	35	20	35	25	30	30	35	40	40	20
Weight of the tablet (mg)	3235	2830	2803	2765	2885	3575	3705	3725	3850	3340

**Appropriate overages were added to compensate the probable loss on storage*

Table.2. Dry granulation formulations

API & Excipients	DG-1	DG- 2	DG- 3	DG- 4	DG- 5	DG- 6	DG- 7	DG- 8	DG- 9	DG-10
Enzyme Premix	400*	400*	400*	400*	400*	400*	400*	400*	400*	400*
Citric Acid (Anhydrous)	400	300	250	400	450	600	400	500	450	350
Tartaric Acid	800	600	500	500	200	-	600	500	800	700
Sodium Bicarbonate	1200	1350	1200	1100	1000	1100	1300	850	1000	1050
Mannitol (Pearlitol 200 SD)	100	100	120	110	120	130	150	175	200	225
Lactose (Pharmatose DCL-21)	50	65	80	100	110	120	50	50	80	75
Crospovidone (Polyplasdon XL)	50	65	80	100	110	100	70	60	80	75
Microcrystalline Cellulose	100	90	100	80	100	120	130	125	150	150
Povidone (PVP-K-30)	50	65	80	65	85	100	80	85	100	90
Acesulfame Potassium	30	40	35	40	45	50	40	55	50	40
Sodium Benzoate	25	30	35	40	45	50	35	40	45	50
Purified Talc	05	10	10	10	10	10	10	10	10	10
Colloidal Silicon Dioxide	05	10	10	10	10	10	10	10	10	10
Orange Flavor	35	40	30	30	50	40	35	50	45	35
Weight of the tablet (mg)	3250	3165	2930	2985	2735	2830	3310	2910	3420	3260

**Appropriate overages were added to compensate the probable loss on storage*

C) Wet granulation[8]

This method comprises four steps- drug granulation, acidic granulation, alkaline granulation and lubrication.

Drug granulation method - Enzyme blend and orange flavor were weighed and passed through sieve no. #40.Tween-80, hypromellose were weighed and dissolved in isopropyl alcohol made a binding to the enzyme blend and obtained wet mass passed through sieve no. # 8 & kept in tray dried at 45^0C for half an hour then the semidried wet mass passed through sieve no. # 20 and the granules were dried until the L.O.D. was observed below 1%.

In acidic granulation weighed the citric acid, tartaric acid were blended and passed through sieve no. #40. Second step simethicone (30%) were weighed and dissolved in organic solvent i.e. methylene dichloride. Granules of acidic component were made with this solution. The obtained wet mass passed through sieve no. # 8 & kept in tray dried at 55^0C for half an hour then the semidried wet mass passed through sieve no. # 20 and the granules were dried until the L.O.D. was observed below 1%. (On IR moisture balance at 105^0C for 5 min.)

In alkaline granulation method weighed sodium bicarbonate, sodium carbonate passed through sieve no.# 40.The granulation were carried out with a binder solution comprises povidone dissolved in isopropyl alcohol. The obtained wet mass passed through sieve no. # 8 & kept in tray dried at 45^0C for half an hour then the semidried wet mass passed through sieve no. # 20 and the granules were dried until the L.O.D. was observed below 1%. (On IR moisture balance at 105^0C for 5 min.)

Lubrication was done with sodium lauryl sulphate and acesulfame potassium was weighed and passed through sieve no. # 40.These ingredients were mixed with above obtained granules passed already through sieve no. # 40.Finally in this blend weighed and add colloidal silicon dioxide, sodium benzoate (passed through sieve no. # 40), purified talc (passed through sieve no. # 60). Taken the trials with above obtained blend using 24.8 mm circular plain punches on single rotary 12 stations tablet compression machine.

Following were the trial formulations prepared with this method shown in Table 3

Table.3. Wet granulation formulations

API & Excipients	**WG-1**	**WG-2**	**WG-3**	**WG-4**	**WG-5**	**WG-6**	**WG-7**	**WG-8**	**WG-9**	**WG-10**
Enzyme Premix	400*	400*	400*	400*	400*	400*	400*	400*	400*	400*
Citric Acid (Anhydrous)	400	-	500	400	800	450	470	480	500	450
Tartaric Acid	800	-	1000	800	-	900	940	960	1000	900
Fumaric Acid	200	600	-	-	-	-	-	-	-	-
Sodium Bicarbonate	1200	1200	1520	1200	1400	1350	1425	1440	1490	1350
Sodium Carbonate	150	200	-	-	-	200	200	210	200	-
Sodium Glycine Carbonate	-	150	-	140	120	-	-	-	-	160
Simethicone 30%	45	50	40	45	35	45	50	60	60	35
Hypromellose (HPMC-5-CPS)	20	30	35	20	25	15	25	15	15	20
Polysorbate-80	15	20	-	20	15	25	20	20	15	10
Sodium Lauryl Sulphate (SLS)	20	-	-	20	20	25	20	20	15	10
Povidone (PVP-K-30)	85	105	100	110	100	110	100	115	100	90
Acesulfame Potassium	15	25	35	30	15	40	45	50	55	-
Sodium Benzoate	20	20	25	15	25	25	25	25	20	20
Purified Talc	5	10	5	10	5	10	5	5	5	10
Colloidal Silicon Dioxide	10	5	5	5	10	5	5	5	5	5
Orange Flavor	35	20	35	25	30	30	35	45	30	20
Iso propyl alcohol	q.s.	q.s.	q.s.	q.s.	q.s.	q.s.	q.s.	q.s.	q.s.	q.s.
Methylenedichloride	q.s.	q.s.	q.s.	q.s.	q.s.	q.s.	q.s.	q.s.	q.s.	q.s.
Weight of tablet (mg)	3420	2835	3700	3240	3000	3630	3765	3850	3910	3480

**Appropriate overages were added to compensate the probable loss on storage q.s.-quantity sufficient.*

Drug - Excipients compatibility and identification of enzymes peak study

The drug-excipients compatibility study was carried out by using Infrared spectroscopy.IR study was conducted using KBr powder mixing method (Shimadzu , Japan) and the spectrums were recorded in the wavelength region of 4000 - 400 cm^{-1}.The individual identification of enzymes can be identified by using Matrix Assisted Laser Desorption Ionization – Time of Flight (MALDI-TOF) instrument.

Evaluation of granules[9]

1) Angle of repose

Angle of repose was determined by Neumann's method and calculated using the formula, for powder as well as granules (lubricated or unlubricated)

$$\text{Tan } \theta = (h / r) \quad \text{or} \quad \theta = \text{Tan}^{-1}(h / r)$$

Where, h = height of pile, r = radius of the pile base

2) Bulk density (g/cm^3)

The bulk density was calculated using equation,

$$Apparent\ Bulk\ \ Density = \frac{Weight\ of\ the\ powder}{Bulk\ Volume}$$

3) Tapped density (g/cm^3)

The tapped density was calculated using equation,

$$Tapped\ density = \frac{Weight\ \ of\ \ the\ \ powder}{Tapped\ \ volume}$$

4) Hausner ratio

Calculated by,

$$HausnerRatio = \frac{TappedDensity}{BulkDensity}$$

5) Compressibility index

Compressibility index of granules determined by,

$$\text{Carrs Index} = \frac{\text{Tapped density - Bulk density}}{\text{Tapped density}} \times 100$$

Evaluation of IPQC parameters of tablets

1) Hardness[10]

Hardness was determined by using Monsanto tablet hardness tester (Campbell Electronics, Mumbai, India). Reading on the scale was noted down in Kg/cm^2.

2) Thickness and Diameter[10]

The thickness and diameter of tablets were measured with vernier caliper. Average thickness and diameter were calculated.

3) Weight variation[10]

20 tablets were weighed individually. Average weight was calculated from the total weight of all tablets. The individual weights were compared with the average weight. The percentage difference in the weight variation should be within the permissible limits (± 5.0%)

$$\% \text{ Weight variation} = \frac{\text{Individual wt. of tablet-Average wt.of tablet}}{\text{Average wt.of tablet}} \times 100$$

4) Effervescent time

Should not more than 5 min. as per pharmacopoeias (I.P. /B.P.)

