RESEARCH ARTICLE

Rutin as an alternative to Paracetamol: Evidence from behavioral, cellular, and inflammatory acute pain responses

Tuhin Bhattacharya[1$], Sabnur Parvage[1$], Kanchan Mukherjee[1], Surajit Sinha[2], Sanjit Dey[1,3,4*]

ABSTRACT

Background: Pain management following acute injury or postoperative procedures is necessary for appropriate recovery and quality of life. Opioids and non-steroidal anti-inflammatory drugs (NSAIDS) have been used for this purpose for an extended period; however, opioids cause addiction and withdrawal symptoms that require additional treatment, whereas NSAIDS have several systemic toxicities. The plant-derived flavonoid rutin has shown promising effects in reducing oxidative stress and inflammation. In this study, our primary objectives are to investigate the antinociceptive and anti-inflammatory effects of rutin as an alternative analgesic with limited adverse effects. *Methods*: Orally administered rutin and its antinociceptive effects were evaluated in a formalin-induced acute pain model in Swiss albino mice. Additionally, the antioxidant and anti-inflammatory effects of rutin were analyzed as preventive and therapeutic strategies in a formalin-induced acute pain model. *Results*: Decreased tail-flick and paw-withdrawal latencies were reversed by preventive and therapeutic Rutin treatment. Rutin treatment depleted reduced glutathione (GSH), reduced catalase and superoxide dismutase 1 (SOD1) activities, and elevated lipid peroxidation at the local level. The levels of the local neuroinflammatory mediator substance P (SP), calcitonin gene-related peptide (CGRP), neurokinin-1 receptor (NK1R), and p65 significantly decreased after Rutin treatment. *Conclusion*: The present evidence shows that rutin is not merely an analgesic but also a comprehensive neuroprotective and anti-neuroinflammatory agent. It corrects the underlying biochemical and cellular pathology that remained unexplored, reiterating the therapeutic efficacy and novelty that fundamentally eclipse the symptomatic relief offered by Paracetamol.

Keywords: Formalin, Rutin, Anti-nociception, Anti-oxidant, Anti-inflammation.

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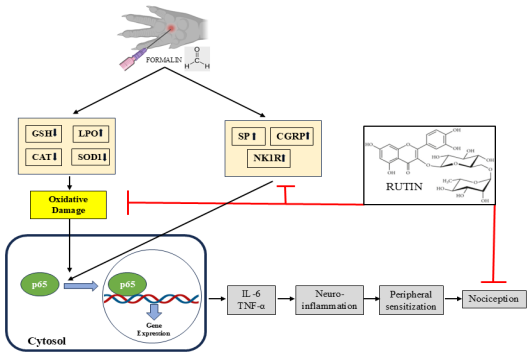

Graphical abstract: Formalin enhanced the oxidative stress and upregulated proinflammatory neuropeptide-mediated events, which enable the translocation of the p65 into the nucleus. This, in turn, induced the inflammatory molecules. The unique chemical structure of rutin scavenges excess reactive oxygen species, neutralizing their lone-pair electrons, thereby showcasing its anti-inflammatory and antinociceptive activities.

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INTRODUCTION

Acute pain signals the organisms to remain aware of the injury and take action to alleviate it, making it essential to address the root cause. Although extensive research supports pain management, the complexity of the origins and effects of pain makes it difficult to develop effective pain relievers without side effects.[1-3]

For many years, practitioners in clinics have administered opioids to treat severe pain. Some opioids, like morphine and opium, have been used for centuries, even though the mainstream use of opioids for pain management started in the 1990s. Efforts to lessen acute, chronic, noncancer, and cancer pain in the late 1990s and early 2000s contributed to the rise in opioid use.[4] Differential use of either delta, mu, or kappa opioid receptors (DOR, MOR, or KOR, respectively) is how morphine or related opioids work. Opioids, however, have a number of negative side effects after extended usage,

including tolerance, dependence, and addiction, as well as withdrawal symptoms upon stopping use.[5] Paracetamol (acetaminophen) is a common NSAID that helps relieve acute pain, reduce fever, and manage related symptoms. Its efficacy in a variety of illnesses is largely responsible for its extensive use. However, there is significant evidence showing the risks linked to long-term or frequent use.[6-8] On the other hand to their reduced toxicity and absence of hallucinogenic effects, plant-derived medications have gained popularity recently. Rutin, a major flavonoid glycoside found in 'rue' (*Ruta graveolens*) and other plants like the buckwheat, passion flower, apple, and tea, has shown promising biological activity.[9,10] It has demonstrated pain-relieving,[11,12] anti-inflammatory, and vasoactive properties, the ability to prevent gastric mucosal ulceration, and a strong capacity to neutralize hydroxyl and superoxide radicals.[13] The antioxidant and anti-inflammatory activities of rutin are well documented. A multitude of beneficial physiological protective phenomena are well known; however, the causal effects are not known exclusively- how rutin is responsible for pain amelioration.

