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BENTHAM SCIENCE

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RESEARCH ARTICLE

Chronic Inflammatory Pain Modulating Potential of Rubiadin: *In-vivo*, *In-vitro* and *In-silico* Investigations

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Abstract:

Background:

The study model of chronic musculoskeletal inflammatory pain, Rubiadin [1,3-dihydroxy-2-methylanthracene-9,10-dione] choice of a drug, aimed to evaluate the anti-hyperalgesic effects.

Objective:

To induce gastrocnemius muscle-stimulated hyperalgesia, 3% carrageenan was injected intraperitoneally.

Methods:

The response to heat and mechanical stimuli was monitored for 9 days. The effect of 1st dose of rubiadin started monitoring after the 14th day of carrageenan injection and continued monitoring until the 22nd day. After the administration of rubiadin intraperitoneally, antihyperanalgesic activity was observed.

Results:

Furthermore, increasing the temperature and mechanical threshold supports histopathological observations with extreme reduction in prostaglandin E2 (PGE2) level.

Conclusion:

The objective is to observe anti-inflammatory and anti-hyperalgesic activity of rubiadin in a pain model that is initiated via supraspinal or spinal neuronal mechanisms, predominantly by inhibition of PEG2. Rubiadin provides a wide range of activities in the treatment of chronic muscle pain and chronic muscular inflammation.

Keywords: Rubiadin, Anti-hyperalgesic activity, Chronic muscle pain, Anti-inflammatroy, *In-silico*, Docking, Cronic musculoskeletal inflammatory pain.

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1. INTRODUCTION

Muscle pain is a globally major symptom and difficult to be encountered with traditional medicaments. Global Burden of Disease (GBD) provides data across a population suffering from muscle pain affiliated issues [1]. To study and understand the pathological mechanisms of chronic inflammation and pain, a number of experimental animal models have been selected over the decades. The experimental animals remain operational field [2, 3]. The various evolutional studies on experimental animals cover a wide range of chronic disorders such as schizophrenia, chronic wounds, neuropathic pain, and chronic inflammation [4 - 6]. In chronic hyperalgesia originated from muscle inflammation in animals, there has been similar relation to human musculoskeletal pain [6].

The plant-derived medicines from traditional medicinal

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plants and Ayurveda haveled tremendous ways to treat chronic pain disease, for example,*Commiphora wightii, Curcuma longa, Zingiber officinale, Tinospora cordifolia,* and *Withaniasomnifera* [7, 8].

In our allopathic medicaments, the therapeutic activity comes along with drug-oriented adverse effects, the short therapeutic window of allopathic medicine responsible for adverse events. To overcome or to provide a wide range of alternative medicines from natural origin, there are imperative requirements to discover and explore medicinal plants, which play a critical role in diseases and provide a wide therapeutical index with minimum or no adverse effects [9].

One of the medicinal plant origin drug molecules is rubiadin; it is an anthroquinone derivative and obtained and extracted from the roots of *Rubia cordifolia* Linn (Family-Rubiaceae) [10]. *Rubia cordifolia* is a medicinal plant which provides a wide range of Ayurveda activities. Rubiadin is an active constituent isolated from *R. cordifolia* medicinal plants, responsible for anti-inflammatory activity [11, 12]. Furthermore, rubiadin possesses antioxidant activity and inhibits lipid peroxidation [13 - 18].

In a recent study Roa *et al*. reported that rubiadin has protective activity against carbon tetrachloride [14]. In another research study Shen *et al* reported that active constituents of *R. cordifolia* L showed moderate anti-inflammatory activity, namely rubiadin, 6-hydroxyrubiadin, alizarin and purpurin [11]. In our line of work, we emphasize chronic pain modulating potential of absolute, isolated and characterized rubiadin in chronic in vivo models in addition to in silico molecular modeling studies to carry forward our current explore (Fig. **1**). Molecular docking provides a supportive technique utilized in our contemporized work [19 - 38] and will also shed more light on the prostaglandin stimulated activity of rubiadin.

2. MATERIALS AND METHODS

2.1. Animals Used

Adult male Wistar rats selected for this study had a weight in the range of 200-340 grams. The animal was situated and maintained in standard environmental conditions and followed by a standard diet and water ad libitum. Food and water were freely available throughout the experiments. A prior approval (Approval number – RCP/18-19/CPCPSEA/P-20) was obtained from the Animal Ethics registered under Committee for the Purpose of Control and Supervision of Experiment on Animals (CPCSEA), Government of India. The experiments were performed according to the ethical guidelines of the International Association for the Study of Pain (Zimmermann, 1983) and the CPCSEA.

2.2. Chemical Agents

The Rubiadin [1,3-dihydroxy-2-methylanthracene-9,10-dione] was purchased from Natural Remedies Pvt. Ltd., Bangalore. The purity of rubiadin was determined by the manufacturer by HPLC area normalization and was certified above 95.0%. Dimethylsulphoxide (DMSO), sodium chloride (NaCl), all from Loba chemicals, were used in this investigation.