5) Friability[10]

The friability of tablets using 10 tablets as a sample was measured using a Roche friabilator. Tablets were rotated at 25 rpm for 4 minutes or up to 100 revolutions. The tablets were taken out, deducted and reweighted. The percentage friability was calculated from the loss in weight as given in equation below. The weight loss should not more than 1%.

$$\% \text{ Friability} = \frac{\text{Initial weight} - \text{Final weight}}{\text{Initial weight}} \times 100$$

6) Carbon dioxide content[11]

I. Refer to the electronic balance user's guide to set up the balance. Be sure to level and zero the unit.

II. Put empty glass beaker on balance and note down the reading (in gm).

III. Add water (app. 3 – 4 ounces) in the weighed beaker and note down the reading (in gm)

IV. Take and note down the accurate weight of tablet / powder (M_3).

V. Note down the weight of glass beaker filled with water and tablet / powder (in gm) before the effervescent reaction (M_2).

VI. Add the weighed tablet / powder in glass beaker filled with water.

VII. After complete effervescent reaction (5 min.) note down the weight of glass beaker (M_1).

VIII. Carbon dioxide content can be calculated by following formula,

$$\text{Carbon dioxide, percent by mass} = \frac{M_2 - M_1}{M_3} \times 100$$

Where,

M_1 – Mass in grams after the test

M_2 – Mass in grams before the test,

M_3 – Mass in grams of the sample taken for test

Stability studies of optimized formulations

Stability testing of drug products begins as a part of drug discovery and ends with the demise of the compound or commercial product. Optimized formulation were packed and stored in stability chambers as per ICH guidelines maintained at 25°C±2°C/60%±5%RH, 30°C± 2°C/65% ±5% RH, 40°C±2°C/75%±5% RH for two months. The tablets were withdrawn periodically and evaluated for thickness, hardness, effervescent time, pH of solution etc. At the end of studies, samples were analyzed for the % drug content.

Drug-excipients compatibility study

The incompatibility between the drug and excipients were studied by IR spectroscopy. The spectral data of pure drug and drug-excipient mixtures indicate that there was no chemical incompatibility between drug excipients used in formulation. The following identical peak was observed within study 2924 - 2918 cm^{-1} (H-C-H Asymmetric and Symmetric stretching), 2854 – 2850 cm^{-1} (Alkyl C-H Stretching), 1658 -1635 cm^{-1} (C-C=C Symmetric stretching), 1546 – 1527 cm^{-1} (N-H bending), 744 – 705 cm^{-1}(Aromatic C- H bending).

Peak identification

The individual peak of enzymes were identified with the help of MALDI-TOF instrument.

1) Papain- (Mole.wt. - 23,406 Da)
2) Bromelain- (Mole.wt.- 24,397 Da)
3) Rutoside Trihydrate- (Mole.wt.- 664.56 Da)
4) Vitamin C- (Mole.wt.-176.12 Da)

