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Effect of Hesperetin Isolated from *Delonixelata* on Adjuvant induced Arthritis in rats - A Radiographic Densitometric analysis

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In the present study, the anti-arthritic effect of oral administration of hesperetin from *Delonixelata* on Freund's adjuvant induced arthritis has been studied in Wistar albino rats. The loss of body weight during the arthritic condition was corrected on treatment with hesperetin at 50 and 100 $mg.kg^{-1}$body weights. The swelling of the paws during the secondary lesions was also markedly reduced on treatment with hesperetin and this result was confirmed using radiographic analysis and the changes in the density of Hind Limb Bone Mass (HLBM) was measured using photodensitometer and aluminium step wedge. The HLBM was significantly reduced on treatment with hesperetin (50 and 100 $mg.kg^{-1}$body weight) and standard drug Indomethacin (10 $mg.kg^{-1}$). From the result we observed that the hesperetin possess potent anti-arthritic activity.

KEYWORDS- Hesperetin, Anti-arthritic, Freund's complete adjuvant, Photodensitometer, Aluminium step wedge.

1. INTRODUCTION

Arthritic affects 0.5-1% of the world population with more women being affected than men [**1]**. The immune system is a well-organized and well-regulated. The deregulation of the immune system may lead to the development of autoimmune diseases such as Rheumatoid arthritis (RA), is proto-type of such groups of illness with chronic, systemic disorders with destructive inflammatory polyarticular joint potentially resulting in progressive destruction of articular and periarticular structure [2]. Persistent inflammation produces swollen joints with severe synovitis, decreased nociceptive threshold [3, 4] and massive subsynovial infiltration of mononuclear cells, which is along with angiogenesis leads to pannus formation [5]. Expansion of the pannus induces bone erosion and cartilage thinning, leading to the loss of joint function [6, 7]. This result in a high degree of morbidity and disturbed daily life of the patient. Corticosteroids have not been able to fully control the incidence because of its limitations and risk of side effects. Many patients and practitioners are seeking alternative approach to provide an effective cure in the treatment of arthritis and to overcome the serious draw backs such as gastro intestinal bleeding [8] on treatment with Corticosteroids. Hence there is an urgent need to find safer drug for the management of rheumatoid arthritis.

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The isolated compound hesperetin from *Delonixelata* at the dose level of 50, 100 $mg.kg^{-1}$ was evaluated for its anti-arthritic activity in animal model using rats. Until now no work has been carried out to assess anti-arthritic activity of hesperetin from *Delonixelata*

Indian medicinal plants are a rich source of bioactive substances, which are claimed to induce para- immunity, the nonspecific immune modulation of essentially granulocytes, macrophages, and natural killer cells and complement factors (7). *Delonixelata* (Family: Caesalpiniaceae) is found in some parts of south India and widely distributed in waste lands. It is a large bush (or) small tree, reaching 9m height with more or less pubescent leaves and branches and the leaves are no specific taste, soft and feathy to touch and pale green in colour (8-10). They are growing in mesic habitats with moderate rainfall and mild temperature. The leaves of the plant are used in inflammation rheumatism. The decoction of the root and leaves of the herb is used in rheumatism, antimicrobial (11) nervous diseases, convalescence of measles, piles, chronic bronchitis (12) *etc*. Hence the present investigation was undertaken to study the anti-arthritic activity of hesperetin due to the fact that was traditionally used for wound healing, fever, infection, pain, edema or rheumatic disorders is taken as an indicator that the plant should be tested for its anti- arthritic properties [13].

2. MATERIALS AND METHODS

2.1 Plant material

Taxonomic identification of the plant was made from Rapinat Herbarium, St.Joseph's college of arts and sciences, Trichy, Tamilnadu, India (Voucher specimen number RH/CP/24A). whole fresh plant leaves of *Delonixelata* were collected from Vatharayanthattu, Cuddalore (Dist), Tamilnadu, India. The leaves were dried under shade, segregated, pulverized by a mechanical grinder and passed through 40 mesh sieves.

2.2 Preparation of extracts

The powdered leaves (500 g) were successively extracted with ethanol (70-80° C) for 24 hrs by continuous hot percolation method using soxhlet apparatus. The fraction was separated from the solvent by distillation under reduced pressure to yield 5.6% w/w solid mass that was stored in a refrigerator and used for further studies.