2.3. Induction of Carrageenan Cronic Inflammatory Muscle Hyperalgesia

2.3.1. Generation of Chronic Inflammatory Muscle Hyperalgesia by Inducing Carrageenan

10 μL injection of 3% carrageenan in normal saline solution was administered in a left gastrocnemius muscle belly of rats along with mild ether anesthesia. The activity was started after 24 hours of intramuscular administration of carrageenan, and local induction of pain was observed in rats. Some evaluations were performed, namely, heat-oriented paw withdrawal latencies (PWLs) and mechanical stimuli, recorded in all groups; the chronic model was activated after 2 weeks of carrageenan and reduction in PWL (paw withdrawal latency) of the non-carrageenan injected contralateral paw.

2.4. Experimental Dosing of Rubiadin and Standard Medicament Protocol

All groups of animals were treated with carrageenan and observed chronic inflammation, as depicted earlier. The activity of inflammatory drugs was evaluated by mechanical and thermal tests and assured hypersensitivity. Two different groups of animals were treated with different dose regiments, specifically 100 and 200 mg/kg (on the basis of previous studies ref 13, 14) were injected to the test animal via the intraperitoneal route from 2nd to 3rd week of chronic pain.

The dose of herbal medicine rubiadin was administrated twice a day from the 1st week of chronic inflammation and pain. In the experimental standard medicament, namely, Aceclofenac was selected as the drug of choice. Aceclofenac is the 1st line drug of choice in chronic pain and shows anti-inflammatory activity by selectively inhibiting COX-2 inhibitor. The dosing of Aceclofenac was given through the intraperitoneal route in a dose of 10 mg/kg. The treatment was discontinued on the 18th and 19th day and again continued on the 20th day to investigate the possible tolerance developed due to discontinuation. The evaluation was performed in 60-80 min nociceptive responses from the 1st day of treatment. After starting treatment, maximum inhibition of acute pain stimuli was observed. In the group of inflammatory control animals, one group of animals was treated with the standard medicament Aceclofenac and another group of animals was treated with medicament Rubiadin in vehicle dimethyl sulfoxide 0.2 mL intraperitoneally. A control group of healthy animals were kept aside to manage normal control to assess the level of muscle inflammation, histopathological features, and changes in prostaglandin E2 levels. Animals were continuously evaluated using different evaluation methods, specifically PWLs to mechanical and heat stimuli before the generation of inflammation by carrageenan injection and continued conducting till the end of the study. The animal was cured after 22 days of treatment.

2.5. Behavioral Testing for Evaluation of Thermal/heat Hyperalgesia-

The inflammatory hyperalgesia was determined by measuring PWL of carrageenan injected paw by dipping it in the water bath maintained at 47 ± 1 °C. The thermal responses

were monitored thrice in 5 minutes intervals, recorded and averaged data. The cut-off time was considered 15 seconds to avoid irreversible damage to the paw of animals. The PWL of the contralateral paw was analyzed in a chronic model after 60-80 min of drug administration. The heat stimuli were monitored before and after intramuscular injection of carrageenan and continuously performed the test every day until the end of the study.

2.6. Behavioral Testing for Evaluation of Mechanical Hyperalgesia

For performing mechanical hyperalgesia and allodynia, the test animals were placed on an elevated metal grid allowing stimulation of the plantar surface of the paw. Before performing the test, the animal was placed in a test environment for 15 minutes. Mechanical hyperalgesia was determined by performing a series of von Frey nylon hair or filaments (2-20 g), which were applied in increasing order until the rat withdrew their hind paws. Each nylon hair was applied 5 times to a lab animal, and the threshold was taken as the minimum force that caused paw withdrawal was monitored at least 3 times out of 5 consecutive stimuli. The calibration of nylon hair was taken 3 times to avoid errors throughout the experiment. The mechanical responses were monitored at the initial time of the study, after administration of the drug molecules and after the intramuscular administration of carrageenan till the end of the study.

2.7. Measurement of Muscle Circumference

The muscle circumference was monitored after 14 days of inflammation and non-inflamed muscle was monitored as a reference using a measuring tape to verify the threshold of inflammation. The circumference was also measured in subsequent groups before and during the administration of drug molecules to correlate the severity of inflammation and PWL. This test was performed by wrapping a piece of cotton thread around the paw of each rat and measuring the muscle circumference with the help of a metal ruler. On 13 days before the generation of chronic inflammation by intramuscular carrageenan, muscle circumference was measured and continued until the 22^{nd} day.