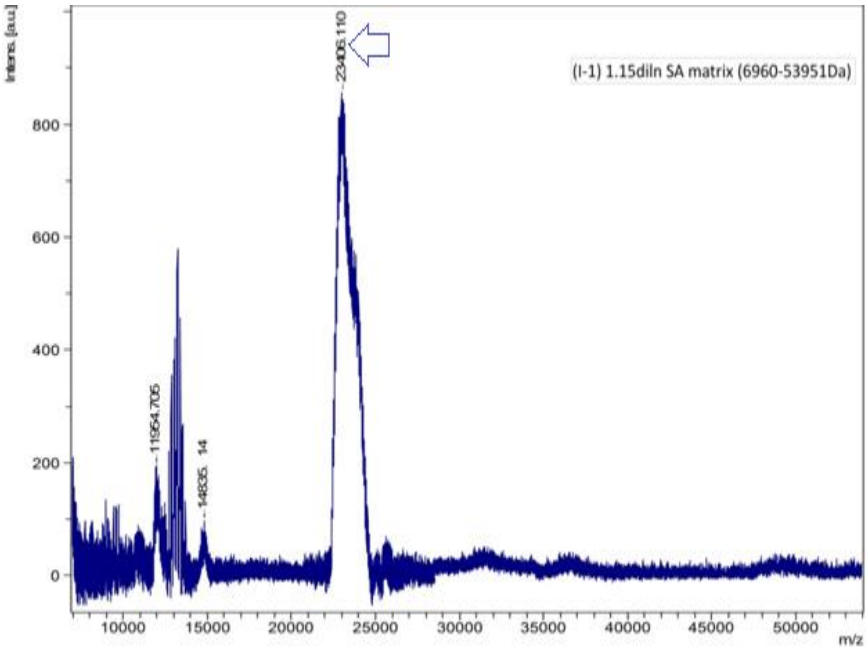


Figure 1. Papain peak

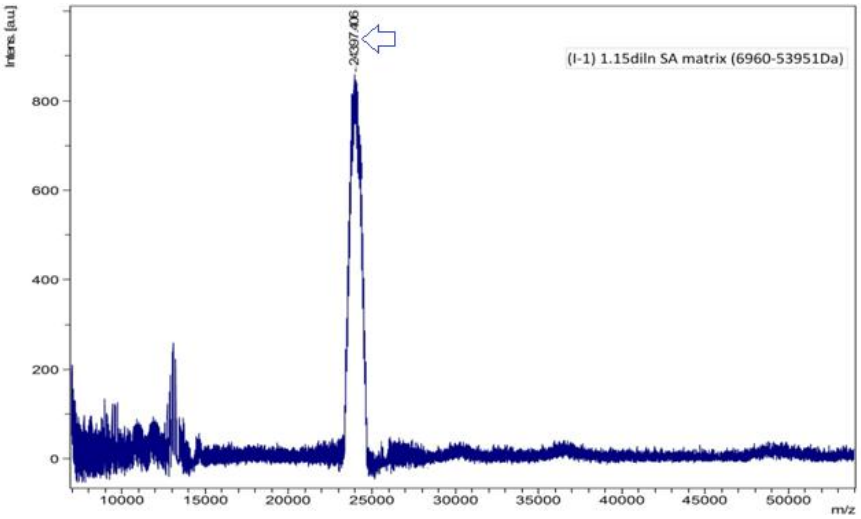


Figure 2. Bromelain peak

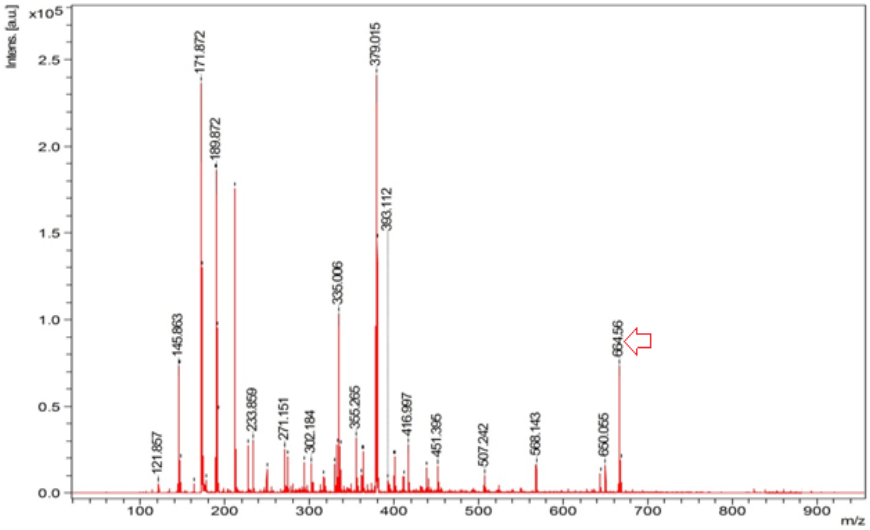


Figure 3. Rutoside trihydrate peak

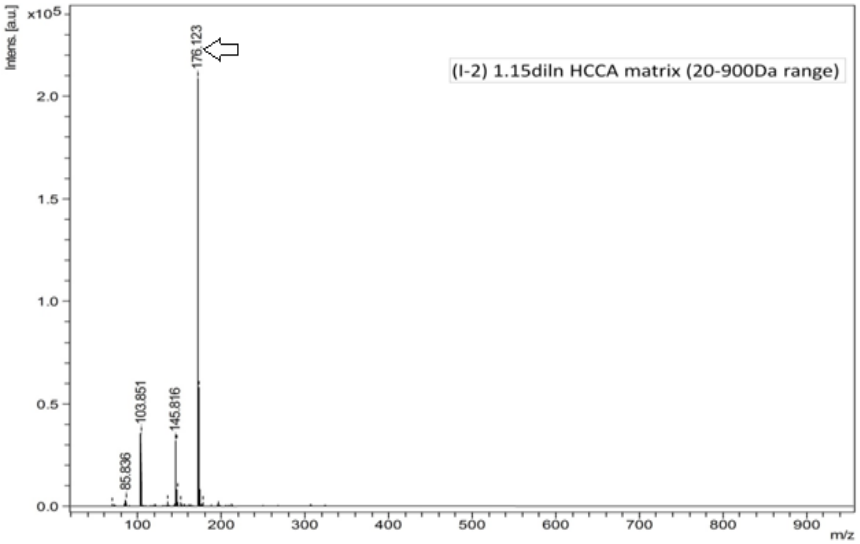


Figure 4. Vitamin C peak

Evaluation of tablet parameters

The formulation with direct compression (DC-6), dry granulation (DG-7) and wet granulation (WG-8) trials were found satisfactory physical parameters as compared to other trials with respective method of tablet preparations. The appearance of tablets was found good in DG-8 as compared to DC-6 and DG-7.

The evaluation of physical parameters of DC-6 (Direct compression), DG-7(Dry granulation) and WG-8 (wet granulation) shown in Table 4, were concluded that the WG-8 trial formulation observed comprehensive better physical characteristics than others.

Table 4. Comparison of best trial formulations physical parameters

Method	Thickness (mm)	Hardness (Kg/cm^2)	Friability (%)	Efferve. time (sec)	pH of solution	L.O.D. (%)	Diameter (mm)	CO_2 gm/tab	Wt. Variation (%)
DC-6 (Direct compression)	6.15 (±0.15)	4 – 5	1.2 %	70 ± 5	6.80 (±0.02)	1.30 %	24.80 (+0.01)	0.414 (11.57%)	3.80 (±0.2)
DG-7(Dry granulation)	5.70 (±0.15)	4 – 5	0.9 %	75 ± 5	6.80 (±0.02)	1.25%	24.80 (+0.01)	0.35 (10.57%)	2.90 (±0.2)
WG-8 (Wet granulation)	5.90 (±0.1)	4 –5	0.8 %	80 ± 5	6.40 (±0.02)	0.85%	24.80 (+0.01)	0.50 (12.98%)	2.40 (±0.2)

(Mean ± SD) n=5

The tablets obtained with wet granulation method were found to be satisfactory as compared to other methods. For stability study the good three trial formulations within each method i.e.DC-6(direct compression), DG-7(dry granulation), WG-8 (wet granulation) were considered. The physical properties of WG-8 (wet granulation) were found good rather than DC-6 and DG-7 trial formulations. The initial nutritional and microbiological analysis of trial formulation WG-8 (wet granulation) was shown in Table 5. The FPS of trial formulation WG-8 (wet granulation) was shown in Table 6.

Table 5. Microbiological, nutritional report of WG-8 wet granulation formulation

Sr. No	Parameter	Result	Method
1.	Salmonella Spp	Absent/25g	IS 5887-Part III
2.	*Escherichia coli*	Absent/g	IS 5887 Part I
3.	Carbohydrates	39.36%	By calculation
4.	Protein	44.28%	IS 7219-1973
5.	Fat	3.15%	AOAC: 920.85
6.	Dietary fibre	24.68%	IS 11062-1984
7.	Colour	Yellow	Sensory Evaluation
8.	Odour	Off odour	
9.	Particle Size	Amorphous	

Table 6. Finished product specifications WG-8 (wet granulation)

Sr.No.	Test	Acceptance criteria	Observed
1	Description	Beige colored circular shaped tablet having plain on both sides.	Beige colored circular shaped tablet having plain on both sides.
2	Thickness	5.80 ± 0.2 mm (5.60 mm to 6.00 mm)	5.70 mm to 5.90 mm
3	Uniformity of weight	3850 mg ± 5.0 % (3830.75 mg to 3869.25 mg)	3835 mg to 3860 mg
4	Effervescent time (sec.)	Not more than 5 minutes	75 to 85
5	Content of actives (Enzyme activity/tablet)	Not less than 2000 FIP units as per FCC 2500 Units / tablet ± 10.0 % of input (2250 Units to 2750 Units / tablet)	2468 Units / tablet (123.4 % with overages)
6	% Content of active (%Enzyme activity/tablet)	Not less than 90.0 % to Not more than 110.0 % of input	98.72 % of input

The three trial formulations DC-6, DG-7, WG-8 were kept for stability as per ICH guidelines in 0.04 mm aluminium-aluminium single tablet strip. The stability study was done up to 2 months. After 2 months stability evaluate the physical and analytical parameters of all stability batches. The comparative evaluations of stability study with all batches were shown in Table 7.

Table 7. Comparative evaluation of stability study after two months

Sr. No.	Trial Formulations	Pack	Physical and Analytical parameters	Initial results	Climatic conditions		
					40^0C/ 75 % RH	30^0C/ 65 % RH	25^0C/ 60 % RH
1	DC-6 (Direct Compression)	Alu-Alu strip (0.04 mm)	Assay	121.9%	100.02%	105.3%	110.2%
			Thickness (mm)	6.10(±0.2)	6.50(±0.2)	6.25(±0.2)	6.11(±0.2)
			Hardness (Kg/cm^2)	4 – 5	3– 4	3.5 – 4.5	4 – 5
			Effervescent time (sec.)	70 ± 5	200 ± 5	190 ± 5	140 ± 5
			pH of solution	6.90 (± 0.03)	6.60 (±0.03)	6.50 (±0.03)	6.70 (±0.03)
2	DG-7 (Dry Granulation)	Alu-Alu strip (0.04 mm)	Assay	122.8%	101.2%	107.8%	115.02%
			Thickness (mm)	5.65(±0.2)	5.85(±0.1)	5.75(±0.1)	5.7(±0.1)
			Hardness (Kg/cm^2)	4 – 5	3 – 4	3.5 – 4.5	4 – 5
			Effervescent time (sec.)	75 ± 5	190 ± 5	155 ± 5	135 ± 5
			pH of solution	6.80 ± 0.03	7.10± 0.03	6.95±0.04	6.85± 0.03
3	WG-8 (Wet Granulation)	Alu-Alu strip (0.04 mm)	Assay	123.4%	113.9 %	117.8 %	119.4 %
			Thickness (mm)	5.80 (±0.2)	6.10(±0.1)	5.95(±0.2)	5.90 (±0.2)
			Hardness (Kg/cm^2)	4 – 5	3.5 – 4.5	4 – 5	4 – 5
			Effervescent time(sec.)	80 ± 5	170 ± 5	150 ± 5	110 ± 5
			pH of solution	6.40 ± 0.03	6.85 ± 0.03	6.70 ± 0.02	6.50 ± 0.03

Preclinical Study

Measurement of Anti-inflammatory activity

To study the acute and sub acute phases of inflammation in rodents (rats and mice), carrageenan is a widely used irritant or inflammogen or a phlogestic agent. Chemically, it is a sulphated polysaccharide from sea weeds (Rhodophyceae). The experimental tissue injury caused by this irritant initiates the cascade of events leading to formation of exudates. The inflammation induced it is biphasic in nature. The first phase is attributed to the release of histamine, 5-HT and kinins, while the second phase is related to the release of prostaglandins. Therefore, to study the effect of test preparation on acute inflammation a well recognized carrageenan induced rat paw edema technique.[12-14] This technique has been successfully used by various researchers.[15-21]