2.3 Animals

The animals for the present study were procured after ethical clearance from the Institutional Animal Ethical Committee (IAEC) in Annamalai University, Annamalainagar, Tamilnadu, India. The animal experiments were carried out according to Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) rules. Inbred Wistar rats (150-200g) were used for testing anti-inflammatory activity. The albino mice (20-25g) were used for testing antinociceptive activity. The animals were housed at the central animal house (Rajah Muthiah Medical College and Hospital, Annamalai University, Tamilnadu, India) under standard conditions of temperature (23 ± 1 °C), relative humidity(55 ± 1%), 12 hrs light and dark cycles and fed with standard pellet diet, and tap water *adlibitum.*

2.4 Drugs and chemicals

All the drugs used in this study were of pharmaceutical grade. Freund's adjuvant was supplied by Genex Pharma, Indomethacin is a gift sample from Cadila Pharmaceuticals, Ahamedabad, India.

2.5 Preliminary phytochemical and compound isolation

The leaf extract of *Delonixelata* was subjected to preliminary phytochemical screening, for various active phytochemical constituents such as carbohydrates, steroids, proteins, flavonoids, amino acids, fat, fixed oil, gum and mucilage (14). The compound was isolated by column chromatography and TLC and these fractions subjected to characterized by using IR, Mass, NMR. After all the above findings I have isolated one compound from the plant, that compound was subjected to further studies named as hesperetin (15).

2.6 Evaluating of paw volume and body weight changes in Freund's induced arthritic animal.

Freund's adjuvant induced arthritis model [16] was used to assess the anti-arthritic activity of hesperitin in Wister rats. Animals were randomly divided into five groups of six animals each (n=6). Group I animals received normal saline (5 $mg.kg^{-1}$) served as control, Group II animals received Indomethacine (10 $mg.kg^{-1}$ p.o.) served as reference standard, Group III animals received (0.01 ml *Freund's* adjuvant) served as an arthritic control and Group IV and V animals received the isolated compound hesperetin (50 and 100 $mg.kg^{-1}$). The paw volume is a indicator of arthritic condition. To assess the anti-inflammatory and anti-arthritic activity of the isolated compound hesperetin was given to the animal 30 minutes before the administration of freund's adjuvant and continued till 28^{th} day. Paw volume was measured on 4^{th}, 8^{th}, 12^{th}, 16^{th}, 20^{th}, 24^{th} and 28^{th} day by using plethismometer and changes in body weight also measured.

2.7 Evaluation of radiological changes in the hind limb paw

Freund's adjuvant induced Arthritis model [16] was used to assess the anti-arthritic activity in Wister rats. Animals were randomly divided into five groups of six animals each (n=6). Group I animals received normal saline (5 $mg.kg^{-1}$) served as control, Group II animals received Indomethacine (10 $mg.kg^{-1}$ p.o.) served as reference standard and Group III animals received (0.01 ml Freund's adjuvant) served as an arthritic control and Group IV and V animals received the isolated

compound hesperetin (50 and 100 mg.kg^{-1}). The hesperetin was administered after 14 days from the day of adjuvant injection for 14 days by intubation and the drug treatment was given until 28th day by oral route. All radiographs were taken with a Wipro GE X-ray instrument set at 45kV and 4 mAs using Laser Orthochromic film. The film -to- source distance was 100cm. X-ray was taken at the joints of the hind paw for the confirmation and evaluation of the severity of arthritis in FAI induced rats. The X-ray examination of Hind Limb Bone Mass of animals in HLBM of Group I – Group V was carried out continuously for 5 weeks [17]. On day 1 the animals were subjected to X-ray examination without any treatment and after 3hrs the animals GP I- IV were induced with 0.1ml of Freund's adjuvant in left hind paw of rats. On 7th day X-ray examination of animals in all groups were carried out. On day 14th day X-ray was taken after treatment with isolated compound hesperetin and indomethacine to the corresponding group of animals. The same treatment was continued until 28th day but the X-ray examination was carried out on 21st and 28th day. Estimation of HLBM using photodensitometer and aluminium step wedge. Densitometer is a device by which we can measure the Optical Density (OD) at a particular area of a film and we can measure the density from 0 to 4 with 0.01 accuracy. The processed X-ray film carries the visible image in terms of metallic silver pattern and in other words the degree of blackness is directly related to the amount of silver present. The film darkness directly depend on intensity of radiation reaching the film which in turn depends on atomic number and density of the tissue through which uniform X-ray beam has passed. By measuring the amount of silver loss or degree of blackening, is indicator of nature of tissue. For radiographic standardization x-ray tube voltage KeV, mAs, developing condition should be as a kept constant. A high level of standardization is required in both projection geometry and image acquisition, which is needed to achieve a precise measurement of density. Radiographic projection geometry is defined by the relative location, and orientation of X-ray source, the object and the film detector. After assessing the nature of tissue, a quantitative measurement was performed using aluminium step wedge. The step of height of aluminium step wedge is 1.5mm to 10.5mm with a width of 3mm was placed on the film cassette and radiograph was exposed. Aluminium was chosen for step wedge since its atomic number is very similar to the effective atomic number of bone. Mineral with similar atomic number will attenuate X-ray in a similar manner. OD of each step of the step wedges was measured and the values were plotted against the corresponding thickness of aluminum. The curve obtained provided the corresponding aluminium equivalent to the measured optical density of the Hind Limb. In this way an indication of the HLBM was obtained.

