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Evaluation of anti-inflammatory, analgesic and immunosuppressant activities of aceclofenac solid dispersions on wistar rats

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ABSTRACT

The present research work was focussed on the preparation as well as the evaluation of Aceclofenac solid dispersions for analgesic, anti-inflammatory and immunosuppressant activities. Solid dispersions were formulated using cros-carmellose sodium as polymer in different ratios. The solid dispersions were prepared using physical mixture, solvent evaporation and kneading techniques. The solid dispersions were found to release the drug faster than the pure drug in dissolution media of phosphate buffer pH 7.0. The formulation ACK4prepared by kneading technique, showed best drug release profile when compared to other formulations. This formulation was used for the evaluation of various pharmacological activities on Wistar rats. Heat induced haemolysis and protein denaturation methods were selected for the evaluation of Ex-vivo anti-inflammatory activity using fresh rat blood. Whereas, the traditional hot plate method is used for evaluation of analgesic action. A novel technique was employed for evaluation of immunosuppression activity using fungi. When compared to the control group, the group of animals treated with ACK4 formulation showed better results. From the test results, a reduction in haemolysis was observed. It also showed reduction in pain perception and immunosuppression. Thus from the present study it was concluded that Aceclofenac solid dispersion could be a promising analgesic, anti-inflammatory and immunosuppressant agent which would be a boon in organ-transplantation and cardiac patients.

Keywords: Aceclofenac, Solid dispersions, Cros-carmellose sodium, *Wistar* rats, Immunosuppression.

INTRODUCTION

Inflammation is a basic way in which a body reacts to infection, irritation or other injury, the key feature being redness, warmth, swelling and pain. As it is a protective response, it is also called as second line of defence in the body [1]. Now-a-days, NSAIDS are mostly used for this purpose, which are commonly known as non-steroidal anti-inflammatory agents. They also have analgesic and antipyretic activities. Drugs like Diclofenac and Aceclofenac are mostly used to relieve pain and inflammation during sprains and other conditions. Apart from anti-inflammatory effect, these drugs also have analgesic effect which is beneficial because these drugs do not exert any dependence or addiction when compared to that of standard narcotics. Pain is an unpleasant sensory and emotional experience associated with actual or potential tissue damage [2]. In general, the inflammatory mediators like histamine, kinins, and prostaglandins increase the sensitization of pain receptors which causes severe pain. NSAIDS like Aceclofenac and Diclofenac blocks the pain sensitizing mechanism which was induced by bradykinins, interleukins and other analgesic agents [3]. Immunosuppression is a mechanism in which the cellular or humoral or both the immune responses were inhibited. NSAIDS like Aceclofenac prevents the chemo taxis of neutrophils at the site of injury. This could a possible chance for proving the immunosuppressant activity of Aceclofenac which was not reported yet [4]. This would be much useful in organ transplantation and auto-immune disease treatment.

The most common and preferred method of drug delivery is oral route, due to its convenience and ease of ingestion. Oral palatability of a dosage form is comfortable and familiar means of taking medication, according to a patient [5].

Though it is much preferred, limited drug absorption which results in poor bioavailability is a major issue that can be observed while delivering an active agent through oral route. This may be attributed to various factors like poor aqueous solubility or membrane permeability of the drug molecule. In biopharmaceutical classification system (BCS), drugs with low aqueous solubility and high membrane permeability are categorized as class–II drugs. One of such drugs which fall under this category is Aceclofenac. One of the approaches which enhance the dissolution rate of poorly water soluble drugs is "solid dispersions". Solid dispersions are a group of solid products consisting of a hydrophilic matrix and a hydrophobic drug [6]. As they increase the dissolution rate of drug at the absorption site, there is a gradual increase in the bioavailability too [7]. Though the advantages like easy in production, prevention of pre-systemic metabolism dominate, the stability issues were a bit nutshell to crack [8]. The solid dispersions were prepared using various techniques like physical mixing, solvent evaporation, kneading etc. The anti-inflammatory activity can be performed ex-vivo using various techniques like protein denaturation and heat induced haemolysis. Whereas the analgesic activity is evaluated using the very standard and most commonly employed hot plate method. A novel technique was implemented for evaluation of immunosuppressant activity which is phagocyte count method. The main objective of the present study is to evaluate the anti-inflammatory, analgesic and immunosuppressant activities which would serve as a boon to treat various conditions like heart diseases, algesia, and tissue grafting and organ transplantation.