The hind paw edema in rats (24 nos.) was induced by injecting 0.1ml (w/v) of 1% carrageenan (prepared in normal saline) in sub planter region. These rats were equally divided in four groups. The rats in different groups received treatments one hour prior to carrageenan administration. The rats in group I received no treatment which served as a control group and rats in group II received indomethacin as a standard drug at the dose rate of 10 mg/kg body weight. Rats in group III, group IV received test preparation at the dose rate of 20 mg/kg body weight and 40 mg/kg body weight respectively. The paw volume, up to the ankle joint, was measured before and at the end of each hour up to three hours after carrageenan injection, by using the plethysmometrically (Model no.PLM-01, Make- Orchid Scientific, Nashik). An increase in paw volume has been expressed as percent increase over control values recorded in terms of unit (ml). The percent inhibition of acute inflammation was calculated by using the formula.

$$\text{Percent Inhibition (\%)} = \frac{Vc - Vt}{Vc} \times 100$$

Where, Vc and Vt represent mean paw volumes in control and treated group, respectively.

Toxicity Study

The experiment was carried out as per Organization for Economic Co-operation and Development (OECD) guideline no. 407 and 425[22-23] with slight modifications. The rats were kept under observation for a period of 28 days during the study. During this time the animals were weighed daily. The blood was collected from any six animals for the hematological and biochemical analysis before the beginning of the experiment on 15th day and on 29th day of the experiment through retro-orbital plexus 24 hr. after the last dose, centrifuged at 3000 rpm at 4°C for 10 minutes to separate serum.[24] The activities of serum glutamate pyruvate transaminase (SGPT)[25], Albumin[26] were assayed. The alkaline phosphatase activity in the serum was also measured. [27] The blood was also used to estimate other hematological parameters of normal, control and treated animal groups.[28-29] The liver was excised, rinsed in ice-cold normal saline followed by cold 0.15 M Tris-HCl (pH 7.4), blotted and weighed. Proteins were estimated by using bovine serum albumin as the standard.[30]

In the toxicity study the following parameters were checked.

General observations**:** During the entire period of the study check whether animals are died or not.

Body weights**:** The weights of the animals recorded daily throughout the treatment period. Biochemical parameters**:** The biochemical parameters were studied on 15th day and 29th day of study and the values were compared to corresponding values from the same animals at 0 day**.** The biochemical parameters were studied to find out the kidney and liver functions of the animals under study.

Hematological parameters: The hematological parameters were studied on day 0 day, 15th day and 29th day of study for any changes. The Hemoglobin, WBC, Platelets, RBCs, etc. parameters were studied.

Histopathological study: On 29th day of study the representative rats were sacrificed and all the vital organs were observed for any macroscopic changes.

Anti-inflammatory effect of test drug on carrageenan induced paw edema

Test drug had shown a significant anti-inflammatory activity against carrageenan induced paw edema in a dose dependent manner. In this carrageenan induced paw edema model Treatment 1(20 mg/kg body weight) and Treatment 2 (40 mg/kg body weight) showed maximum percent inhibition at 3rd hr as follows: 28.23 % and 47.56 % respectively. During present study the standard drug indomethacin showed maximum percent inhibition at 4th hr 73.83 % at a dose of 10 mg/kg body weight. Percent anti-inflammatory activity in different groups of rats based on an increase in paw volume at different time interval is shown in (Tables 8, 9 and Figure 5)

Table 8. Average increase in paw volume (ml) at different hours in various groups and percent Anti-inflammatory activity as compared to control (n=6)

Group	Time (hr)				
	1	2	3	4	5
Control	0.533	1.069	1.449	1.357	1.172
Standard	0.405	0.518	0.413	0.355	0.313
Treatment 1 (20 mg/kg body weight)	0.548	0.813	1.040	0.998	0.955
Treatment 2 (40 mg/kg body weight)	0.490	0.720	0.760	0.813	0.890
% activity - Standard	23.82	51.52	71.48	73.83	73.26
% activity –Treatment 1 (20mg/kg body weight)	-3.07	24.01	28.23	26.47	18.49
% activity –Treatment 2 (40mg/kg body weight)	7.84	32.66	47.56	40.11	24.04

Table 9. Showing increase in paw volume (Mean ± SE) in different group of animals at the end of different hours (n=6)

Group	1 hr		2 hr		3 hr		4 hr		5 hr	
	Mean	S.E.(±)	Mean	S.E.(±)	Mean	S.E.(±)	Mean	S.E.(±)	Mean	S.E.(±)
I (Control)	0.533*	0.022	1.070*	0.084	1.449*	0.097	1.357*	0.108	1.172*	0.061
II (Standard)	0.405*	0.036	0.518*	0.065	0.413*	0.053	0.355*	0.031	0.313*	0.039
III (Treatment 1)	0.542*	0.019	0.813*	0.087	1.042*	0.095	0.998*	0.092	0.955*	0.094
IV (Treatment 2)	0.487*	0.046	0.722*	0.034	0.762*	0.020	0.813*	0.014	0.892*	0.014
C.D.	0.096		0.211		0.219		0.217		0.177	
S.E.M.	0.032		0.071		0.074		0.073		0.060	
C.V.	16.112		22.232		19.659		20.336		17.523	

C.D. - Critical Difference, S.E.M. - Standard Error Mean, C.V.- Coefficient of Variation

**p<0.01 significantly different as compared to control (n=6 in each group) .The test drug were compared with the Indomethacin group. - Data are represented as Mean ± S.E.M. Statistical analyze was done by one-way ANOVA*

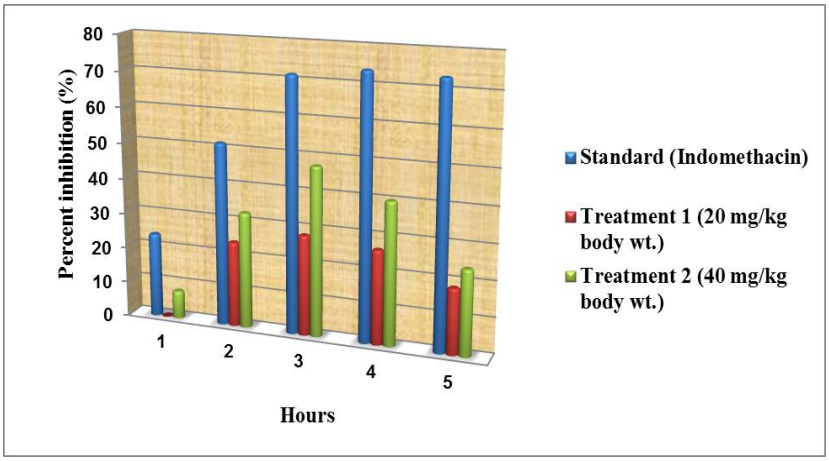


Figure 5. Percent inhibition of inflammation by Standard (Indomethacin), Treatment 1, Treatment 2 in carrageenan induced inflammation in rats

Table 10. Average body weights (gm) of the rats during the study period of 28 day