The pain-relieving effects of rutin have not been explored, and its molecular target sites and mechanisms of action remain largely unknown. The formalin test, which triggers a two-phase response in mice, is used to evaluate persistent acute pain.[14,15] Behavioral changes in rodents manifest the initial response to pain and neurogenic inflammation. The most visible changes in paw withdrawal, tail-flick latency, and paw licking are indicative of pain development, whereas the latter response is linked to central mechanisms.[16,17] Formalin induced pain model is effective for examining the impact of medications on one or both phases and the role of ion channels in the response.[18] Thus, the pain-ameliorating activity of rutin can be evaluated using this acute inflammatory pain model. The specific duration of antinociception in the biphasic formalin reaction was also studied.

Rutin, on the other hand, has been explored relatively recently. It has been widely tested for health-beneficial activities.[19] Rutin, owing to its modest bioavailability and C ring, confers exceptional chemical advantage to the acceptor of free lone electrons. Based on the chemistry, it neutralizes formaldehyde or reactive species quite efficiently.[20] Thus, by showcasing its antioxidant role, it confers neuroprotection, hepatoprotection, and other salubrious effects, maintaining cellular and metabolic harmony. Therefore, we identified a range of first-phase causative factors and second-phase downstream effectors of inflammation.

In this study, we examined the dose-dependent anti-inflammatory, antioxidant, and pain-relieving effects of rutin on formalin-induced acute inflammatory pain. This study explored the preventive and therapeutic aspects of pain alleviation. We also investigated the biomolecules involved in developing an effective alternative treatment strategy for acute pain.

MATERIALS AND METHODS

Chemicals

Rutin, thiobarbituric acid (TBA), trichloroacetic acid (TCA), acrylamide-bisacrylamide, bovine serum albumin (BSA), phenyl-methyl-sulfonyl fluoride (PMSF), and Tween 20 were purchased from Sigma-Aldrich (St. Louis, MO, USA). Pre-stained protein markers were procured from Abcam (Cambridge, UK). Primary and secondary antibodies were procured from Cell Signaling Technology (Danvers, Massachusetts, USA). Mouse-specific ELISA kits were obtained from MyBioSource (San Diego, CA, USA). All other reagents were purchased from Merck (Darmstadt, Germany). All reagents were of the highest analytical grade.

Animals and ethical statement

Ten- to twelve-week-old male Swiss albino mice (approximately 20–25 g) were maintained under standard environmental conditions (25 ± 2°C, humidity 50 ± 2%), with a 12 h light-dark cycle and food and water *ad libitum*. The animals were acclimated for at least one week before testing. Animal use and care for this study were approved by the Institutional Animal Ethics Committee (IAEC) of the University of Calcutta, Kolkata, India [IAEC-V/T/SD-12/SABNUR PARVAGE/2024]. For this experimental study using an animal model, the researcher followed the standard Animal Research: Reporting of in vivo Experiments (ARRIVE) guidelines.

Experimental design and induction of acute pain

To manifest the clinical nature of acute pain, 2.5% v/v formalin was administered to the plantar surface of the right hind paw of mice, as described previously.[21,22] To evaluate the effect of rutin, ten- to twelve-week-old male Swiss albino mice (N=3) were orally administered rutin (50, 100, and 150 mg/kg) 30 min before the formalin injection. Based on the experimental data, a 100 mg/kg dose was selected as the optimum dose for further experiments. Oral administration of drugs was performed by oral gavage using a feeding needle following standard protocols.[23] We designed our study as both a preventive and an immediate curative treatment with rutin after formalin administration, since the timing of analgesic administration following injury or noxious stimuli is a significant concern for clinicians, as is the preventive strategy. A total of 36 animals were divided into six groups:

Group 1: Control (20 µL saline in the paw);
Group 2: Formalin (20 µL 2.5% v/v formalin in the paw).
Group 3: Rutin (100 mg/kg Rutin oral administration).
Group 4: R+F (100 mg/kg Rutin, 30 min before 20 µL 2.5% v/v formalin in the paw).
Group 5: F+R (100 mg/kg Rutin, immediately after 20 µL 2.5% v/v formalin in the paw).
Group 6: F+P (100 mg/kg paracetamol, immediately after 20 µL 2.5% v/v formalin in the paw).