MATERIALS AND METHODS

Aceclofenac was a gift sample from M/s. Shreya's Laboratories, Vijayawada. Cros-carmellose sodium was a gift sample from M/s. NATCO Pharma. Ltd., Hyderabad. Nutrient broth was procured from Hi-Media, Mumbai. Methanol, disodium hydrogen phosphate and potassium dihydrogen phosphate were procured from S.D. Fine Chemicals, Mumbai. All other materials used were of analytical grade and procured commercially.

Experimental Animals:
Healthy adult male albino rats (*Wistar strain*) weighing 250–300g, housed in polypropylene cages, maintained under standardized condition i.e., 12:12 hour light/dark cycle at 25 ± 2^0C with paddy husk bedding at the animal house, Chebrolu Hanumaiah Institute of Pharmaceutical Sciences, Guntur, India were provided with standard pellet food and had free access to purified drinking water. The guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Social Justice and Empowerment, Government of India were followed and prior permission was soughted from Institutional Animal Ethics Committee (IAEC) for conducting the study.

Preparation of Aceclofenac Solid Dispersions by Physical Mixing Method:
The physical mixtures were prepared by weighing the calculated amount of drug and carriers and then mixing them in a glass mortar by triturating. The resultant physical mixtures were passed through 44-mesh sieve and stored in desiccators until used for further studies.

Preparation of Aceclofenac Solid Dispersions by Solvent Evaporation Method:
The solvent evaporation method was employed for the preparation of solid dispersions. Specified quantity of Aceclofenac and carrier were taken in a china dish and to that few ml of methanol was added and slightly heated until both drug and polymer dissolves. Then it is subsequently allowed to evaporate. The obtained mixture was dried, passed through the sieve no.80, packed in a wide mouthed amber colored glass container and was hermetically sealed and stored [9].

Preparation of Aceclofenac Solid Dispersions by Kneading Method:
The calculated amounts of drug and carriers where weighed and mixed together with suitable amount of water. The damp mass obtained was passed through a 44-mesh sieve; the resultant granules were dispersed in Petri dishes and dried at 60^0C under vacuum, until a constant weight was obtained. The granules obtained were stored in desiccators for further studies [10].

***Invitro* Drug Release Studies of Aceclofenac Solid Dispersions:**
The dissolution test for the solid dispersions was carried out in USP Apparatus Type II (paddle) with 900ml of Phosphate buffer pH 7.5 as the dissolution medium which is maintained at 37 ± 0.5^0C. The samples were drawn at 5, 15, 30 and 60 minutes. Fresh volume of the medium was replaced with the withdrawn volume to maintain the sink conditions and constant volume throughout the experiment. Samples withdrawn were suitably diluted with same dissolution medium and the amount of drug dissolved was estimated by ELICO double beam spectrophotometer at 272 nm and subsequently analyzed for the cumulative percentage of drug released.

Acute Toxicity Study:
The acute oral toxicity study was performed according to the OECD toxicity guidelines. The animals were fasted overnight prior to the experiment. Test doses of 0.1 and 0.4g/kg body weight were given orally to rats. During first four hours after drug administration, the animals were observed for gross behavioural changes such as hyperactivity, grooming, convulsions, sedation, hypothermia, body weight and mortality were observed up to 14 days [11].

Pharmacological Evaluation:
Evaluation of Ex-Vivo Anti-Inflammatory Activity:

Heat Induced Haemolysis:
20µL of uncoagulated fresh rat blood was added to vials containing 1 mL of 0.1 M PBS (Phosphate Buffered Saline, pH 7.4). ACK4 and pure drug were added to the vials (in triplicate), so as to achieve the final concentrations of 100, 150, 200, 250, 300, 350 and 400 µg/ml of each sample. PBS (1ml) and rat blood was added to control vials. Then the drug solutions were subjected to centrifugation at 3000 rpm in centrifuge for 10 minutes to make sure that all the materials had completely dissolved. No residues were ever observed after centrifugation indicating complete solubility of the drug preparations used. After mixing, the contents in vials were pre-incubated at 37^0C for 15 minutes. Then the mixtures were heated for 25 minutes at 54^0C. After spinning down the precipitate, the absorbance of the supernatant was measured at 540 nm in a spectrophotometer [12]. The percentage inhibition of haemolysis of test should be compared with respect to the control.