Ani. No.	Days of Treatment																										
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28
1	181	209	208	208	212	214	210	215	222	231	235	232	235	235	232	223	216	213	216	217	220	226	232	240	232	232	232	235
2	200	228	232	230	228	226	217	229	230	240	238	243	250	252	245	246	240	241	243	246	246	248	249	246	243	248	242	243
3	190	216	220	220	224	236	228	246	245	251	252	246	245	250	249	232	242	238	241	241	243	244	250	254	242	243	246	233
4	191	219	220	219	219	220	214	222	226	236	237	238	243	244	239	235	228	227	230	232	233	237	241	243	238	240	237	239
5	194	221	224	223	224	227	220	232	234	242	242	242	246	249	244	238	237	235	238	240	241	243	247	248	241	244	242	238
6	214	236	232	230	235	240	230	231	230	234	229	225	226	230	219	213	228	228	220	221	226	223	227	229	218	223	220	220
7	204	219	220	225	226	229	226	228	227	226	224	225	227	218	218	217	224	220	215	220	218	224	230	226	219	219	215	221
8	217	227	221	230	228	227	224	221	214	224	228	228	221	225	222	216	230	231	229	232	229	232	235	230	227	224	229	230
9	209	228	226	228	232	235	228	230	229	230	227	225	227	224	219	215	226	224	218	221	222	224	229	228	219	221	218	221
10	212	227	224	228	230	232	227	227	224	228	227	226	225	224	220	215	227	226	221	224	224	226	231	228	221	222	221	224
Avg.	182.88	202.83	202.76	204.08	205.73	208.35	202.70	208.05	208.08	213.79	213.56	212.88	214.23	214.94	210.98	205.94	210.45	209.24	208.11	210.26	211.18	213.53	217.47	217.77	211.29	212.88	211.68	211.95
S.D.	12.02	7.71	6.90	6.98	6.61	7.67	6.87	8.24	8.06	8.29	8.69	8.43	10.62	12.55	12.59	11.66	7.83	8.43	10.62	10.22	10.02	9.58	8.86	10.15	10.39	11.11	11.29	8.59
S.E.	3.804	2.440	2.185	2.208	2.093	2.427	2.174	2.607	2.552	2.624	2.750	2.669	3.360	3.971	3.986	3.689	2.476	2.667	3.362	3.235	3.172	3.033	2.802	3.213	3.289	3.515	3.571	2.717

S.D.- Standard Deviation S.E.-Standard Error

Table 11. Various Biochemical parameters at 0 day, 15th day and 29th day of study

Treat - ment	Blood Urea Nitrogen		Creatinine		SGPT		Alkaline Phosphatase		Total Protein		Albumin		Globulin		A:G Ratio	
	Mean	S.E.(±)	Mean	S.E.(±)	Mean	S.E.(±)	Mean	S.E.(±)	Mean	S.E.(±)	Mean	S.E.(±)	Mean	S.E.(±)	Mean	S.E.(±)
0 day	45.508*	2.114	1.075*	0.116	93.520**	13.249	129.963	14.224	6.262**	1.003	4.822*	0.650	1.542*	0.174	2.788*	0.503
15th day	52.275*	3.753	1.285*	0.079	76.500**	5.207	147.325	5.793	8.482**	0.139	6.285*	0.159	2.197*	0.094	2.928*	0.189
29th day	76.100*	7.363	2.122*	0.227	129.675**	18.428	141.525	9.575	6.097**	0.264	3.362*	0.174	2.735*	0.185	1.357*	0.172
C.D.	14.981		0.470		40.895		N/A		1.839		1.214		0.475		0.991	
S.E.M.	4.925		0.154		13.444		10.449		0.604		0.399		0.156		0.326	
C.V.	20.814		25.322		32.965		18.335		21.313		20.270		17.722		33.83	

*C.D. - Critical Difference, S.E.M. - Standard Error Mean, C.V.- Coefficient of Variation **p<0.01 and **p<0.05 significantly different at 15th day and 29th day compared to control 0 day (n=6 in each group) - Data are represented as Mean ± S.E.M. -Statistical analyze was done by one-way ANOVA followed by completely randomized design.*

Toxicity study

During the entire toxicity study (28 days), none of these animals was died and all the animals were found clinically healthy with normal feeding and watering. The weights of the animals were recorded daily are depicted in the Table 10. In the toxicity studies we have compared 15th day and 29th day observed biochemical and hematological parameters with 0 day (Tables 11, 12 and Figure 6, 7, 8, 9).

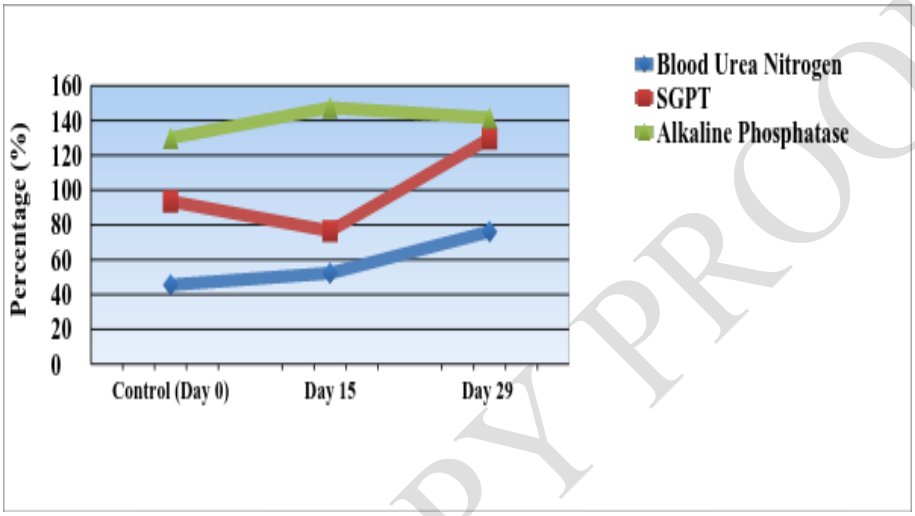


Figure 6.Effect of oral administration of test drug on BUN, SGPT, Alkaline phosphatase in rats

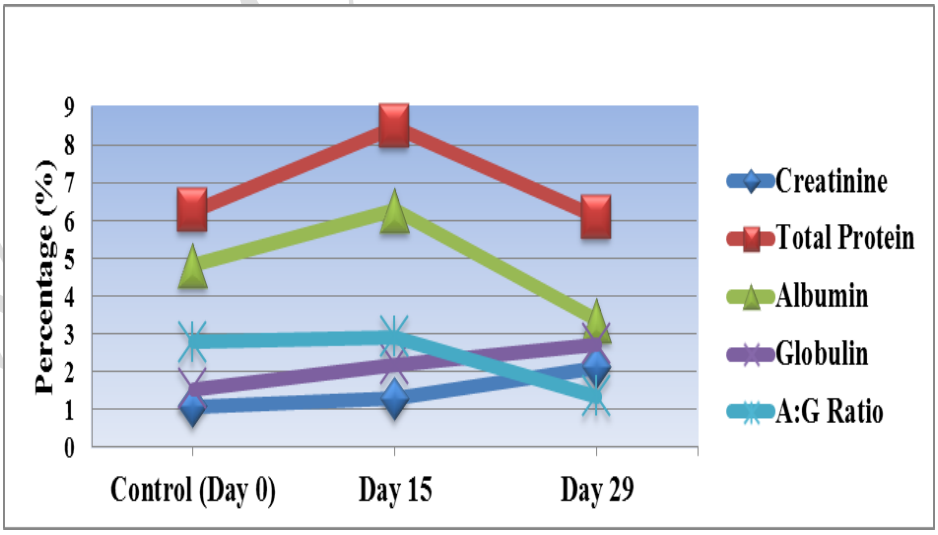


Figure 7. Effect of oral administration of test drug on Creatinine, Total Protein, Albumin, Globulin, A: G Ratio in rats

Table 12. Various Hematological parameters at 0 day, 15th day and 29th day of study

Treatment	Hemoglobin (Hb)		WBC		Platelets		RBC		HTC (Hematocrit)		MCV (Mean Corpu-scular Volume)		MCH (Mean Corpuscular Hemoglobin)		MCHC (Mean Corpuscular Hemoglobin Concentration)	
	Mean	S.E.(±)	Mean	S.E.(±)	Mean	S.E.(±)	Mean	S.E.(±)	Mean	S.E.(±)	Mean	S.E.(±)	Mean	S.E.(±)	Mean	S.E.(±)
0 day	13.000	0.056	11.135 **	0.476	7.168	0.333	7.677 *	0.078	41.583	0.498	54.155 **	0.265	17.017 *	0.171	31.473 *	0.288
15th day	13.275	0.165	11.625 **	0.880	8.012	0.383	7.690 *	0.132	42.117	0.580	54.800 **	0.762	17.217 *	0.117	31.483 *	0.271
29th day	14.733	0.986	14.383 **	1.158	8.205	0.350	5.600 *	0.486	43.575	2.989	46.642 **	3.954	24.608 *	1.256	33.100 *	0.341
C.D.	N/A		2.687		N/A		0.895		N/A		7.087		2.235		0.918	
S.E.M.	0.578		0.883		0.356		0.294		1.781		2.330		0.735		0.302	
C.V.	10.361		17.478		11.187		10.309		10.284		11.003		9.178		2.308	