2.8. Histopathological Studies

The two animals were selected as a control and drug treatment after 2 weeks of carrageenan injection. The ipsilateral knee joints were dissected and fixed in 10% formalin. The dissected muscle was embedded in paraffin. The embedded tissue section was stained with hematoxylin and eosin (H and E) and inspected under a bright field microscope. The descriptive analysis of histological findings was performed and the report was analyzed by a pathologist.

2.9. Measurement of Prostaglandin E-2

2 ml Potassium hydroxide-methanol solution (0.5mol/L) was prepared and mixed with 0.5ml supernatant of inflammatory immersion. The solution was kept at 50 °C for 20 min in a water bath for isomerization. Then 5 ml methanol was added, and the mixture was mixed thoroughly. After mixing, the absorbance of the mixture was measured at 278nm using UV spectrophotometer. The absorbance value was correlated with inflammatory exudate and prostaglandin E-2 (PGE2) concentration.

2.10. *In-silico* Analysis

We validated the *in-silico* predictions of molecular properties and drug-likeness of rubiadin for understanding passive intestinal absorption and brain penetration as a part of drug transportation (lipophilicity) and solubility (hydrophilicity) using the system-generated tool “Molecular Editor v 1.5.1”.

2.11. Statistical Analysis

The statistical data was interpreted by using Graphpad Prism 9 software version 6.01. We generated all data in terms of ± SEM (standard error of the mean). The control value was statistically correlated with statistical significance (P-value < 0.01). In addition, we also performed one-way ANOVA for statistical analysis and treatment with Dunnett’s multiple comparison test.

3. RESULTS

3.1. Effect of Carrageenan Injection on Gastrocnemius Muscle Inflammation-

The intramuscular injection of 3% carrageenan was injected in the left gastrocnemius muscle of the rat to produce inflammation. The inflammation was observed after 24 hours of administration, and the inflammation lasted for 2 weeks in the chronic model. The significant reduction in paw withdrawal latency to heat and mechanical stimuli denoted the generation of inflammation.

3.2. Spontaneous Pain Behavior

The test animals were revealed to have acute pain by guarding the inflamed paw and losing weight bearing capacity on the contra lateral paw in the initial 48 hours. After 48 hours, the pain stimulus was comparatively low except for that continued curling of the paw ipsilaterally which indicated progression of chronic pain and lasted for 2 weeks.

3.3. Effect of Rubiadin on Heat Hyperalgesia-

All groups of animals were placed in the chamber to monitor basal withdrawal latencies; the approximate value was observed around 8.53 ± 0.15 seconds (n = 30). After administration of carrageenan intramuscular injection, the withdrawal latencies were remarkably reduced to 2.95 ± 0.23 seconds. The graphical representation illustrates the effect of carrageenan injection on test animals by heat hyperalgesia in Fig. (**1**).