Noninvasive hyperalgesic and allodynic behaviors were

noted immediately after treatment completion. 24 hours after treatment, all mice were euthanized in accordance with CCSEA standard guidelines using isoflurane, and hind paws, serum, and spinal L4-L6 segments were isolated and stored at -80°C until further use.

Determination of tail flick latency and paw withdrawal latency

To assess nociceptive responses and determine the antinociceptive activities of rutin, a tail-flick test was performed. The time taken to flick the tail in response to radiant heat was recorded and is represented as tail-flick latency. Three independent tail flicks/mouse were recorded at a given time point, and the average values are presented. The hot plate test assessed the subject's sensorimotor activity during painful stimuli by placing the subject on a metal test plate preheated to 52 ± 1°C. Paw withdrawal latency was recorded as the time elapsed until the subject licked or flicked its hind paw.[24]

Study of paw licking behavior

Mouse paw licking behavior was recorded in phase I (up to 10 min) and phase II (10 min onwards) after formalin infusion. Recorded events were measured as licking per minute for each mouse. Antinociceptive behavior of rutin was recorded in both the first and second phases of formalin-induced nociception, as reported earlier.[25]

Open Field Test (OFT) and Elevated Plus Maze (EPM)

Mice underwent testing in two behavioural setups: a circular open field surrounded by walls and the elevated plus maze (EPM). These tests took place at two different times. Before the experiments, the animals underwent at least 7 days of acclimatization. Each test day, mice spent 1 hour acclimating to the soundproof experimental room next to the housing area. We kept the experimental room at the same conditions as the housing area, including the light-dark cycle, temperature, and humidity.

We recorded the mice's behavior on video and evaluated them using the automated tracking system ANY-Maze™ (Stoelting, USA). In the open field test, the software created track plots to assess exploratory activity, focusing on ambulation (horizontal movement). In the EPM, we analysed occupancy plots to see how long they spent in different areas, indicating anxiety-like behavior. After each trial, we cleaned the equipment thoroughly with 70% ethanol to avoid any olfactory interference.[26]

Antioxidant assay

Estimation of reduced glutathione (GSH) level

The paw homogenate was treated with 0.1 mL of 25% TCA, and the resulting precipitate was obtained by centrifugation at 3900 × g for 10 minutes. Free endogenous sulfhydryl groups were assayed in a total volume of 3 mL by adding 2 mL of 0.5 mM DTNB prepared in 0.2 M phosphate buffer (pH 8) to 1 mL of the supernatant. GSH reacted with DTNB to form yellow complexes. Absorbance was measured at 412 nm.[27]

Catalase activity

To study catalase activity, the absorbance of H_2O_2 was first measured at 240 nm and then by evaluating the decrease in absorbance after the addition of the homogenate, indicating the elimination of H_2O_2 by the action of catalase. Potassium phosphate buffer (50 mM, pH 7.0), 100 mM hydrogen peroxide, and paw tissue homogenate were mixed in a total reaction volume of 1.0 mL. The reaction was then performed at 20°C, and only the initial linear rate of absorbance was used to estimate catalase activity.[28]

Superoxide dismutase 1 (SOD 1) activity

The activity of SOD-1 was estimated by the auto-oxidation of pyrogallol. Tris-cacodylic acid buffer (62.5 mM) was added to the paw tissue homogenate, followed by the addition of 4 mM pyrogallol. To monitor the autoxidation of pyrogallol, the initial absorbance was measured at 420 nm, followed by the absorbance of the test samples at specific time intervals at the same wavelength.[29]

Estimation of lipid peroxidation

The formation of thiobarbituric acid reactive substances (TBARS) in the homogenate was calculated using a standard protocol to determine the tissue levels and cellular stress. Briefly, the homogenate was mixed with TCA (15%), TBA (0.35%), and 5 N HCl, then incubated at 95°C for 15 minutes. After cooling, all mixtures were centrifuged, and the absorbance of the supernatant was measured at 535 nm against an appropriate blank. The amount of lipid peroxidation in each sample was expressed as TBARS in nanomoles/g tissue, estimated using a value of $e = 1.56\times10^5/M/cm$.[27]

Paw edema measurement

Formalin-induced paw edema was measured using a screw gauge and a Vernier caliper. The thickness of the paw dorso-ventrally was measured by the reading from the Vernier caliper at the middle thickest part, and the increase in paw diameter was represented in mm (unit) as a marker of inflammatory edema.[30]