C.D. - Critical Difference, S.E.M. - Standard Error Mean, C.V.- Coefficient of Variation N/A-Not Applicable ***p<0.01 and* ****p<0.05 statistical significance at 15th day and 29th day compared to control 0 day (n=6 in each group) - Data are represented as Mean ± S.E.M. - Statistical analyze was done by one-way ANOVA followed by completely randomized design.*

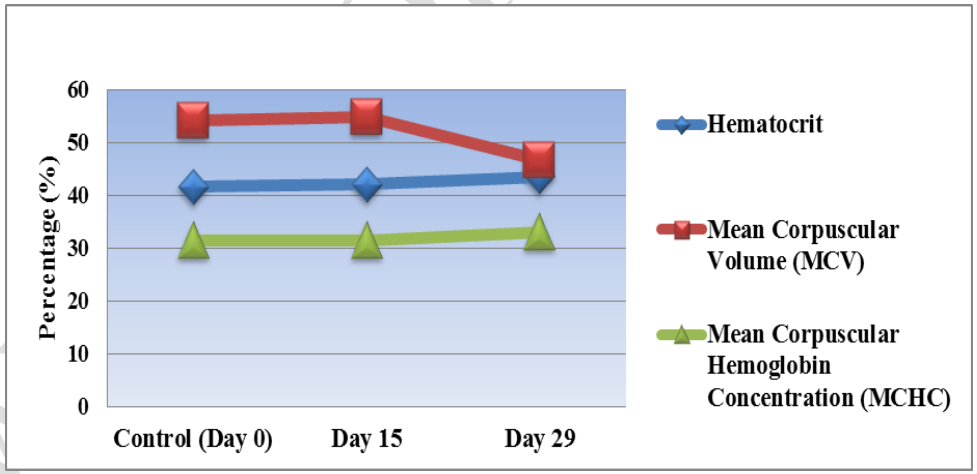


Figure 8. Effect of oral administration of test drug on Hematocrit, MCV, MCHC in rats

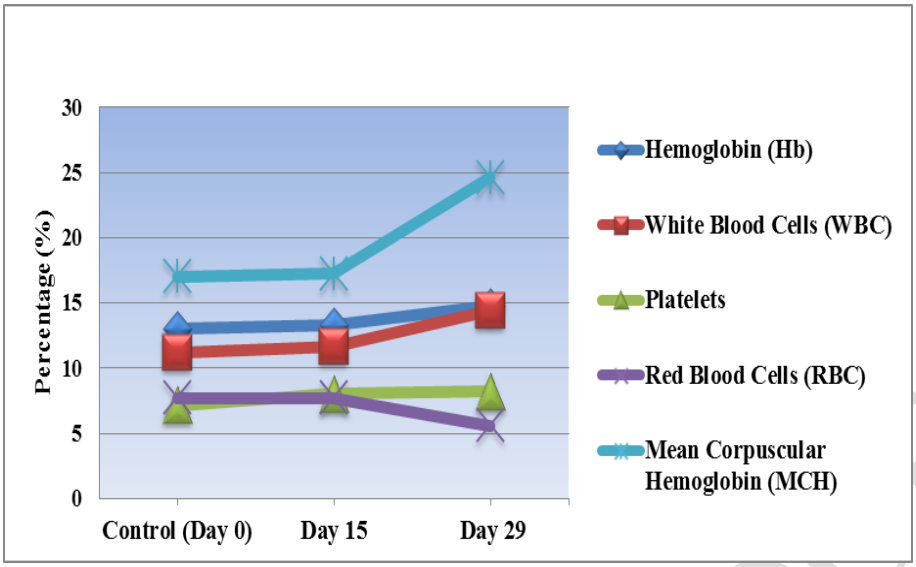
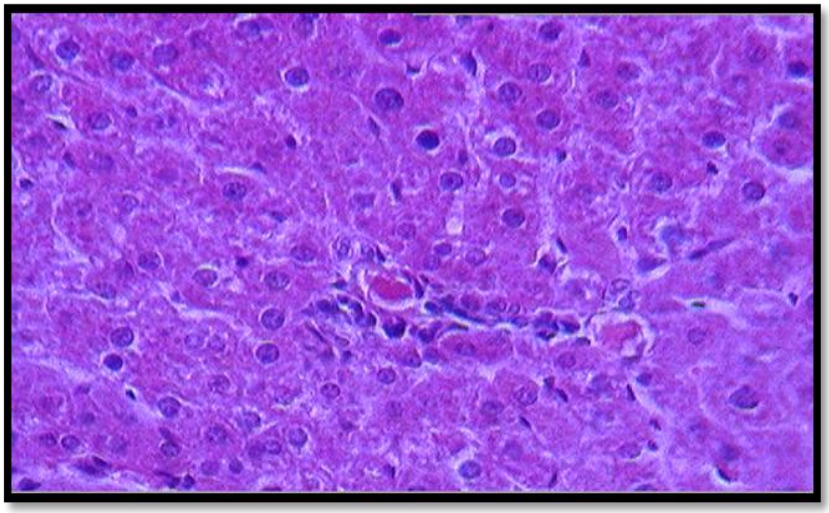
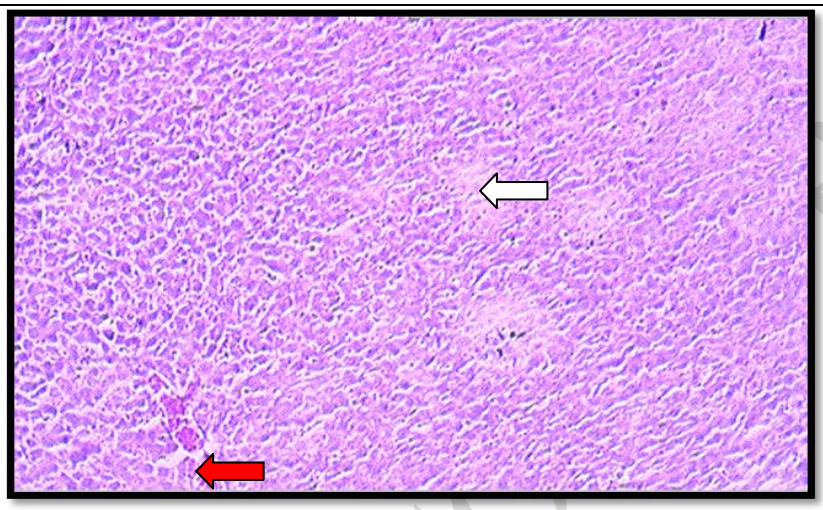
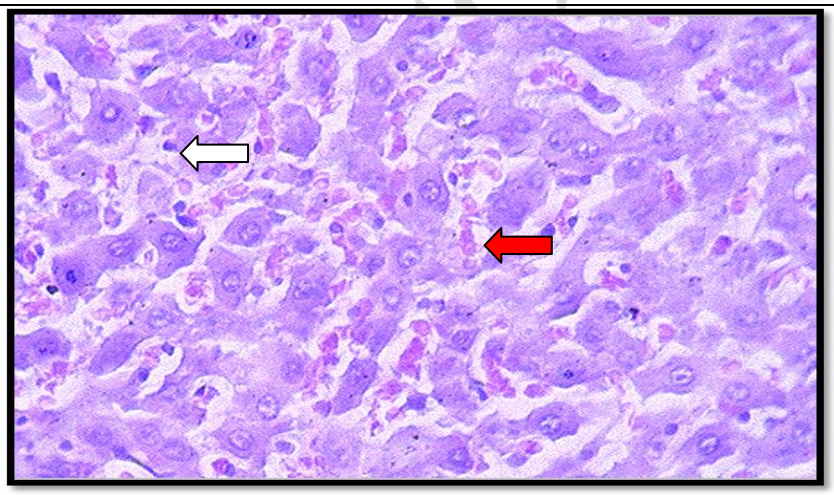