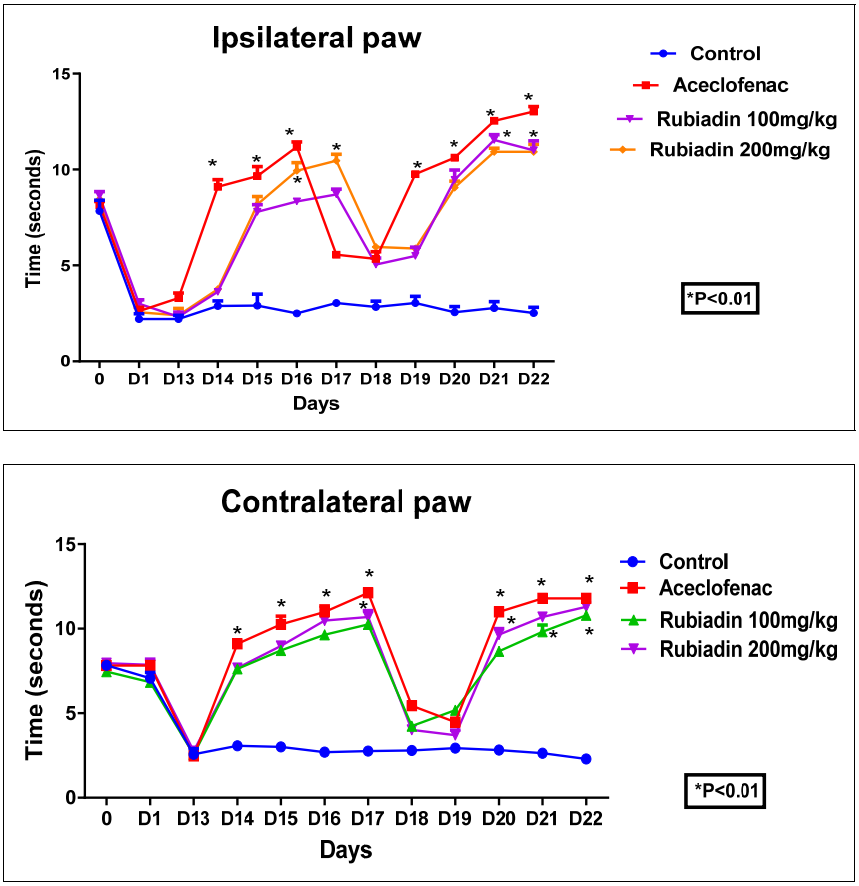

Fig. (1). The comparative effects of Rubiadin on paw withdrawal latency to heat stimuli in chronic inflammatory muscle hyperalgesia on the ipsilateral (**A**) and contralateral side (**B**). Effects of Rubiadin, aceclofenac, and vehicle (inflammatory control) administered post carrageenan injection on thermal hyperalgesia. The mean thermal withdrawal latency (in seconds) was measured for both the paws ipsilateral and contralateral in rats (n = 6 for each group). Each point represents mean ± standard error of mean of the paw withdrawal threshold (in seconds) to stimulation by heat. Data were analyzed by one-way analysis of variance using Dunnett's multiple comparison test. $p < 0.01$ was considered significant as compared with the inflammatory control.

After starting treatment of rubiadin, a consequential reverse in chronic inflammation was observed. Rubiadin has shown activity at the inflammatory site and generated anti-inflammatory activity and analgesic activity and normalized stimuli within 1 hour. The drug molecule avoided spontaneous pain stimuli and heat stimuli, and the state was depicted as hyperalgesia. A significant difference was observed in PWL to heat in different groups of animals, namely, in Rubiadin treated animals. The most positive result was observed in rubiadin treated animals within 60-90 minutes and inflammation started decreasing thereafter. The effect of Rubiadin on chronic hyperalgesia was graphically demonstrated in Fig. (**1**).

3.4. Effect of Rubiadin on Mechanical Hyperalgesia

Rubiadin medicament was injected into the test animals in two suitable doses, namely, 200 mg/kg and 400 mg/kg. The medicament miraculously reduced mechanical hypersensitivity and pain stimuli originated from Carrageenan. The treatment of rubiadin was discontinued from the 2nd day, particularly to the 18th day of carrageenan injection, to initiate mechanical hypersensitivity. The Rubiadin treatment was continued after 2 days, especially on the 20th day. The effect of rubiadin was notably observed on mechanical sensitivity after the continuation of treatment. The reason behind discontinuation in medicament treatment is to monitor the development of tolerance. The effect of rubiadin on thermal hypersensitivity in chronic inflammation is iconographical represented in Fig. (**2**).

3.5. Effects of Rubiadin on Muscle Inflammation-

In the control group, carrageenan induced muscle inflammation in the last for 3 weeks which exhibits an inflammatory response. In Rubiadin treated group noticeably exhibited inhibition of muscle inflammation. The significant reduction in muscle inflammation directly represents anti-inflammatory activity of Rubiadin (Fig. **3**).