Immunoblot assay

Equal amounts of protein (40 µg) were loaded into each lane for 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a PVDF membrane. The membrane was blocked with a 5% bovine serum albumin (BSA) solution overnight at 4°C. Immunoblotting was performed with primary antibodies against mouse p65 and TNF-α. β-actin and histone 3 (H3) were used as loading controls for the cytosolic and nuclear extracts, respectively. Immunoblots were captured using a Bio-Rad Chemidoc (MP imaging system), normalized, and analyzed using ImageJ (NIH) and Molecular Analyst version 1.5 software (Bio-Rad Laboratories, Hercules, CA). All reagents and chemical manufacturers are listed in the supplementary file.[26]

ELISA from Paw tissue

The levels of Substance P, Calcitonin gene-related peptide (CGRP), Neurokinin-1 receptor (NK1-R), and IL-6 were measured according to the manufacturer's protocols (MyBiosource, USA) with the respective catalog no. MBS8800512, MBS8800040, MBS7606597, and MBS2023471 from the paw tissue lysate.[26]

Data analysis and statistics

All data are presented as the mean ± standard deviation. The significance of differences between the treated and untreated groups was assessed using one-way analysis of variance (ANOVA) followed by Dunnett's test in GraphPad Prism 8. Statistical significance was set at $p < 0.05$.

RESULTS

Optimum antinociceptive dose of rutin against formalin-induced acute pain

Formalin-induced tail-flick latency(sec), paw withdrawal latency (sec), and paw licking (events/min) behavior were measured to establish the hyperalgesic threshold and antinociceptive activity. First, dose-dependent studies (50, 100, 150 mg/kg) of rutin were performed for optimal dose selection, and significant improvements were observed both in the first phase and second phase of tail flick latency (Figure 1A & 1B). Paw withdrawal latency in phase I was significantly ameliorated (Figure 1C). Paw withdrawal latencies in the second phase of the formalin reaction showed similar effects (Figure 1D). Paw licking events were significantly ameliorated in Rutin treatments as compared to formalin injection (Figure 1E). These results showed that the 100 mg/kg dose of rutin was the most effective antinociceptive dose, and the effects plateaued beyond this dose. Hence, 100mg/kg Rutin was selected as the standard dose.

Rutin alters nociceptive behavioral pattern after acute pain induction

Formalin-induced hyperalgesic behaviors were evaluated by challenging the neuroinflammatory onset with preventive and therapeutic intervention of rutin. Paracetamol was used as the control in this set of experiments. Formalin (2.5% v/v) administration decreased tail-flick latency(sec) (TFL) and paw withdrawal latency(sec) (PWL) in both phase I and 2. Preventive treatment of rutin exerted its antihyperalgesic activity in both phase I and phase II, whereas curative treatment of rutin and Paracetamol showed anti-hyperalgesia in phase II of formalin administration (Figure 2A, 2B, 2D & 2E). Formalin injection significantly elevated the paw licking events/min count. Rutin exerted its antinociceptive activity by lowering paw licking events both preventively and curatively, like Paracetamol (Figure 2C).

Rutin ameliorates restricted locomotion and anxiogenic behaviour after acute pain.

Locomotor activity in animals is typically suppressed under painful conditions. To evaluate the effect of rutin on pain-induced locomotor deficits, the open field test (OFT) was used. Important parameters, including distance travelled and mean speed, were measured through monitoring the animals' movements in a circular open field both before and after treatment. Formalin administration significantly reduced locomotor activity, indicating nociceptive-induced behavioral suppression. However, treatment with rutin effectively restored locomotion, both when administered preventively and therapeutically, demonstrating efficacy comparable to that of Paracetamol (Figure 3C). These findings were also substantiated by track plots, which visually confirmed the reversal of formalin-induced hypoactivity upon treatment with Rutin (Figure 3A). To evaluate the potential anxiolytic effect of rutin, the elevated plus maze (EPM) test was utilized. In this test, the time spent in the open arms served as an index of anxiety-related behavior, with values measured and recorded both before and after treatment. Formalin administration resulted in a marked reduction in time spent in the open arms, indicating increased anxiety. However, treatment with rutin significantly increased open arm exploration in both preventive and therapeutic paradigms. This increase in open arm time indicates a robust anxiolytic effect of rutin, comparable to that of Paracetamol (Figure 3D). These behavioral findings were