2. RESULTS

2.1 PAW VOLUME

An oral administration of isolated compound hesperetin (50 and 100 mg.kg^{-1}) showed a marked inhibition of edema of adjuvant induced chronic arthritic rats and the maximum effect was observed on 28th day and the effect is very similar to the standard drug "Indomethacine". The results are shown in Fig. 2.

3.2 BODY WEIGHT CHANGES

The isolated compound hesperetin (50 and 100 mg.kg^{-1})inhibits the loss of body weight on adjuvant induced arthritic animal than compared to vehicle control animals, when the loss of body weight is predominant and indomethacine could ameliorate the weight loss occurred during arthritis. The results are shown in **Table.1**

3.3 X-RAY ANALYSIS (DENSITOMETRY)

Radiographs were taken in the left hind paw once in a week and this procedure was followed for 5 weeks before and after treatment of isolated compound hesperetin (50 and 100 mg.kg^{-1}) and standard drug indomethacin. Adjuvant – induced group shows severe bone swelling. The bone density changes were evaluated using photodensitometer and quantitative measurements were made using aluminium step wedge and HLBM was calculated. HLBM of rats was increased by Freund's adjuvant as compared to control group. Interestingly the arthritic animals treated with isolated compound hesperetin (50 and 100 mg.kg^{-1}) showed significant reduction in bone

density which is similar to that of standard drug Indomethacin. From the aluminium step wedge we can measure the accurate bone mass density