$$\%\text{Inhibition of haemolysis} = \frac{\text{Absorbance in control group} - \text{Absorbance in test group X 100}}{\text{Absorbance in control group}}$$

Protein Denaturation:
0.2 mL of egg albumin was added to vials containing 2.8 mL of 0.1 M PBS (Phosphate Buffered Saline, pH 6.4). 2 ml of ACK4 and pure drug were added to the vials (in triplicate), so as to achieve the final concentrations of 100, 150, 200, 250, 300, 350 and 400 µg/ml of each sample. PBS (2.5 ml) and egg albumin was added to control vials. Then the mixtures were incubated at 37 ± 2^0C in a BOD incubator for 15 minutes and then heated at 70^0C for 5 minutes. After cooling, their absorbance was measured at 660 nm using vehicle as blank [13]. The percentage inhibition of protein denaturation of test should be compared with respect to the control.

$$\%\text{Inhibition of denaturation} = \frac{\text{Absorbance in control group} - \text{Absorbance in test group X100}}{\text{Absorbance in control group}}$$

Analgesic Activity by Hot Plate Method:
Male rats were selected, weighed and grouped. The control group received only saline. Whereas the standard group received the standard Aceclofenac and the test group receives the ACK4 at a dose of 10mg/kg body weight. The basal reaction time was noted by observing hind paw licking or jump response (whichever appears first) in animals when placed on the hot plate maintained at constant temperature (55^0C). Normally animals show such response in 6-8 sec. A cut off period of 15 sec is observed to avoid damage to the paws. The standard drug was administered to animals and the reaction time of animals on the hot plate was noted at 0, 15, 30, 60, 90, 120 and 180 min after the drug administration. As the reaction time increases, 15 sec is taken as maximum analgesia and the animals are removed from the hot plate to avoid injury to the paws [14]. The percent increase in reaction-time was noted (as index of analgesia) at each time interval.

It can be calculated by using the formula,

% Increase in basal reaction time

$$= \frac{\text{Basal reaction time in test group} - \text{Basal reaction time in control group X 100}}{\text{Basal reaction time in test group}}$$

Immunosuppression Activity:
Preparation of Candida albicans Suspension:
Candida albicans was incubated in nutrient broth overnight and then centrifuged and the supernatant was discarded [15]. If necessary, the suspension was diluted.

Slide Preparation:
Rat blood was obtained in required quantity by retro-orbital puncture method. 0.2 ml of blood was placed on glass slide. The slides were prepared for control, standard Aceclofenac and ACK4. The concentrations of 100, 200 and

300 µg/ml were made with standard and with ACK4. Slides in triplicate were incubated at 37^0C for 25 minutes to allow clotting. The blood clot was removed and the slide was drained carefully with normal saline. Monolayer of polymorph nuclear leucocytes was flooded with the standard and ACK4 solutions for 15 minutes at 37^0C. Then these slides were covered with *Candida albicans* suspension for 1 hour. Then the slide was drained, fixed with methanol and stained with Giemsa stain. The number of leucocytes and phagocytes in each slide were observed by taking the count of 100 cells in the visible area and an average was made. From this, the percentage immunosuppression was calculated using the formula;

$$\% \text{ Immunosuppression} = \frac{\text{Granulocytes in test} - \text{Granulocytes in Control X 100}}{\text{ranulocytes in Control}}$$

RESULTS AND DISCUSSION

Preparation of Aceclofenac Solid Dispersions by Various Methods:

Aceclofenac solid dispersions were prepared in various ratios using cros-carmellose sodium as a polymer. The solid dispersions were prepared by various methods like physical mixing, solvent evaporation and kneading. They were prepared by keeping the drug concentration constant and by increasing the polymer concentration. Different concentrations of solid dispersions with various drug to polymer ratios were placed in table – I.