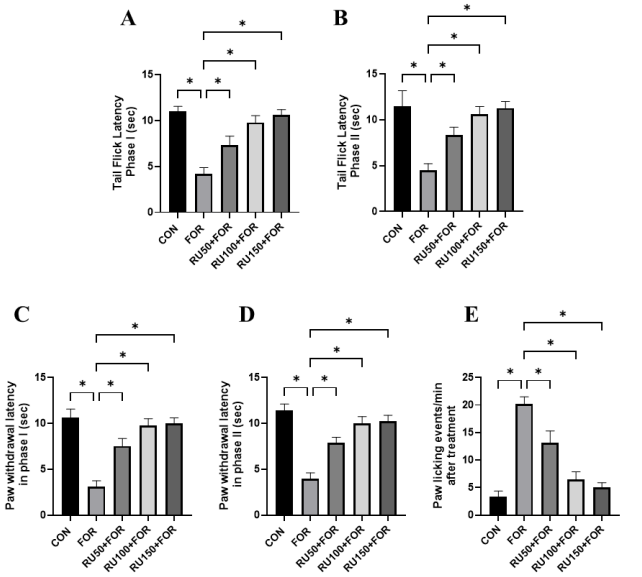

Figure 1: Effective dose of rutin in assessment of pain sensitivity. (A) Tail flick latency (measured in seconds) in Phase I after formalin and increasing rutin treatment. (B) Tail flick latency (measured in seconds) in Phase II after formalin and increasing rutin treatment. (C) Paw withdrawal latency (measured in seconds) in Phase I after formalin and increasing rutin treatment. (D) Paw withdrawal latency (measured in seconds) in Phase II after formalin and increasing rutin treatment. (E) Paw licking events (events/per minute) after formalin and increasing rutin treatment. Formalin was injected in the plantar region at a dose of 2.5% v/v bearing a volume of 20µl. Phase I denotes up to 10 mins of time window from formalin injection. Rutin was administered orally at a dose of 50, 100 and 150 mg/kg dose. Mean ± SD data were represented after One-way ANOVA followed by Dunnett's post hoc analysis. (n=3, $p<0.05$, * indicates significant difference with formalin treated group).

further corroborated by heat map analyses, which showed enhanced occupancy in the open arms following Rutin treatment (Figure 3B).

Rutin neutralizes formalin induced alteration in oxidative milieu

To determine whether Rutin treatment quenched formalin-induced oxidative stress in paw lysates, the antioxidant activity was measured. Reduced glutathione (GSH) content (measured in µmoles/mg of protein) showed elevated levels in both the Rutin treatment and paracetamol treatment in comparison to formalin only treated group(Figure 4A). Superoxide Dismutase 1 (SOD1), a metalloprotein that protects cells from oxidative damage, also showed exaggerated activity (measured in U/mg of protein) on rutin and paracetamol treatments compared with formalin treatment (Figure 4D). Catalase (CAT) activity (measured in µmol of H_2O_2 reduced/mg of protein), which is crucial for the neutralization of reactive oxygen species (ROS) through H_2O_2 decomposition, also increased upon treatment with rutin and Paracetamol, and was significantly greater than that in mice treated with formalin (Figure 4C). Thio-barbituric acid reactive substrates (TBARS), which are formed as a side product of lipid peroxidation (LPO), did not increase after treatment with rutin or Paracetamol compared to the formalin-treated groups (Figure 4B).

Rutin neutralizes the altered inflammatory milieu after formalin injection.

The redox imbalance promoted by formalin injection triggers an inflammatory cascade, leading to the release of several proinflammatory mediators in the local tissue and systemic circulation. The neurotransmitter substance P is important for the transmission of pain information to the central nervous system and is known to generate an inflammatory pain response. Levels of substance P(pg/mg) in the paw after formalin treatment were significantly increased with respect to the control mice and were restored to the normal levels in rutin and Paracetamol-treated mice (Figure 5A). Neurokinin-1 (NK-1) receptor expression (pg/mg)