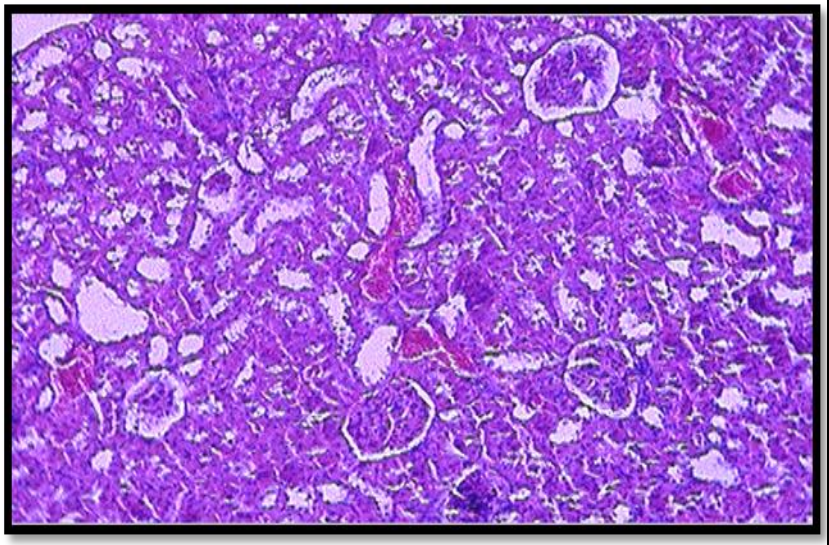
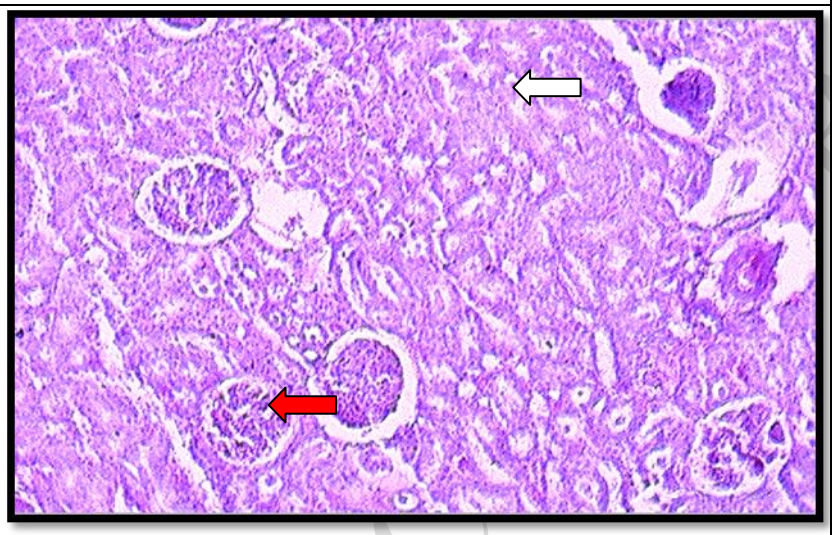
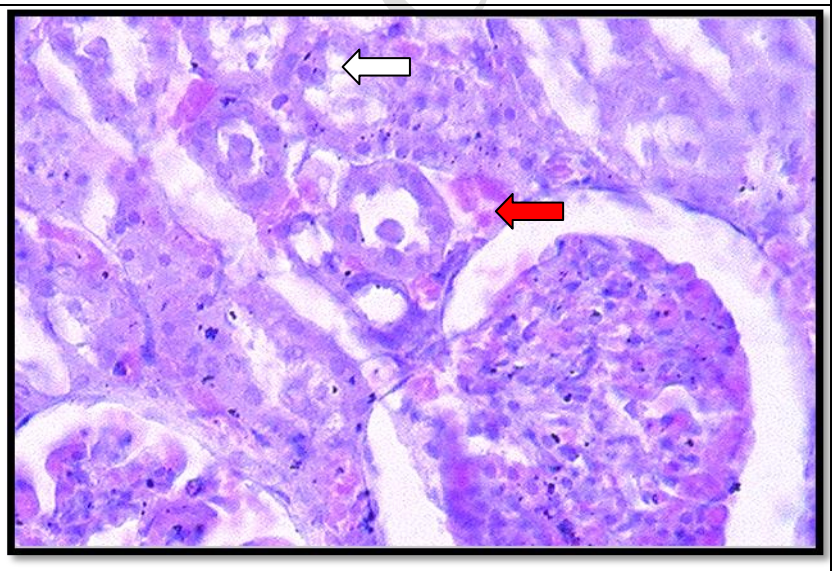
Figure 9.Effect of oral administration of test drug on Hb, WBC, Platelets, RBC, MCH in rats

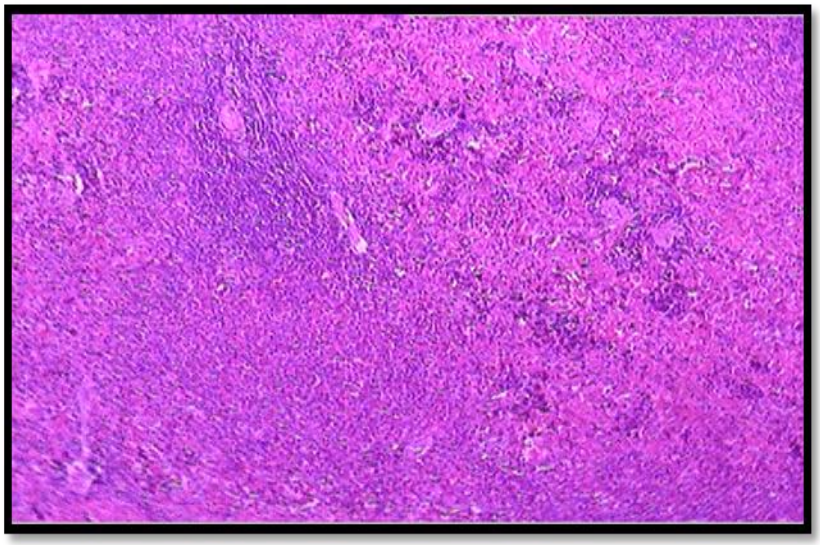
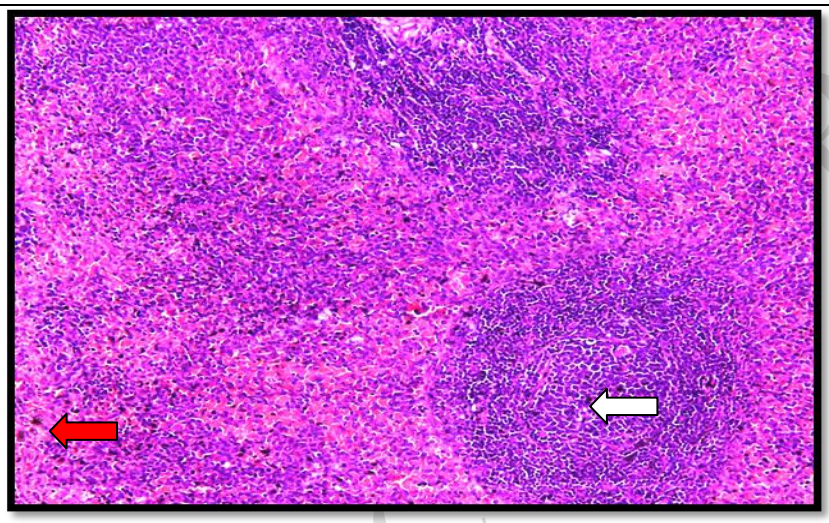
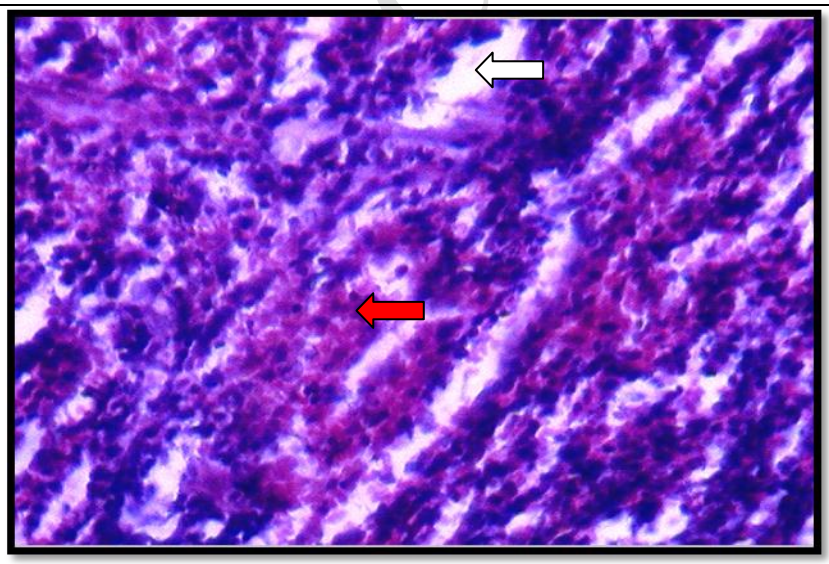
Microscopical observations