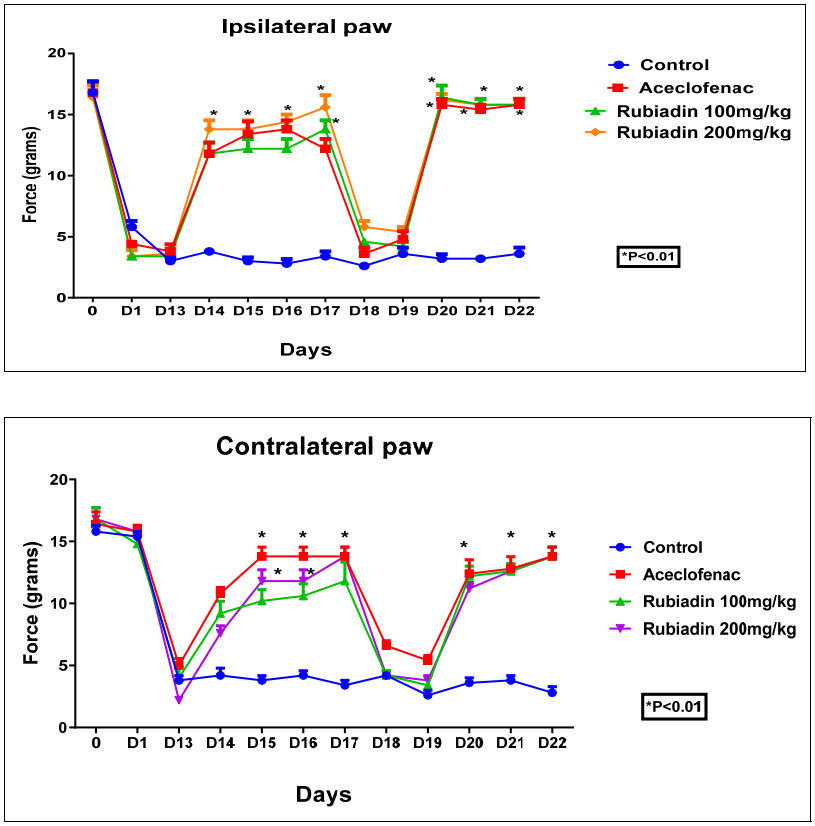

Fig. (2). The comparative effects of Rubiadin on paw withdrawal latency to mechanical stimuli in chronic inflammatory muscle hyperalgesia on the ipsilateral (**A**) and contralateral side (**B**). Effects of Rubiadin, aceclofenac, and vehicle (inflammatory control) administered post carrageenan injection on mechanical hyperalgesia. The mean mechanical withdrawal latency (in grams) was measured for both the paws ipsilateral and contralateral in rats (n = 6 for each group). Each point represents mean ± standard error of mean of the paw withdrawal threshold (in grams) to stimulation by von Frey filaments. Data were analyzed by one-way analysis of variance using Dunnett's multiple comparison test. $p < 0.01$ was considered significant as compared with the inflammatory control.

3.6. Effects on the Concentration of PGE2 Level-

The treatment with rubiadin significantly decreased PGE2 levels in the edema exudates as compared with the control group. The inhibitory potency of rubiadin was also better compared to the control (Fig. **4**).

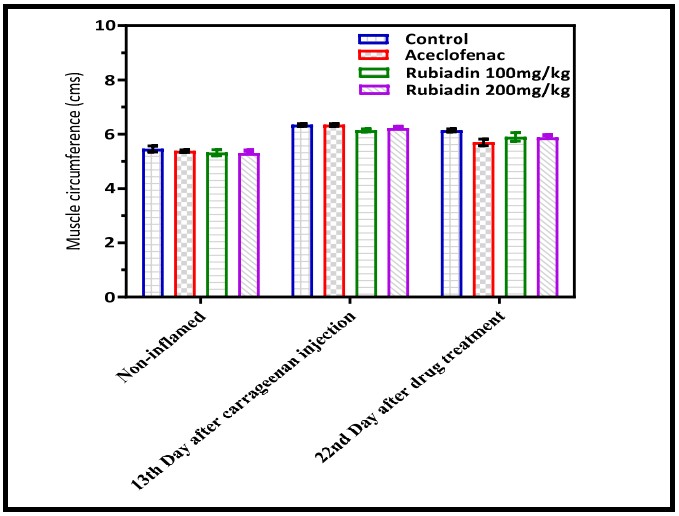

Fig. (3). Effect of Rubiadin (100 and 200 mg/kg), aceclofenac, or vehicle on muscle inflammation in chronic inflammatory hyperalgesia. Muscle diameter was measured only ipsilaterally. Each point represents the mean ± standard error of mean of muscle thickness/diameter (in centimeters) before carrageenan injection (baseline) or at the times (13th and 22nd day) after intramuscular injection of Carrageenan.

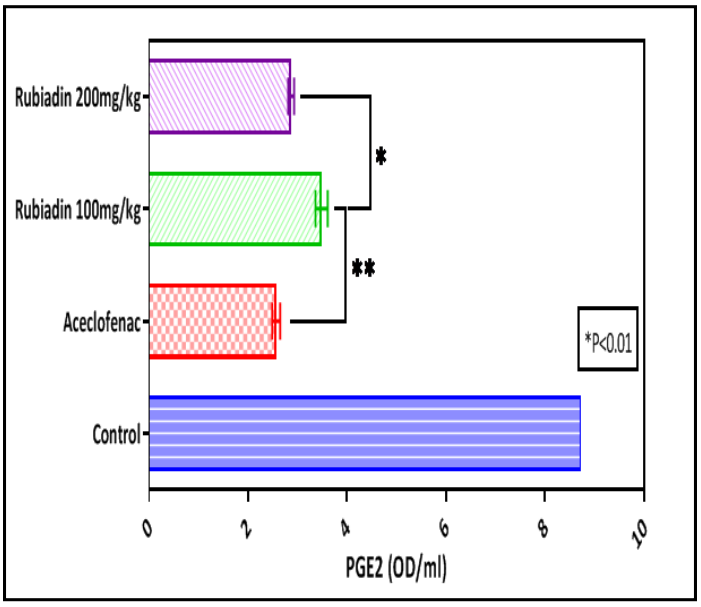

Fig. (4). Effects of Rubiadin on PGE2 concentration in muscle exudates on 22nd day after intramuscular injection of carrageenan in rats. PGE2 concentration was measured only ipsilateral carrageeenan-injected muscle. Each bar represents the mean ± standard error of mean of the PGE2 concentration (in optical density/mL).

3.7. Histopathological Studies

Histological changes were observed throughout the process, and the changes in muscle histology were different in different experimental stages. The histology of Carrageenan injected group animals was changed after the administration of Rubiadin. In Fig. (**5**) pictographic representation of arrows and rectangle foci of muscle necrosis, bunch of cell infiltrates, macrophages in chronic hyperalgesic control and subsequent drug-treated groups under a light microscope (Magnification 40x).