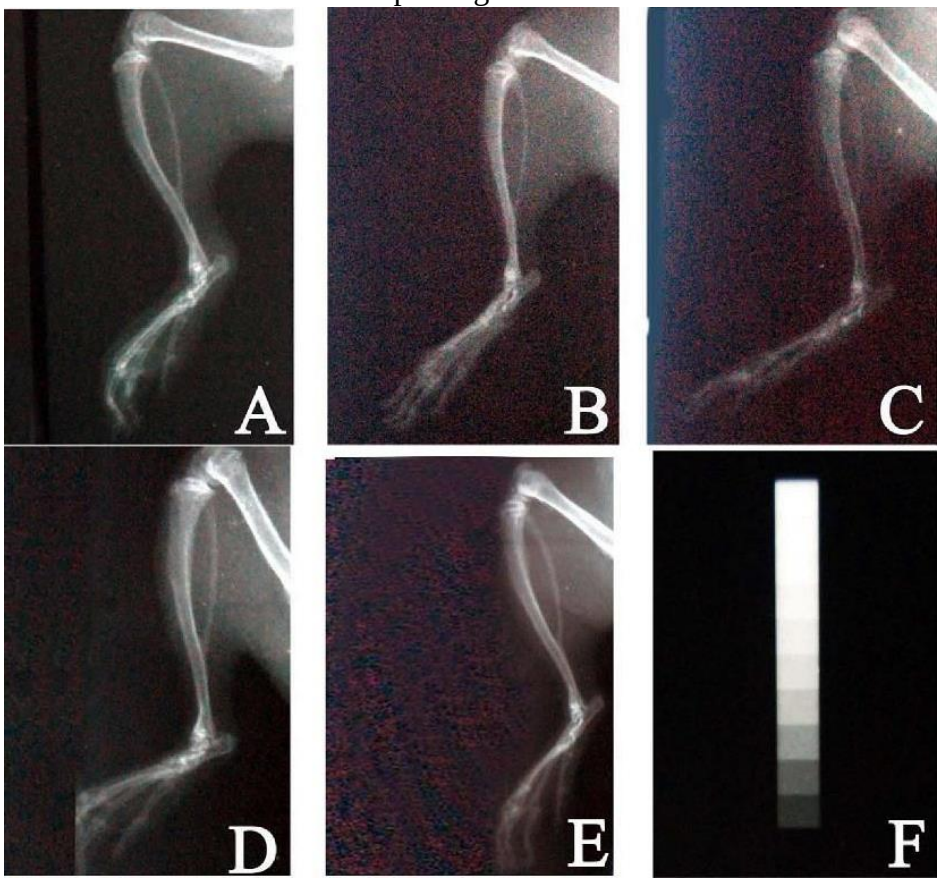

Fig.1 Anti-arthritic effect isolated compound hesperetin on rats measured using optical densitometer of X-ray films on 28th day where the control animals with normal saline (A); Animal induced with adjuvant and treated with standard drug "Indomethacin" (B) ; Animal induced with arthritis using adjuvant (C); Animal induced with adjuvant and treated with 50mg\kg of hesperetin (D); Animal induced with adjuvant and treated with 100 mg\kg of hesperetin (E); Aluminium foil step wedge to measure the optical bone density (F).

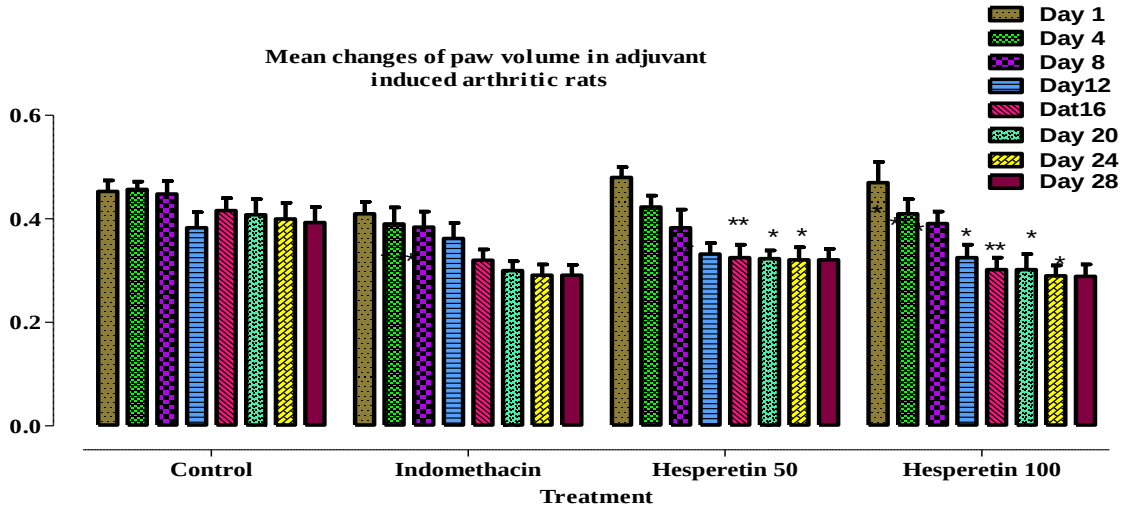

Fig.2.The anti-arthritic effect of isolated compound hesperetin (50 and 100 mg.kg^{-1} body weight) was tested by Freund's adjuvant paw edema in rat. The indomethacin (10 mg.kg^{-1} body weight) was used as a standard drug. The control animal was induced with saline (5 ml.kg^{-1} body weight). The anti- arthritic effect was tested in different time interval such as 1, 4, 8, 12, 16, 20, 24 and 28days. Each value represents mean ± S.E.M, n=6. The statistical analysis was carried out using one way ANOVA method, where **P < 0.01, * P< 0.05

Table. 1: Body weight changes in adjuvant induced arthritis in rats

Group	Mean body weight (gm)		Mean changes in body weight (±SEM)
	Before induction	After treatment (On 28th day)	
Control (Normal saline 5 mg.kg^{-1})	158.6	166.4	8.33±1.667
Standard (Indomethacine 10 mg.kg^{-1})	155.1	195.5	40±2.582**
Hesperetin 50 mg.kg^{-1}	151.2	174.2	23.33±4.595**
Hesperetin 100 mg.kg^{-1}	151.8	162.7	10.83±1.537

Table.1 The anti-arthritic effect of isolated compound hesperetin (50 and 100 mg.kg^{-1} body weight) was tested by measuring the change of body weight. The Indomethacine (10 mg.kg^{-1} body weight) was used as a standard drug. The control animal was treated with saline (5ml.kg^{-1} body weight). The arthritis was induced with 0.1 ml of FCA. The anti-arthritic effect was tested by before induction and after induction of arthritis. Each value represents mean ± S.E.M, n=6. The statistical analysis was carried out using one way ANOVA method, where **P< 0.001**P < 0.01.