On 29th day of study the representative rats were sacrified and all the vital organs were observed for any macroscopic changes. All the organs were found to be normal as per the gross observations. The organs such as liver, kidney, spleen and lungs were then collected and examine for the histopathological study compared to the normal structures. The results are presented in the slides 1, 2, 3, 4, 5, 6, 7, 8 and 9. The both liver and kidney sections showed the mild degeneration and congestion of pathological grade '+' (H & E stain 400 X). The spleen sections showed mild congestion and depopulation of Pathological grade'+' (H &E stain 400 X).Whereas, lung did not show any changes in the histoarchitecture. Following slides shows the changes of treated animal organs with healthy animal organs.

In the present study histopathological changes that observed in the liver were of mild degree and may be found as the incidental changes; however lesions in spleen were indicative of mild depopulations in some of the animals, which indicate mild immune suppression. The lesions in kidneys and lung were of not related to drug administration.

	**Slide 1. Normal structure of Liver**
	**Slide 2. Liver photograph showing mild degeneration (white arrow) and congestion (red arrow) H&E stain 100X Pathological grade : +**
	**Slide 3. Liver photograph showing mild degeneration (white arrow) and congestion (red arrow) H&E stain 400X Pathological grade : +**

	**Slide 4. Normal structure of Kidney**
	**Slide 5. Kidney photograph showing mild degeneration (white arrow) and congestion (red arrow) H&E stain 100X Pathological grade : +**
	**Slide 6. Kidney photograph showing mild degeneration (white arrow) and congestion (red arrow) H&E stain 100X Pathological grade : +**

	**Slide 7. Normal structure of Spleen**
	**Slide 8. Spleen photograph showing mild degeneration (white arrow) and congestion (red arrow) H&E stain 100X Pathological grade : +**
	**Slide 9. Spleen photograph showing mild degeneration (white arrow) and congestion (red arrow) H&E stain 400X Pathological grade : +**

DISCUSSION

Pharmacological Study: Anti-inflammatory activity

The result of this study shows that the test drug (combination of Bromelain, Papain, Bacterial Protease, with Rutin and Vitamin C) showed significant anti-inflammatory activity in Carrageenan induced paw edema method.

Toxicity study

During the entire toxicity study (28 days), none of these animals was died and all the animals were found clinically healthy with normal feeding and watering. The biochemical parameters as well as hematological parameters were studied to find out the effect of test drug on the kidney, spleen, liver and lung functions of the animals under study. All parameters were studied on 15th day and 29th day of study and the values was compared to corresponding values from the same animals at control 0 day.

Biochemical parameters

From the results it was observed that blood urea nitrogen which showed 45.508 ± 2.114 was non-significantly increased to 52.275 ± 3.753 on 15th day of study and further increased significantly to 76.100 ±7.363 by the end of study. Similarly, creatinine was increased non-significantly to 1.285 ± 0.079 and 2.122 ± 0.227 on 15th day and 29th day respectively as compared to its initial value of 1.075 ± 0.116.

The Serum Glutamate Pyruvate Transaminase (SGPT) levels were also increased non-significantly and reached to 129.675 ± 18.428 on 29th day of study compared to its initial values of 93.520 ± 13.249. Alkaline Phosphatase also increased non-significantly by the end of study to 141.525 ± 9.575 over its initial level of 129.963 ± 14.224. Similarly, Total Protein 6.262 ± 1.003 increased significantly to 8.482 ± 0.139 on 15th day of study and thereafter declined to significantly from this level to 6.097 ± 0.264 on 29th day of study showing non-significant difference to the initial Total Protein levels. Albumin levels increased significantly to 6.285 ± 0.159 on 15th day of study and again declined significantly to 3.362 ± 0.174 on 29th day of study and these levels were significantly less even to that of the initial albumin level of 4.822 ± 0.650. However, the globulin levels which were 1.542 ± 0.174 increased significantly to 2.197 ± 0.094 and 2.735 ± 0.185 respectively on 15th day and 29th day of study.

Hematological parameters

From the results It was observed that, Hemoglobin, Platelets and Hematocrit increased non-significantly by the end of study to 14.733 ± 0.986, 8.205 ± 0.350, 43.575 ± 2.989 over its initial level of 13.000 ± 0.056, 7.168 ± 0.333 and 41.583 ± 0.498 respectively. WBC, MCH,

MCHC which showed 11.135 ± 0.476, 17.017 ± 0.171, 31.473 ± 0.288 was non-significantly increased to 11.625 ± 0.880, 17.217 ± 0.117, 31.483 ± 0.271 on 15th day of study and further increased significantly to 14.383 ± 1.158, 24.608 ± 1.256, 33.100 ± 0.34 respectively by the end of study.

Similarly, RBC and MCV showed 7.677 ± 0.078, 54.155 ± 0.265 was non-significantly increased to 7.690 ± 0.132, 54.800 ± 0.762 on 15th day of study and thereafter declined significantly to 5.600 ± 0.486, 46.642 ± 3.954 respectively at the end of study.

CONCLUSION

Effervescent tablet containing Enzymes with Vitamin C can be prepared successfully by using direct compression, dry granulation (slug) and wet granulation method. The effervescent tablets with wet granulation method were found to be most feasible with good stability among all the formulations with all different method, WG-8 after two months stability shows assay of tablet 113.9%, 117.8% and 119.4 % compare to DC-6 (100.02%, 105.3%, 110.2%) and DG-7 (101.2%,107.8%,115.02%) at 40^{0}C/75 % RH, 30^{0}C/65 % RH, 25^{0}C/60 % RH respectively. Stability studies revealed that there were no significant changes in hardness, thickness, effervescent time of WG-8 compared to DC-6 and DG-7 trial formulations.

Also from the results obtained in the present study it can be concluded that, the test preparation is having significant anti-inflammatory activity which was observed to be dose dependent. Also, from the present study it was concluded that, the test material if given in the dose of 2.5gm/kg in rats did not show any remarkable toxic effects except some increase in the biochemical and hematological parameters which were also confirmed by the histopathological study. However, as per the observed data report histopathological changes that observed in the liver were of mild degree and may be found as the incidental changes; however lesions in spleen were indicative of mild depopulations in some of the animals, which indicate mild immune suppression. The lesions in kidneys and lung were of not related to drug administration.

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