Table – I: Composition of Aceclofenac Solid Dispersions Prepared by Various Methods

S. No	Drug : Polymer Ratio	Formulation		
		Physical Mixing	Solvent Evaporation	Kneading
1	1:0.5	ACP1	ACS1	ACK1
2	1:1	ACP2	ACS2	ACK2
3	1:1.5	ACP3	ACS3	ACK3
4	1:2	ACP4	ACS4	ACK4

In-Vitro Drug Release Studies of Aceclofenac Solid Dispersions:

The dissolution studies were performed for different formulations of Aceclofenac solid dispersions along with the Aceclofenac pure drug using cros-carmellose sodium as polymer by U.S.P paddle method (apparatus II) with 7.5 pH phosphate buffer as a medium, maintained at a temperature of 37 ± 0.5^0C. The absorbance values were noted at 272 nm using ELICO double beam spectrophotometer. The results thus obtained were tabulated in table – II and indicated in figure – I. The formulation ACK4 with composition of 500mg of Aceclofenac and 2000 mg of cros-carmellose sodium, prepared by Kneading method showed best dissolution profile when compared to all other formulations. The cumulative percentage drug release for ACK4 after 60 minutes is 92.86%. All the formulations clearly indicated that as the carrier concentration increases, there is an increase in the dissolution rate of the drug. This is due to increased wettability of the drug by the polymer, thus leading to enhanced solubility [16]. This process favours the absorption of the drug in gastro-intestinal tract. Hence, ACK4 was selected for further pharmacological evaluation.

Acute Toxicity Studies:

The acute toxicity study revealed the non-toxic nature of the formulation. The doses used, i.e., 0.1 and 0.4 g/kg were found to be safe. Hence the oral test dose needed to be administered in such a way that it lies in between $1/10^{th}$ and $1/50^{th}$ of the maximum safe dose i.e., in the range of 8 – 40 mg/kg.

Table – II: Dissolution Profiles of Aceclofenac Solid Dispersions Prepared by Various Methods

S. No	Time (mins)	Cumulative % Drug Released												
		APD	ACP1	ACP2	ACP3	ACP4	ACS1	ACS2	ACS3	ACS4	ACK1	ACK2	ACK3	ACK4
1	5	**6.26**	13.25	21.13	26.37	**32.98**	15.22	22.55	34.43	**36.23**	22.41	34.22	40.44	**44.84**
2	15	**16.25**	17.90	28.39	32.21	**40.30**	22.41	29.95	40.08	**44.69**	33.31	43.05	51.85	**59.66**
3	30	**21.20**	24.39	35.17	40.10	**47.09**	29.95	37.11	47.02	**55.26**	42.77	55.29	64.31	**81.99**
4	60	**25.16**	32.83	40.72	55.31	**63.15**	39.97	46.10	57.28	**70.41**	50.25	65.38	80.02	**92.86**

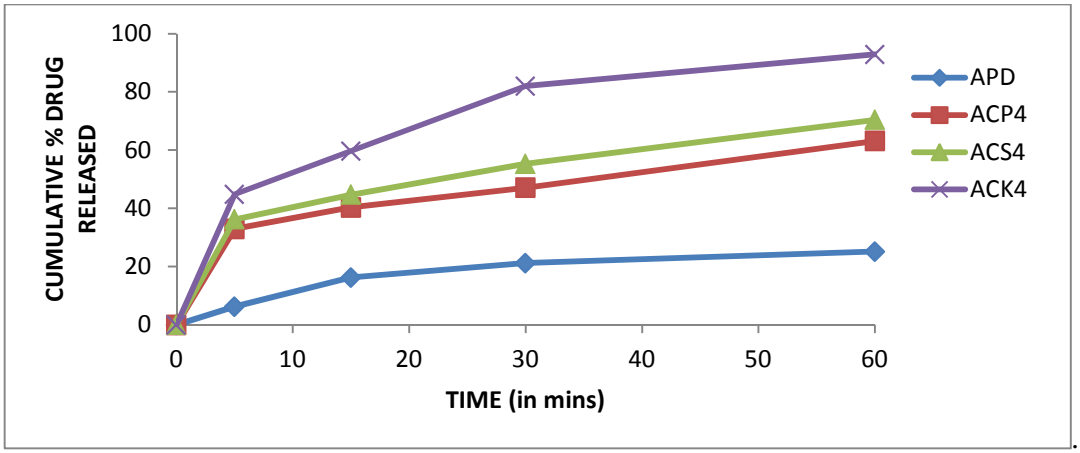

Figure – I: Drug Release Profiles of Aceclofenac Solid Dispersions Prepared by Various Techniques