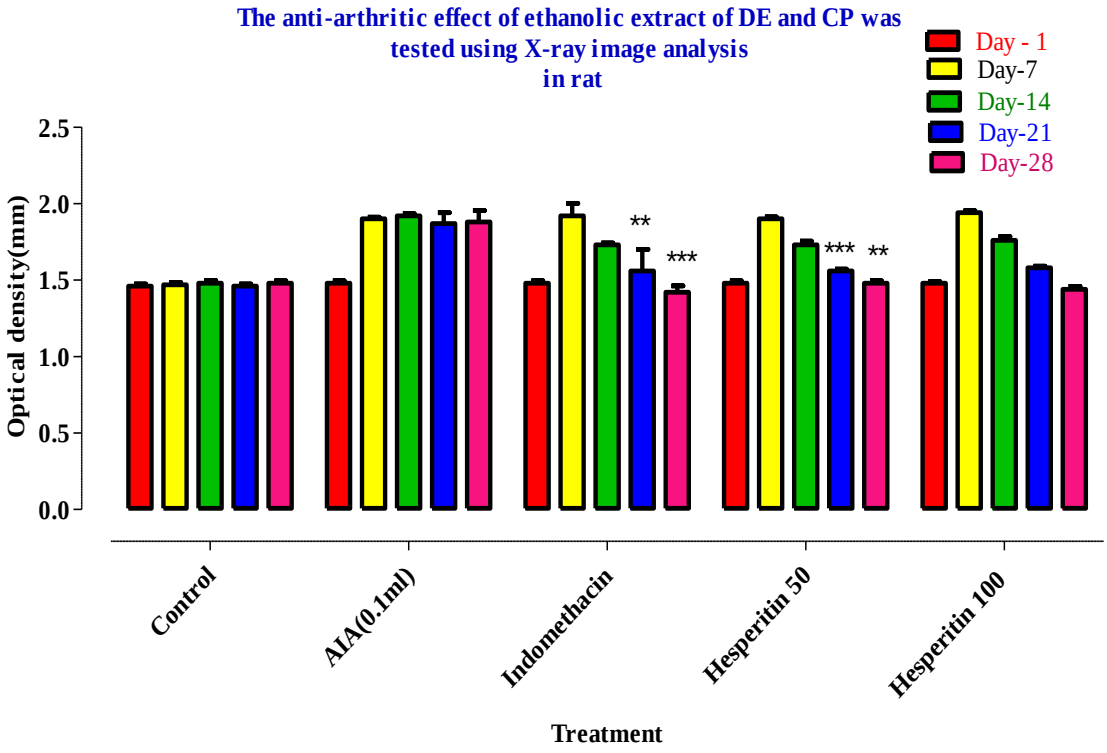

Fig.3.The anti-arthritic effect of isolated compound hesperetin (50 and 100 mg.kg^{-1} body weight) was tested using X-ray image analysis (optical density) in rat. The Indomethacin (10 mg.kg^{-1} body weight) was used as a standard drug. The control animal was treated with saline (5 mg.kg^{-1} body weight). Arthritis was induced with Freund's adjuvant (0.1 ml). The anti-arthritic effect was tested in different time interval such as 1, 2, 3, 4 and 5 weeks. Each value represents mean ± S.E.M, n=6. The statistical analysis was carried out using one way ANOVA method, where ***P < 0.001, **P<0.01 n=6.

Treatment	Day – 1		Day - 7		Day - 14		Day - 21		Day – 28	
	O.D	**Step wedge Thickness**	**O.D**	**Step wedge Thickness**	**O.D**	**Step wedge Thickness**	**O.D**	**Step wedge Thickness**	**O.D**	**Step wedge Thickness**
Control	2.8 0±0.0075	1.7± 0.0248	2.9±0.0.236	1.6± 0.00752	2.8±0.00816	1.8± 0.0075	2.9±0.0236	1.9± 0.0136	2.7±0.0075	1.72±0.0248
Standard	2.6±0.0542	1.6± 0.0075	3.6±0.0816	2.16±0.0.00816***	3.4±0.108	2.13±0.0116***	2.8±0.0075	1.89±00089***	2.5±0.0543	1.68±0.01505***
Adjuvant induced Arthritis	2.9±0.0236	1.76±0.0150	3.8±0.0816	2.17±0.00816***	3.7±0.516	2.11± 0.0116*	3.6±0.0547	2.18±0.0081*	3.5±0.0547	2.18±0.00816**
D.Elata 250mg/kg	2.8±0.0075	1.81±0.0075	3.7±0.0516	2.18±0.00812***	3.2±0.109	2.11 ±0.0116*	2.9±0.0236	1.88±0.0075***	2.6±0.0547	1.71 ±0.0248**
D.Elata 500mg/kg	2.8±0.0.00752	1.72±0.0248	3.7±0.00248	2.16± 0.00814***	3.3±0.108	2.14±0.0082***	3±0.1085	1.86 ±0.015**	2.4±0.0150	1.68 ±0.01505***