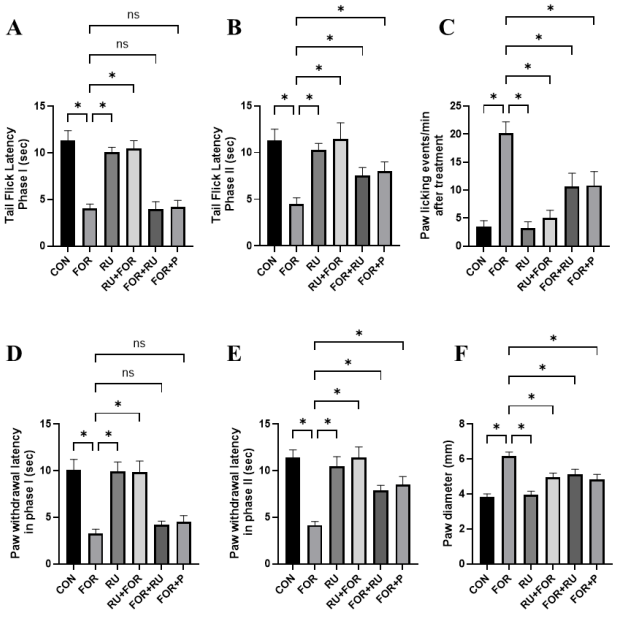

Figure 2. Pain sensitivity and inflammation assessment: (Tail flick test, Paw licking events, Hot plate test) and Paw diameter measurement. (A) Tail flick latency (measured in seconds) in Phase I after formalin and rutin treatment. (B) Tail flick latency (measured in seconds) in Phase II after formalin and rutin treatment. (C) Paw licking events (events/per minute) after formalin and rutin treatment. (D) Paw withdrawal latency (measured in seconds) in Phase I after formalin and rutin treatment. (E) Paw withdrawal latency (measured in seconds) in Phase II after formalin and rutin treatment. (F) Paw diameter (measured in mm) after treatment. Formalin was injected in the plantar region at a dose of 2.5% v/v, bearing a volume of 20µl. Phase I denotes up to 10 mins of the time window from formalin injection. Rutin was administered orally at a dose of 100 mg/kg. Paracetamol was orally administered at a 100mg/kg dose. Mean ± SD data were represented after One-way ANOVA followed by Dunnett's post hoc analysis. (n=6, p<0.05, * indicates significant difference with formalin treated group).

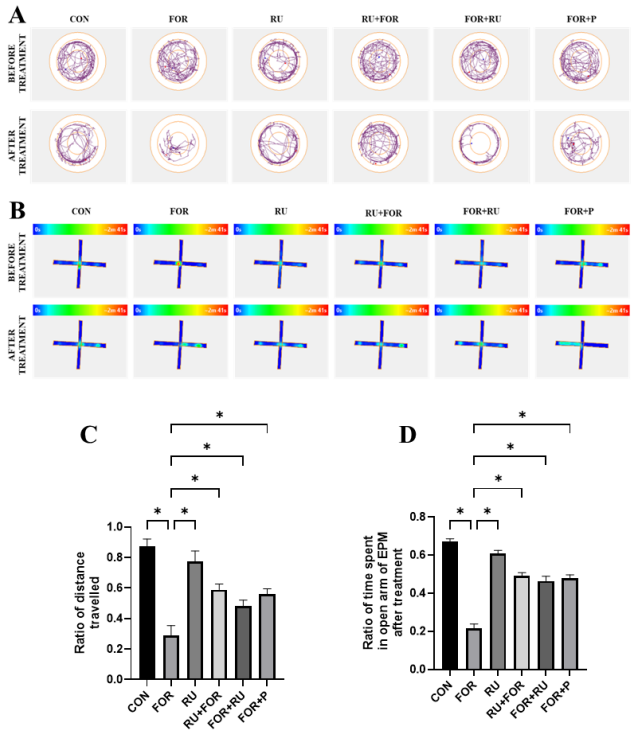

Figure 3: Locomotor activity and anxiety-like Behaviour study in open field and elevated plus maze. (A) Track plot of an animal showing locomotor activities before and after treatment in a circular open field. (B) Heat map showing the movement of animals in the elevated plus maze before and after treatment. (C) Ratio of distance travelled (measured in meters) before and after formalin and rutin treatment. (D) Ratio of time spent in the open arm of the elevated plus maze, before and after formalin and rutin induction. Formalin was injected in the plantar region at a dose of 2.5% v/v, bearing a volume of 20µl. Rutin was administered orally at a dose of 100 mg/kg. Paracetamol was orally administered at a 100mg/kg dose. Mean ± SD data were represented after One-way ANOVA followed by Dunnett's post hoc analysis. (n=6, *p* <0.05, * indicates significant difference with formalin treated group).