***Ex-Vivo* Anti-Inflammatory Activity:**

Effect on Heat Induced Haemolysis:

The ACK4 formulation was subjected to heat induced haemolysis test in various concentrations using fresh rat blood. The ACK4 showed 85.52% inhibition of haemolysis on erythrocyte membrane at a maximum dose of 400µg/ml. It is more effective when compared to that of the control and as the dose increases, there is an increase in the percentage inhibition. In this, lysosomes play a major role in the inflammatory reaction [17]. The vitality of cells depends upon the integrity of their membrane, exposure of RBCs to injurious substances such as hypotonic medium results in lysis of its membrane accompanied by haemolysis and oxidation of haemoglobin [18]. Compounds with membrane stabilizing properties are well known for their ability to interfere with the release of phospholipases that trigger the synthesis of inflammatory mediators. These results were indicated in table – III and figure – II.

Table – III: Effect of APD and ACK4 on Heat Induced Haemolysis of Erythrocyte Membrane

S. No	Concentration(µg/ml)	% Inhibition of Heat Induced Haemolysis *(Mean ± S.E.M)*	
		APD	ACK4
1	100	35.68 ± 1.818	55.86 ± 0.704
2	150	52.34 ± 1.240	66.69 ± 1.047
3	200	64.95 ± 1.521	70.66 ± 0.807
4	250	68.21 ± 1.496	74.23 ± 0.742
5	300	71.41 ± 0.553	76.47 ± 0.471
6	350	75.25 ± 0.978	80.69 ± 1.149
7	400	78.05 ± 1.176	85.52 ± 1.710

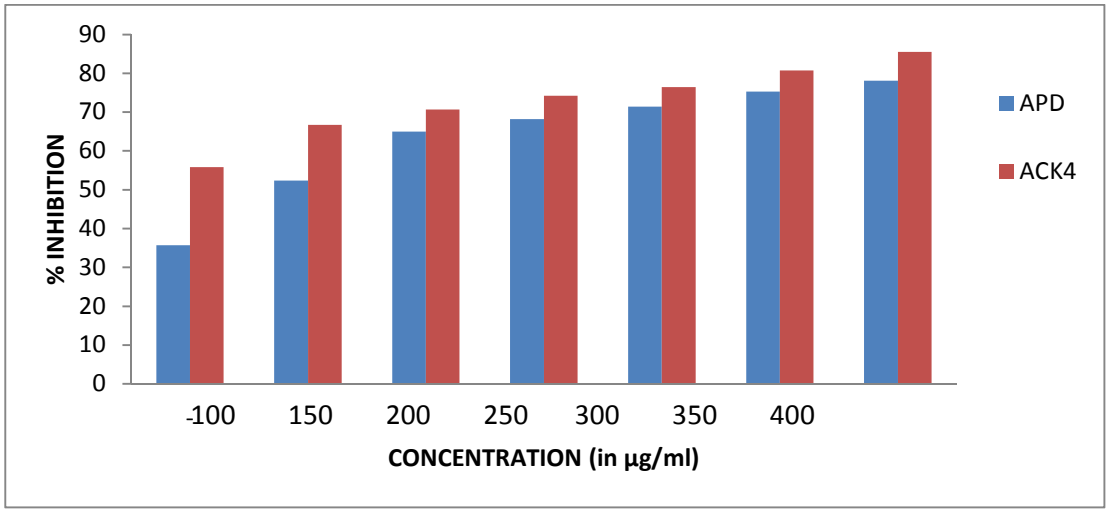

Figure – II: Effect of APD and ACK4 on Heat Induced Haemolysis of Erythrocyte Membrane

Effect on Protein Denaturation:

The ACK4 formulation was subjected to protein denaturation in various concentrations using fresh egg albumin. The ACK4 showed 83.18% inhibition of protein denaturation at a maximum dose of 400 µg/ml. It is more effective

when compared to that of the control and as the dose increases, there is an increase in the % inhibition. Denaturation of proteins is a well documented cause of inflammation [19]. Several anti-inflammatory drugs have shown dose dependent ability to inhibit thermally induced protein denaturation. Ability of ACK4 to bring down thermal denaturation of protein is possibly a contributing factor for its anti-inflammatory action. These results were shown in table – IV and figure – III.

