Research Article

Evaluation of Anti-granulation Effect of Apigenin - A Plant Flavonoid in Wister Albino Rat

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ABSTRACT

Several plant bioactive compounds have exhibited functional activities that suggest they could play a remarkable role in preventing a wide range of chronic diseases. The largest group of naturally-occurring polyphenols are the flavonoids, including apigenin. The anti-inflammatory activity of Apigenin (10, 20 and 40 mg/kg p.o) has been evaluated in cotton pellet-induced granuloma in Wister albino rats. Apigenin has reduced inflammation as evidenced by decreased weight of cotton pellet in cotton pellet-induced granuloma in rats. The optimal anti-inflammatory effect of apigenin can be seen at dose 20mg/Kg in cotton pellet-induced granuloma model ($P<0.001$) in comparison to control and respective group. The results demonstrate the anti-inflammatory properties of Apigenin and the effects were comparable to diclofenac sodium, a standard non-steroidal anti-inflammatory drug.

Keywords: Apigenin, Flavonoids, cotton-pellet, inflammation.

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INTRODUCTION

Inflammation is a pathophysiological local response of living tissue to injury due to any agent[1]. In chronic inflammatory and granulomatous disease, macrophage activation is known to contribute towards both maintenance and progression of the disease state as is evident by the elevated levels of predominantly macrophage-derived pro-inflammatory cytokines viz TNF-α, IL-6 & IL-1, Prostaglandins, Histamine in these conditions[2]. The specific chemical mediators vary with the type of inflammatory process[3]. Although modern drugs are effective in the management of inflammation and associated conditions, but their use is often limited because of side effects especially in chronic debilitating disease like rheumatoid arthritis, osteoarthritis etc[4].

In recent era there is growing realization that apart from being safer, economical and easily available, nutraceutical, plant flavonoid can influence the course of inflammation. Therefore, it would be scientifically rational to evaluate the plant flavonoid used for their potential use in inflammatory diseases. An increasing importance is being given today to alternative medicine and dietary approach in prevention and treatment of chronic inflammatory disease.

Apigenin (4', 5, 7,-trihydroxyflavone) is a non-toxic and non-mutagenic dietary flavonoid, which is abundantly present in common fruits and vegetables, such as oranges, grapefruits, parsley, onions, chamomile, wheat sprouts, and some seasonings[5]. Different epidemiologic studies suggest that a diet rich in flavones is related to a decreased risk of chronic diseases such as cancer, as well as neurodegenerative, metabolic and heart diseases[6,7,8]. It has been suggested that apigenin may be protective in other diseases that are affected by oxidative process, such as cardiovascular and neurological disorders, more research needs to be conducted in this regard[9]. Although few evidence exists in the use of apigenin a naturally occurring plant flavone in the treatment of inflammation, but not much is known about its anti-inflammatory mechanism and action.

Therefore, In the present study we investigated the anti-inflammatory activity of Apigenin in cotton pallet induce granuloma model to evaluate the involvement of anti-inflammatory activity at the doses tested as it is depictive of circulating macrophage activation and aggregation.

MATERIALS AND METHODS

Description of plant flavonoid

Apigenin was procured from Sigma pharmaceuticals pvt.ltd. as a powder form.

Selection of animals, caring and handling

A total of 21 healthy Wistar albino rats (90–150 g), of either sex obtained from the Central Animal house IGIMS, Patna India were used in the experiments. Male & female Rats separated in different cages. Rats were housed in polypropylene cage containing sterile paddy husk

(procured locally) not more than 3 animals per cage under standard conditions of temperature (23±3°C), humidity and 12 hours natural light and 12hours Natural dark cycle. Animals were acclimatized for 7 days to the laboratory conditions before experiment. Animals were given standard dry pellet diet and tap water ad libitum. One day before the experiment animals were deprived of food as this is known to enhance their motivation to perform the test. For easy identification of animals in every group tail had been colored. The study was undertaken after obtaining approval of Institutional Animal Ethics Committee (IAEC approval Letter No. 125/2018/Pharma/IAEC/IGIMS dated 14/12/2018).