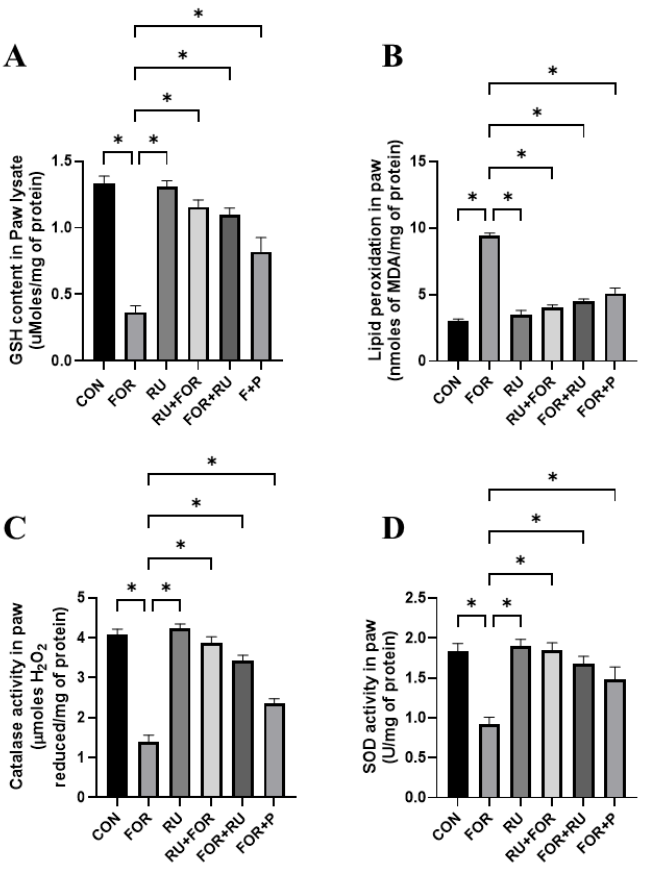

Figure 4: Antioxidant status in paw lysate post sacrifice (24 hours). (A) Reduced glutathione levels (GSH, measured in µmoles/mg of protein). (B) Lipid Peroxidation (LPO, measured as nmoles of TBARS/ mg of protein). (C) Catalase activity (measured in µmoles of H_2O_2 reduced/ mg of protein). (D) Superoxide Dismutase 1 activity (SOD 1, measured in U/ mg of protein). Formalin was injected in the plantar region at a dose of 2.5% v/v, bearing a volume of 20µl. Rutin was administered orally at a dose of 100 mg/kg. Paracetamol was orally administered at a 100 mg/kg dose. Mean ± SD data were represented after One-way ANOVA followed by Dunnett's post hoc analysis. (n=6, p <0.05, * indicates significant difference with formalin treated group).

showed a trend similar to that of SP at the local level (Figure 5B). On the other hand, calcitonin gene-related peptide (CGRP), which plays a key role in pain modulation, was significantly decreased by rutin and paracetamol treatment (Figure 5C). Inflammatory edema after formalin injection was measured using a screw gauge. Paw diameter (mm) did not increase in rutin and paracetamol-treated groups after formalin administration (Figure 2F). Substance P can induce p65 activation, its nuclear translocation, and downstream upregulation of proinflammatory cytokines, such as IL-6 and TNF-α. Western blot analysis of paw tissue samples showed increased expression of these inflammatory markers after formalin treatment, and these levels were lowered by the administration of rutin and Paracetamol. The levels of nuclear p65 were significantly downregulated in the rutin- and paracetamol-treated groups compared with those in the formalin-only treatment group (Figure 6 and Figure S1).

DISCUSSIONS

The present study demonstrated that formalin-induced inflammatory pain in mice was effectively prevented or alleviated by dietary rutin. The novelty of our study is the unique strategy using both preventive and therapeutic approaches, which gives an additional edge in understanding the administration of rutin in the treatment regimen as an oral antinociceptive agent. In our experimental groups, we found that the formalin-injected mice experienced significantly more pain, thus showing hypersensitivity. Moreover, inflammation-inducing parameters were enhanced compared with those in the control group. This model effectively mimicked acute inflammatory pain. The formalin-induced acute pain model is widely used to investigate the pain-relieving activity of various drugs in rodent models.[31] The injection of formalin into the paw resulted in two distinct phases. The first phase, lasting up to 10 min, is characterized by nocifensive behaviors such as paw licking, lifting, and flinching. This followed a second phase lasting up to 60 minutes.[32,33] Several local pain-relievers, including morphine and lidocaine, have been shown to effectively reduce pain in both phases of a formalin-induced pain model.[34,35] When formalin is injected into the paw, it immediately triggers inflammation by activating certain ion channels and releasing neuropeptides such as substance P, CGRP, and neuropeptide Y.[25,36,37] Previous research on rodent models of inflammatory pain has revealed decreased antioxidant activity and increased reactive oxygen species (ROS). This was evidenced by an increase in lipid peroxidation, a process marked by the formation of malondialdehyde (MDA), a marker of inflammatory pain. Additionally, reduced glutathione levels, an important antioxidant, were lower both locally and in the spinal cord. These changes helped drive the progression from neurogenic inflammation (Phase I)