Table – IV: Effect of APD and ACK4 on Protein Denaturation

S. No	Concentration (µg/ml)	% Inhibition of Protein Denaturation *(Mean ± S.E.M)*	
		APD	ACK4
1	100	30.32 ± 1.158	51.55 ± 1.055
2	150	37.46 ± 0.493	61.06 ± 1.359
3	200	49.65 ± 0.519	65.73 ± 0.792
4	250	57.14 ± 0.907	71.14 ± 1.378
5	300	65.24 ± 1.162	74.16 ± 0.898
6	350	69.40 ± 1.612	78.49 ± 1.047
7	400	73.43 ± 1.548	83.18 ± 1.476

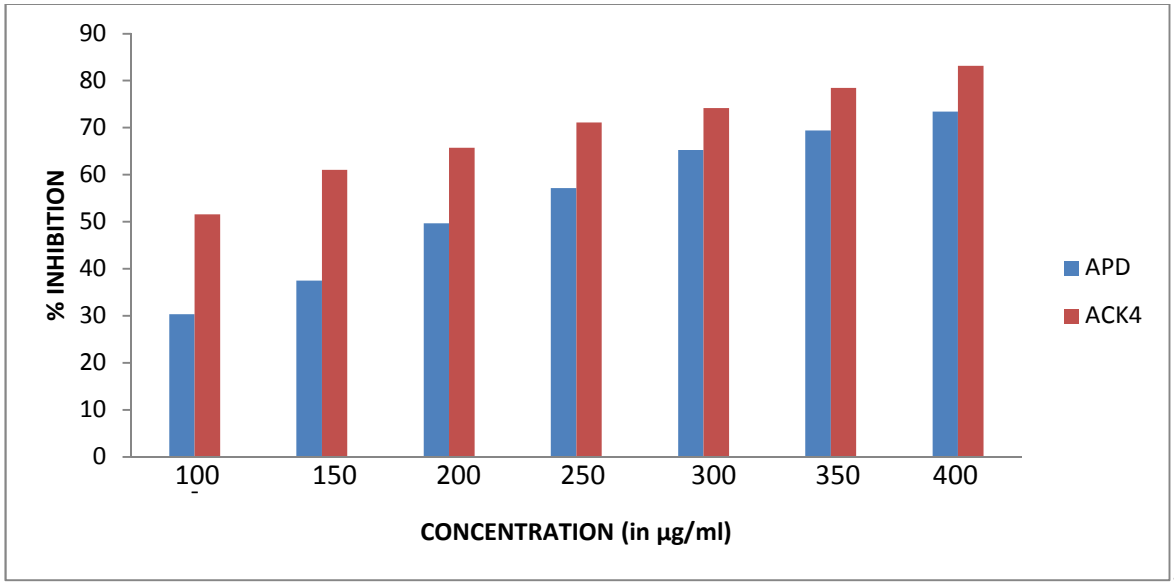

Figure – III: Effect of APD and ACK4 on Protein Denaturation

Analgesic Activity by Hot Plate Method:

The ACK4 formulation was subjected to analgesic activity by Eddy's hot plate method on male Wistar rats. The basal reaction time which is the time taken by the animal to jump out of the hot plate from its initial placing time is noted down. The ACK4 showed a maximum of 62.88% increase in basal reaction time after 90 minutes of drug ingestion. This is higher when compared to that of the control and APD which showed only 52.63%. Decreased sensitisation of pain receptors reduces the pain. By this mechanism, Aceclofenac shows its analgesic property [20]. These results were shown in table – V and figure – IV.