Study design

The rats were randomly allocated into seven groups of three rats each for this Anti-inflammatory experimental model.

Grouping of Animals: -

Group I (control) received 2 ml normal saline of each rat per-orally through intragastric tube.

Group II received standard drug Diclofenac sodium 100 mg/kg per-orally according to different body weight.

Group III received apigenin 10mg/kg per orally

Group IV received apigenin 20mg/kg per orally

Group V received apigenin 40mg/kg per orally

Group VI received Diclofenac 50mg/kg per orally (Sub-therapeutic dose of standard drug)

Group VII received Combination dose of 20mg/kg + Diclofenac-50mg/kg per orally

MATERIALS

Drugs

Diclofenac Sodium, Normal saline, Ether (Sigma chemical Co. St Louis, USA, Apigenin.

Chemicals for Histopathological Study

Formalin, haematoxylin and eosin stain (2% w/v), alcohol, Xylene, Ammonia water, Distilled water, Paraffin wax.

Instruments

Needle holder, dissecting forceps (straight-15.5cm & curved), skin hook, surgical tray, cotton, Surgical blade, surgical needled suture, tissue store container, microtome machine, slides, coverslip.

Determination of the drug dosage and dosing schedule

Doses were selected and determined according to the previous acute toxicity studies[10]. Three different doses of Apigenin were selected 10 mg/kg, 20 mg/kg and 40 mg/kg. Before starting the experiment preparation of stock solution of apigenin (2mg/ml,4mg/ml & 8mg/ml) was done by dissolving the apigenin powder in normal saline and administered according to animal body weight.

METHODS

Cotton pallet induced granuloma model

This model represents the pathological events in sub-acute inflammation. It is the most suitable method for studying the efficacy of drugs against the proliferative phase of inflammation in rat by the method of swingle and shideman (1972)[11,12].

On day 1, the abdomen was shaved cleanly with surgical blade. swabbed with 70%(v/v) ethanol & with aseptic precautions sterile cotton pellets (10±1 mg) were implanted subcutaneously, along the flanks of axillae under ether anaesthesia by using a curve dissecting forceps. Throughout the experiment aseptic condition was maintained to avoid infection. All drugs were given orally to the respective group of rats in daily for six consecutive days from day 1. On the 7th day, the animals were anaesthetized, and the pellets together with the granuloma tissues were carefully excised and made free from the surrounding tissues. Weight of the cotton pellet before implantation was subtracted from weight of the dissected dried pellets. The granuloma tissues were fixed in 10% formalin solution for histopathological study in tissue store container.

Histopathological Study: - This process includes following steps

1. Fixation (10% formaldehyde)- In this process tissue had been preserved over night for 24hr. in 10% formaldehyde solution which was prepared freshly[13].

2. Dehydration process- This process Includes removal of alcohol from tissues and is replaced by fluid which was miscible with wax with which tissue must be impregnated. Tissue sample was dipped into increase Concentration of alcohol i.e.,70% alcohol for 1hr, 90% alcohol for 1hr, 95% alcohol for 1hr, Absolute alcohol-I for 1hr, Absolute alcohol-II for1hr[14].

3. Clearing process: - Dehydrated tissue had been dipped into xylene-I solution for 15 minutes followed by another 15minute dipping. This ensures the removal of water from the tissue sample[15].

4. Embedding: - It is the process in which the tissues or the specimens are enclosed in a mass of the embedding medium using a mould. Since the tissue blocks are very thin in thickness, they need a supporting medium in which the tissue blocks are embedded. This supporting medium is called embedding medium. For this experiment paraffin wax had been selected. Paraffin with melting point (56-62°C) was used for embedding. Melted paraffin had been pour inside the L-mould and tissue had been embedded inside it. Mould had been kept on cooling plate at -24°C to ensure the formation of block. The block had been labelled for easy identification of tissue embedded sample.

5. Sectioning process: - embedded tissue had been cut into sections by microtome which was pre-set to cut at 5 µm thicknesses. Blocks were carefully trimmed to expose

tissue. The tissue ribbons were carefully transferred to a warm water bath to float on the surface. First Slides were treated with egg albumin, rubbing it to make the slide sticky then the cut section was put on center of the slides. Slides should be clearly labeled and then allowed to dry upright at 37°C for 30 minutes to gently melt the excess paraffin wax by leaving the tissue section intact.