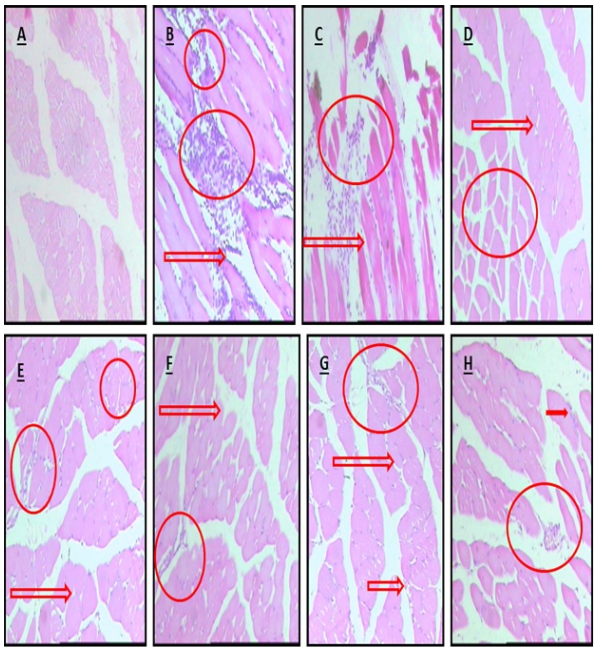

Fig. (5). (**A**) Histological slide of normal gastrocnemius muscle of untreated rat. (**B**) Hemorrhage, edema and inflammatory cell infiltrates mostly neutrophils are seen signifying acute inflammatory response accompanied by myonecrosis. (**C**) Chronic inflammatory response showing primarily the macrophages and few scattered mast cells. (**D**) Aceclofenac decreased the chronic macrophagic response as very few macrophages are seen with no fibrinous exudates. (**E** & **F**)Rubiadin (5 mg/kg) treatment inhibited inhibited the macrophagic response to better extent, while less leukocyte infiltration with was observed. (**G** < **H**) Rubiadin (10 mg/kg) treatment inhibited inhibited the macrophagic response to better extent, while less leukocyte infiltration with was observed.

4. DISCUSSION

4.1. Anti-hyperalgesic Effects of Rubiadin in Chronic Pain Model

In our current research, we performed and monitored the systemic effect of medicament, namely, Rubiadin. For evaluation of the medicament effect, thermal and mechanical hyperalgesia in the chronic model was performed. Interestingly, all major reported data deal with the preventive aspects of hyperalgesia, but to date, nobody has evaluated the effects of Rubiadin on established thermal hyperalgesia in more pronounced spinally mediated muscle hyperalgesia in preclinical settings. Supra-spinal and or spinal neuronal mechanisms play a crucial role in balancing the state of chronic thermal hyperalgesia in the current models of inflammatory muscle pain. On the basis of research outcomes, it is clear rubiadin depicts anti-inflammatory activity against carrageenan induced chronic hyperalgesia.

Our past research clearly evidences that nonselective and COX-2 inhibitors express similar activity against carrageenan induced muscle hyperalgesia. On the contrary, COX-2-selective inhibition was more beneficial in the attenuation of hyperalgesia in the present chronic model as compared with the nonselective COX-2 inhibitors. Spinal COX-2 plays an important role in the maintenance of thermal hyperalgesia induced by Carrageenan [15 - 18]. Thermal and mechanical hyperalgesias are induced in the current pain models via neuronal mechanisms, as an outcome of central and peripheral changes observed at the spinal and superspinal sites. As per histopathological examination, there were no significant contralateral inflammation was observed after rubiadin treatment. The consequence of our current research is to validate and discover the anti-inflammatory and analgesic activity of thermal-originated medicament rubiadin. Moreover, on the basis of tolerance study and histopathological examination, we clearly conclude that rubiadin is a more effective and safer medicament in treatment.

4.2. *In-silico* Methodology (Please refer Supplementary)

Nowadays, molecular docking is playing a crucial role in the process of drug discovery. Varieties of designed/known compounds can be virtually screened through the receptors to identify probable binding modes of compounds or to screen out the hit molecules [19 - 38]. The molecular docking values are based on the predictive potential of the molecules through specific receptor Prostaglandin E Synthase. The 2D and 3D ligand structure of Rubiadin Spectrometric testing methods were suggested.

4.2.1. The Lipinski's' Filter Analysis

Lipinski's filter review indicated that it is a nontoxic compound. While the Lipinski's Filter evaluated the individual compound H-bond donor and acceptor count with its LOGP and Molar Refractivity values as shown in Table **1**.