Table – V: Effect of APD and ACK4 on Basal Reaction Time in Rats

S. No	Group	Basal Reaction Time (in sec)						
		0 min	15 min	30 min	60 min	90 min	120 min	180 min
1	Normal	7.2	---	---	---	---	---	---
2	APD	---	7.4	9.5	12.4	15.2	8.4	7.3
3	ACK4	---	7.8	10.6	15.8	19.4	9.1	7.6
4	% Increase In Basal Reaction Time (APD)	---	2.70	24.21	41.93	52.63	14.28	01.37
5	% Increase In Basal Reaction Time (ACK4)	---	7.69	32.07	54.43	62.88	20.87	5.26

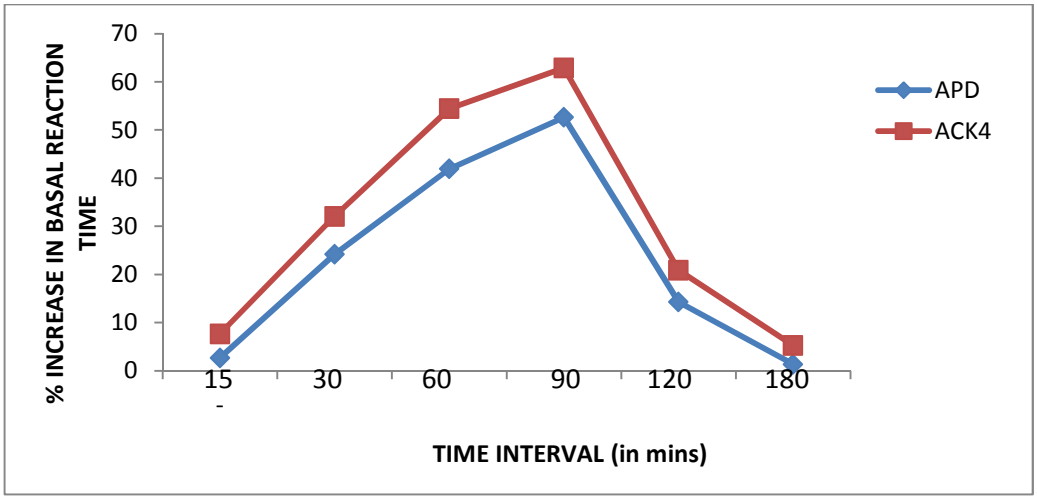

Figure – IV: Effect of APD and ACK4 on Basal Reaction Time in Rats

Immunosuppression Activity:

The ACK4 formulation along with the APD in increasing concentrations was subjected to immunosuppressant activity using *Candida albicans*. As the dose increased, there is an increase in the number of granulocytes and decrease in the number of phagocytes. ACK4 showed a maximum survival of 91 granulocytes at its higher dose of 300 µg/ml. The percentage immunosuppression of ACK4 at its highest dose was found to be 90.77 which are more than APD and it is indicated in table – VI. The Aceclofenac here prevents the process of phagocytosis which is the resultant of immune system activation. The decreased number in phagocytes indicates that Aceclofenac is having the immunosuppression property which is advantageous during graft rejection reactions [21].

Table – VI: Effect of APD and ACK4 on Number of Granulocytes and Phagocytes

S. No	Treatment	Concentration (µg/ml)	Number of Granulocytes	Number of Phagocytes	% Immuno suppression
1	**Control**	----	12	88	----
2	**APD**	100	66	34	80.80
		200	82	18	84.36
		300	89	11	86.51
3	**ACK4**	100	78	22	84.61
		200	86	14	87.03
		300	91	09	90.77

CONCLUSION

As the polymer concentration increased, there is an increase in the cumulative drug release in the Aceclofenac solid dispersions which are prepared by kneading method using cros-carmellose sodium as polymer. The formulation ACK4 which drug release was further subjected to pharmacological evaluation. From the obtained results, it was concluded that there is a significant inhibition of heat induced haemolysis of 85.52% when compared to that of the standard. The formulation ACK4 also showed a drastic analgesic action at 90 minutes after drug administration with 62.88% increase in basal reaction time. Approximately, 90% of immunosuppression was observed when the rat blood was subjected to fungal organisms. Thus, from the current work, it was concluded that the Aceclofenac solid dispersions prepared by Kneading method using cros-carmellose sodium as polymer shows better anti-inflammatory, analgesic and immunosuppression activities.

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