Table.2 Anti-arthritic effect of isolated compound hesperetin by X-ray image analysis (optical density Vs Step wedge thickness) The anti-arthritic effect of isolated compound hesperetin (50 and 100 mg.kg^{-1} body weight) was tested by x-ray image analysis technique in rat. The Indomethacine (10 mg.kg^{-1} body weight) was used as a standard drug. The control animal was treated with saline (5mg.kg^{-1} body weight). The arthritis was induced with 0.1 ml 0f AIA. The anti-arthritic effect was tested in different time interval such as 1, 2, 3, 4 and 5weeks. Each value represents mean ± S.E.M, n=6. The statistical analysis was carried out using one way ANOVA method, where **p< 0.001**p < 0.01, * p< 0.5

of control and drug treated groups. There was a statistically significant ($p<0.001$) difference in mean values of densitometry reading was observed and the value was found to be 0.01. The results of present study indicate that the plant extract treatment successfully suppressed the RA induction. The results are shown in Table.2, Fig.1 and Fig.3.

4 DISCUSSION

Arthritis is a chronic inflammatory disorder and the inflammation involves the release of mediators like cytokines (IL-IB and TNF-α), GM-CSF, interferons and PGDF. These are responsible for the pain, destruction of bone and cartilage that can lead to severe disability [18]. The determination of paw swelling is apparently simple, sensitive and quick procedure for evaluating the degree of inflammation and assessing of therapeutic effects of drugs. In the present study, the rat was selected as an animal model since they develop a chronic swelling in multiple joints with an influence of inflammatory cells and followed by erosion of cartilage in joints and destruction of bones. The rat model is a close resemblance to rheumatoid arthritis of human beings [19]. From our study we observed that the isolated compound hesperetin (50 and 100 $mg.kg^{-1}$) and indomethacine significantly suppressed the swelling of the paws of rats. A change in body weight of rats was also measured as one of the parameter to assess the course of the disease and the response to therapy of anti-inflammatory and arthritic drugs [20]. As the incidence and severity of arthritis increased, a decrease in body weights of the rats also occurred during the course of the experimental period and this observation was supported by the findings of C.V.Winder [21] on alterations in the metabolic activities of diseased rats. In addition to the absorption of ^{14}C- glucose and ^{14}C- leucine in rat's intestine was reduced in the case of inflamed rats [22] put on the treatment with anti-arthritic drugs, the decrease in absorption was nullified [23] and it shows that the anti-arthritic drugs correct the decreased/deranged absorption capacity of intestine during arthritis. The increased body weight during treatment of indomethacine and isolated compound hesperetin may be due to the restoration of absorption capacity of intestine. By using image analysis techniques of radiographs, we measured bone swelling, optical density of all groups of tibio tarsal joints of rats using photodensitometer and aluminium step wedge thickness (mm). This method provides a more sensitive and quantitative approach for radiological image analysis as compared with conventional observation. From the study observed a change of bone swelling by measuring OD in terms of aluminium equivalence (mm) after treatment with isolated compound hesperetin and indomethacine treatment. It has been previously demonstrated that these measurements are positively correlated with the results of conventional radiological and histological evaluation [18]. The radiographic analysis of the tibio tarsal joint in arthritic and drug treated animals further supported and confirms the potent anti-arthritic effect of hesperetin (50 and 100 $mg.kg^{-1}$)in a dose dependent manner and it suppress the pathological changes such as pannus formation [24] and bone destruction [25].

5 CONCLUSION

The isolated compound hesperetin has anti-arthritic and peripheral analgesic on acute and possibly chronic inflammatory processes. The claim made by tradipractitioners [26] that hesperetin use to treat various pains and arthritic diseases is found.

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