4.2.2. Possible Inhibition Mechanism of Rubiadin Against Microsomal Prostaglandin E Synthase Type 2 (mPGES-2) (PDB ID: 1Z9H)

As the study showed, a variety of proteins are encoded by the Prostaglandin E Synthase Type 2 (mPGES-2) receptor, we reproduce the gene, a major component of the encoded Prostaglandin E. Crystal Structure and Possible Catalytic Mechanism of Microsomal Prostaglandin E Synthase Type 2 (mPGES-2) were chosen for the present research arachidonate metabolites (PGD2, PGE2, and PGF2) by the action of three groups of enzymes as the docking target for the work. Rubiadin, with the basic moiety name, was discovered through virtual screening as a potent binder to the Prostaglandin E Synthase catalytic pocket. Predicted free binding energy, inhibitory constant, of Rubiadin is -7.9kcal / mol, with Crystal structure of Prostaglandin E Synthase receptor (PDB ID:1Z9H) receptor. Table **2** depicts the unique interactions between the enzyme active site Rubiadin and the highest binding energy compounds.

In the meantime, the inhibitor forms three residue hydrogen bonds near the catalytic binding pocket shown in SI. Fig. (**6**) and Table **2**. Rubiadin with the THR174 amino main A chain atom forms two hydrogen bonds with distances of 9.82 A°. Logically, then, Rubiadin binds to residues on the main interaction, with a site of the hydrogen bond, and thus substantially undermines the binding affinity.

Binding to LEU102, ARG99L, TRY175, ARG13, HOH1, ARG146, IMN379, TYR107, and VAL148 is seen from the crystal structure of Prostaglandin E Synthase in the long loop region (residues 102 to378), isolated from the active sites of mPGES-2 with their respective residues in SI. (Figs. **6** and **7**).

Table 1. Toxicity of Rubiadin was evaluated through Lipinski's filter.

Ligand	Mass	Hydrogen Bond Donor	Hydrogen Bond Acceptors	LOGP a	Molar Refractivity b
Rubiadin	254	2	4	1.578800	64.743385

Note: a) High lipophilicity (expressed as LogP, acceptable range: <5). b) Molar refractivity should be in between 40-130.

Table 2. Molecular docking of Rubiadin and its interactions with the amino acids of PGE receptor.

Dock Energy	Interaction of Amino Acid with Binding Distance									
	LEU D:102	HIS D:377	ARG D:99L	TRY D:175	ARGA:137	HOH A:1	ARG A:146	IMN A:379	TYR A:107	VAL A:148
-9.3	6.28	5.50	5.94	11.42	6.03	1.23	0.73	6.76	1.41	4.61

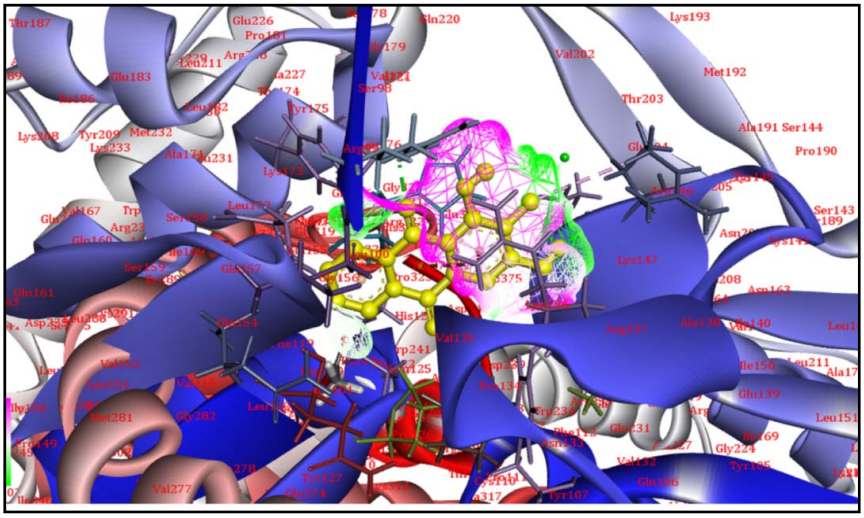

Fig. (6a). 3D dock pose of Rubiadin against 1Z9H receptor.