6. Hydration: - Deparaffinize Tissue slides were then subjected to hydration by dipping the slides into different graded alcohol starting with absolute alcohol for 1min 30 sec. Rectified alcohol for 1min 30sec, 90% alcohol for 1 min 30 sec and 70% alcohol for 1min 30 sec.

7. Staining: - Slide was rinsed with tap water. Slide was kept into Haematoxylin stain bottle for 5-15 minute. Washed the slide under tap water then transferred the slides into 5-10% acid alcohol for 2-6 sec to differentiate the nucleus. Slide was subjected to ammonia water dipping followed by washing under the tap water. Next was the counter staining of the slide with eosin stain for 2min followed by washing under tap water.

8. Dehydration: - Process of removal of alcohol from the tissue by subjecting the stained slide to different grade alcohol i.e.,70% Alcohol ,90% alcohol 95% alcohol followed by absolute alcohol. After Dehydration process the stained slide was finally rinsed in xylene for better visibility. Apply DTX/glue onto the tissue & keep the coverslip and watch under microscope.

Evaluation

The percentage inhibition of granuloma was calculated for each group with respect to its vehicle-treated control group. Percentage inhibition of granuloma= (Mean Dry wet of granuloma of Control- Mean Dry wt. of granuloma of Drug treated)/ Mean Dry wet of granuloma of Control ×100[16].

Statistical analysis

The results were analysed for mean±SEM, SD statistical significance using One way ANOVA, followed by Scheffe's test. A P-value <0.05 was considered significant.

RESULTS

Effect of Apigenin on granuloma weight in the cotton pellet induced granuloma

The dry weight of cotton pellet granuloma in control, three different doses of apigenin, standard drug Diclofenac, subtherapeutic dose of diclofenac (diclofenac 50mg/kg), combination of most effective anti-inflammatory dose of apigenin (Apigenin-20mg/kg) and subtherapeutic dose of diclofenac treated groups is shown in the Table 1. All Apigenin doses significantly reduced (P<0.001) the formation of inflammatory exudate and granuloma formation compare with control. Standard drug with proven anti-inflammatory property (Diclofenac-100mg/kg) showing maximum inhibitory effect in exudate and granuloma formation includes 59.69% (P<0.001) in comparison with control treated group. Among the three doses of apigenin maximum inhibitory action can be seen with Apigenin-20mg/kg with 58.87% which can be comparable with standard drug diclofenac response alone. Subtherapeutic dose of standard drug diclofenac (50mg/kg) showing more inhibitory effect in exudate and granuloma formation than low dose of apigenin(10mg/kg) but less than two higher doses of apigenin i.e., Apigenin-40mg/kg and Apigenin-20mg/kg respectably. While we combined most effective anti-inflammatory dose of Apigenin(20mg/kg) with sub-therapeutic dose of standard drug (Diclofenac-50mg/kg) showing near about 68.93% of inhibitory effect in exudate and granuloma formation which can compete the anti-inflammatory efficacy of standard dose of diclofenac(100mg/kg).

Effect of Apigenin on Histopathological changes in the cotton pellet induced granulomic rats

Anti-granulation effect of standard, subtherapeutic dose of standard drug and three different doses of apigenin, and combination of most effective dose of apigenin with subtherapeutic dose of standard drug was evaluated by doing histopathological study on Hematoxylin and eosin staining and observed under Penta head microscope. As we know peritoneum is "policeman" of abdomen".