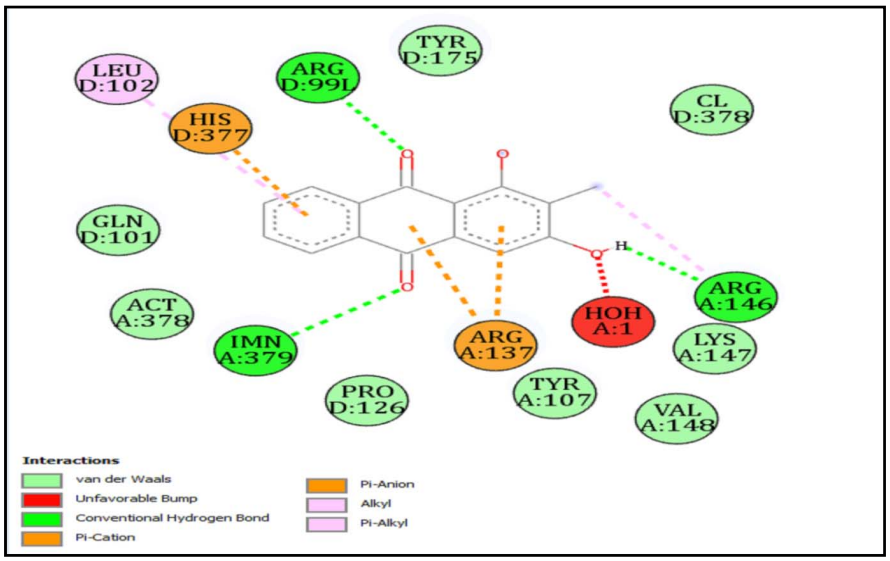

Fig. (6b). 2D dock pose of Rubiadin against 1Z9H receptor.

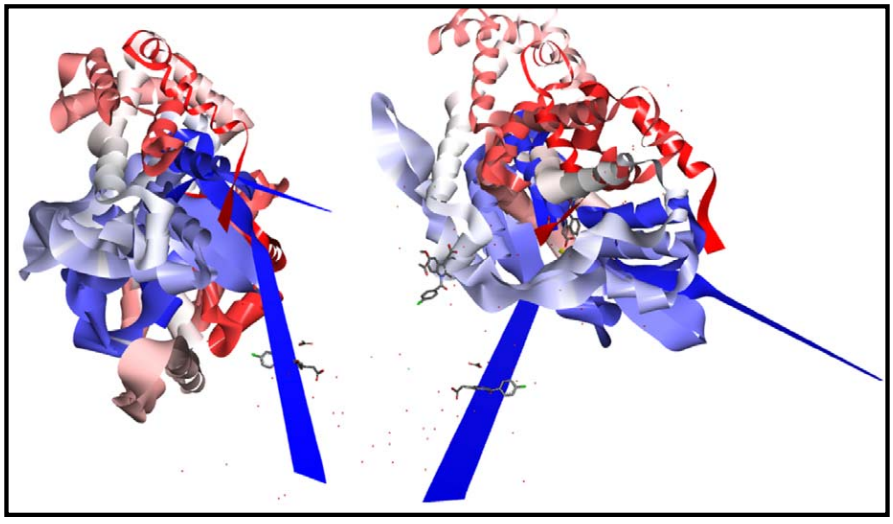

Fig. (7). Crystal structure and possible catalytic mechanism of microsomal prostaglandin E synthase type 2. (mPGES-2) (PDB ID: 1Z9H).

In-silico molecular docking of Rubiadin with Microsomal Prostaglandin E Synthase Type 2 (mPGES-2) (PDB ID: 1Z9H): Docking between selected marked compounds and the targets they reported. A. Prostaglandin E Synthase Type 2 (mPGES-2) receptor as a docking target and Docking is performed with Chimera with autodock Vina, as outlined in Start Method mPGES-2. The files for the protein structure are given in (Table **2**). And the ligand, Rubiadin has the strongest interaction with Prostaglandin E (PDB ID: 1Z9H) receptor crystal structure. The protein domains are shown in the solid ribbon configuration, while the compounds are shown in yellow. The labeled amino acids were those that bind to compounds.

CONCLUSION

To summarize the study model of chronic musculoskeletal inflammatory pain, rubiadin [1,3-dihydroxy-2-methylanthracene-9,10-dione] choice of drug was evaluated for its antihyperalgesic effects. Moreover, our in-silico analysis provided plausible mechanisms. In this context, rubiadin can be explored further via more in-depth in-vivo models for its natural pain-reducing ability.

LIST OF ABBREVIATIONS

GBD	=	Global Burden of Disease
PWLs	=	Paw Withdrawal Latencies

AUTHORS' CONTRIBUTION

VS, MB, PS: conceptualization, methodology, software; AC: conceptualization, methodology, data curation, writing-original draft preparation; AC: visualization, investigation; SM and MB: software, writing- reviewing and editing.

ETHICAL STATEMENT

No humans were used that are the basis of this study. The animal experiments were performed according to the ethical guidelines of the International Association for the Study of Pain (Zimmermann, 1983) and the Animal Ethics registered under Committee for the Purpose of Control and Supervision of Experiment on Animals (CPCSEA), Government of India RCP/18-19/CPCPSEA/P-20).

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

The data of this research will be available from the corresponding author [S.N.M] upon request.

FUNDING

None.

CONFLICT OF INTEREST

Suraj N. Mali is an editorial board member of the journal Current Indian Science.

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SUPPLEMENTARY MATERIAL

Supplementary material is available on the publisher's website along with the published article.

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