Table 1: Anti-inflammatory effect of Apigenin on the cotton pellet-induced granuloma in rat

Drugs	Dose/Route	Mean weight of Exudate (mg)	Mean dry weight of Granuloma	% Inhibition of Granuloma
Control (Normal saline)	2ml/P.O. each Rat	108.3±0.63	24.56±0.64[a]	
Standard (Diclofenac sodium)	100mg/kg po.	59.36±0.22	9.9±0.08*	59.69
Apigenin-10	10mg/kg po.	87.31±0.55	29.49±0.47*	20.07
Apigenin-20	20mg/kg po.	62.69±0.60	10.1±0.07*	58.87
Apigenin-40	40mg/kg po.	77.86±0.39	10.46±0.30*	57.41
Diclofenac sodium	50/kg po.	56.33±0.28	16.72±0.28*[a]	31.92
Apigenin-20+Diclofenac-50	AP-20+Dc50 po.	56.33±0.28	7.63±0.20*	68.93

*P<0.001 Control vs respective group, [a]P<0.001 Apigenin-20+Diclofenac-50 vs respective group. One-way ANOVA; SEM = Standard error of mean, SD=Standard deviation

Subcutaneous implantation of cotton pellet in flanks of axillae part of rat includes inflammation. In histopathological observation of control group treated with normal saline, we can find lots of connective tissue which represent the greater number of collagen fibers occurs due to infiltration of neutrophils. This represents inflammation in the group. Where as in the standard drug diclofenac treated group we can find more fat-globules with less amount of collagen which represent the anti-inflammatory action by standard drug. In combination therapy by subtherapeutic dose of standard drug with most anti-inflammatory dose of apigenin represent comparative effective that of standard drug alone.

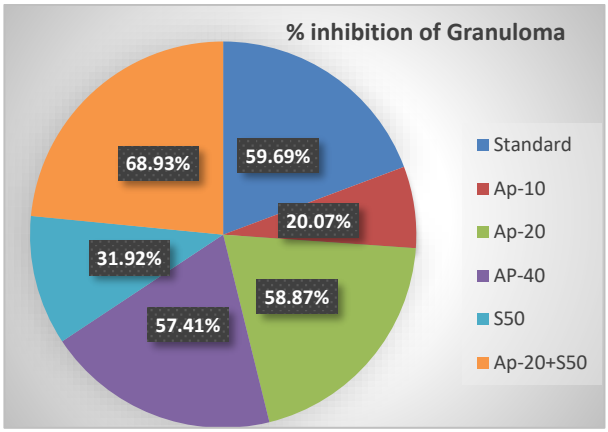

Figure 1: Percentage inhibition of Granuloma

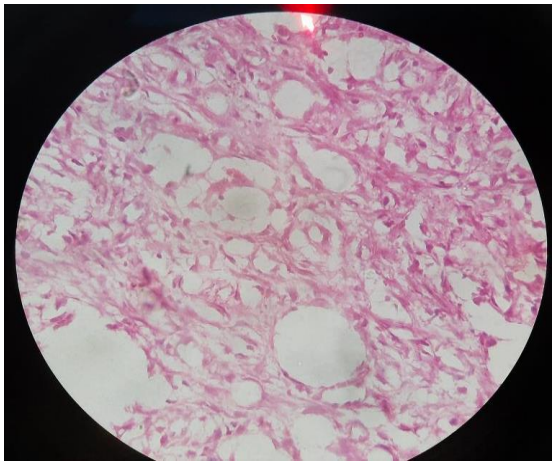

Figure 2: Control treated Group

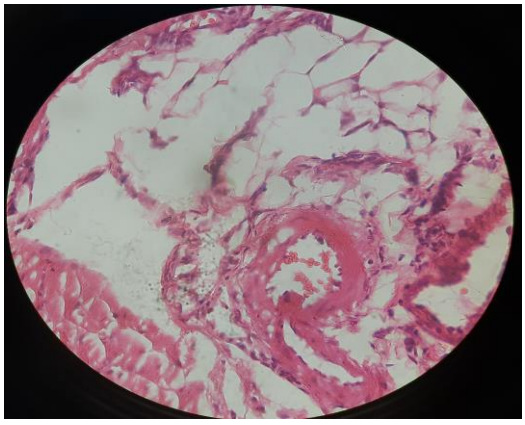

Figure 3: Standard (Diclofenac-100mg) treated Group

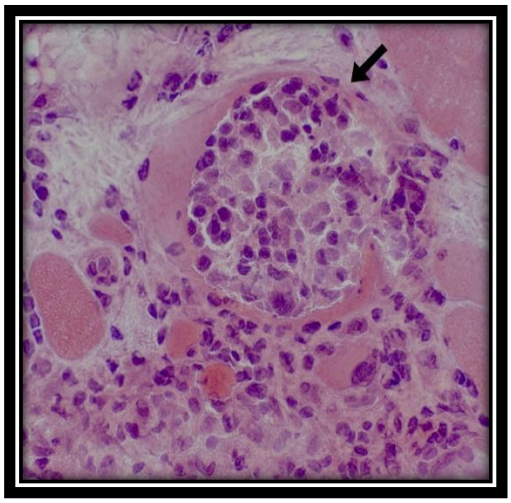

Figure 4: Standard (Diclofenac-100mg) treated Group

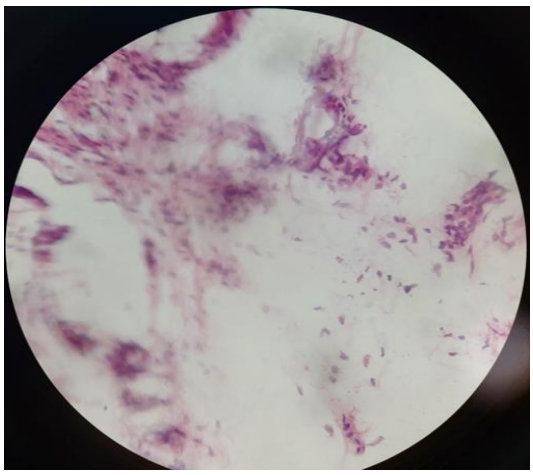

Figure 5: Apigenin-10mg treated Group

DISCUSSION

Cotton pellet granuloma model was used to evaluate the anti-inflammatory activity of Apigenin in sub-acute inflammation. Three phases of the inflammatory response to a subcutaneously implanted cotton pellet in the rats have been described: (A) a transudative phase, that occurs during the first 3 h; (B) an exudative phase, occurring between 3 and 72h after implanting the pellet; (C) a proliferative phase, measured as the increase in dry weight of the granuloma that occurs between 3 and 6 days after implantation[16]. The suppression of proliferative phase of sub-acute inflammation could result in decrease in the weight of granuloma formation[17]. Standard drug diclofenac showing maximum inhibitory effect in exudate and granuloma formation which can also be corelated with histopathological finding on the basis of decrease connective tissue deposition. According to the study by Smith and Dewitt diclofenac sodium act by inhibiting the prostaglandins synthesis at the late phases of inflammation. This effect may be due to the cellular migration to injured sites and accumulation of collagen, an important mucopolysaccharide[18,19]. Decreasing granuloma tissue, prevention of occurring of the collagen fiber and suppression of mucopolysaccharides are indicators of the antiproliferative effect by NSAIDs[19]. The dry weight of

cotton pellet granuloma was significantly reduced (P<0.001) by different dose of Apigenin and maximum by 20mg of apigenin. However, the antiproliferative effect of Apigenin was lesser than that of the standard drug but can be comparable. In the combination therapy of Apigenin with subtherapeutic dose of diclofenac shows higher efficacy than standard diclofenac alone. Hence, increased Antiproliferative efficacy of combination therapy can be alternative choice over diclofenac therapy in chronic disease which can result into improve patient compliance. The cotton pellet implantation model is considered to be a reliable in-vivo system for studying macrophage function, and efficacy in this model is depictive of inhibitory activity on macrophage activation, infiltration and aggregation[20]. This can also be prescribed as an adjuvant therapy in chronic debilitating diseases with a comorbidity of acute renal failure which cause due to long term traditional non-steroidal anti-inflammatory drug[21,22].

CONCLUSION

Present study thus demonstrate the anti-inflammatory activity of Apigenin as it decreased granuloma formation. These finding contribute towards validating the traditional use of Apigenin in the management of chronic granulomatous disorder. Further in vitro and in vivo studies are recommended especially to inflammatory mediators to investigate the detailed action and mechanism of actions as well as to increase the efficacy of apigenin for clinical application.

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