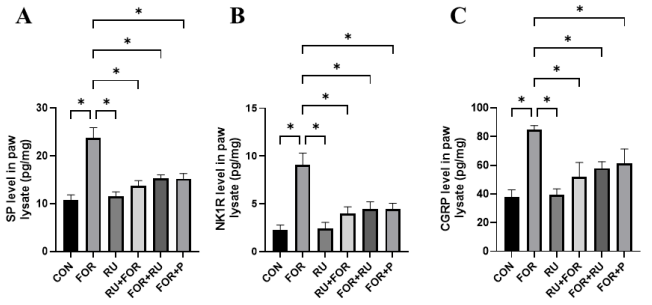

Figure 5: Evaluation of neuroinflammatory markers in paw lysate post-sacrifice (24 hours). (A) Substance P levels (measured as pg/ mg of protein) in paw tissue after treatment. (B) Neurokinin-1 receptor (NK1R) levels (measured as pg/ mg of protein) in paw tissue after treatment. (C) Calcitonin gene-related peptide (CGRP, measured as pg/mg of protein) in paw tissue after treatment. Formalin was injected in the plantar region at a dose of 2.5% v/v, bearing a volume of 20 µL. Rutin was administered orally at a dose of 100 mg/kg. Paracetamol was orally administered at a 100 mg/kg dose. Mean ± SD data were represented after One-way ANOVA followed by Dunnett's post hoc analysis. (n=6, p <0.05, * indicates significant difference with formalin treated group).

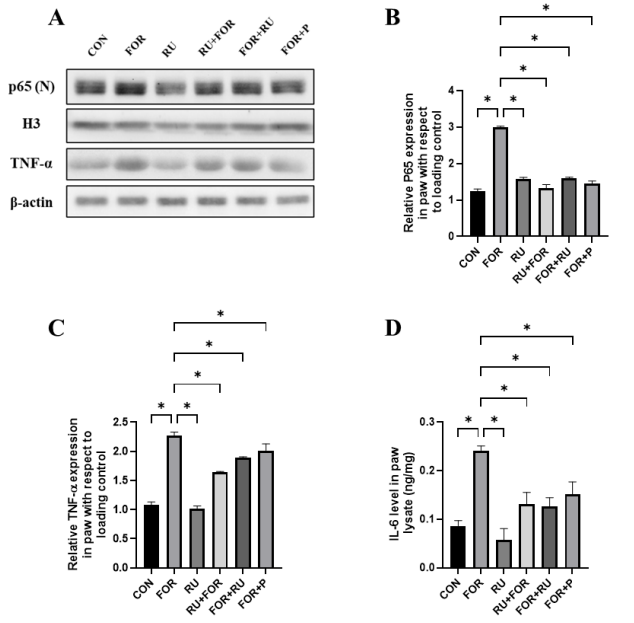

Figure 6: Expression of inflammatory markers from paw tissue post-sacrifice (24 hours). (A) Immunoblot of p65(nuclear), H3, TNF-α, and β-actin after formalin and rutin treatment. (B) Relative densitometric analysis of the nuclear fraction of p65 with respect to H3 after treatment. (C) Relative densitometric analysis of TNF-α expression with respect to β-actin (D) IL-6 levels (measured as ng/ mg of protein) in paw tissue. Formalin was injected in the plantar region at a dose of 2.5% v/v, bearing a volume of 20µl. Rutin was administered orally at a dose of 100 mg/kg. Paracetamol was orally administered at a 100 mg/kg dose. Mean ± SD data were represented after One-way ANOVA followed by Dunnett's post hoc analysis. (n=6, p <0.05, * indicates significant difference with formalin treated group)

to central sensitization (Phase II). We worked on the two hit mechanisms of the pain generation hypothesis. One is the first phase, with rapid entry of calcium and other ions. This influx of cations is only possible once the membrane and channels undergo structural alterations by formalin at the site of the paw tissue where it is injected.[38] Formalin is a tiny aldehyde with powerful free lone pairs of electrons in oxygen, which can easily form bonds with the unpaired electrons of neighboring molecules. Thus, membrane lipids and their associated proteins are affected, triggering tissue injury.[39,40] Thus, events downstream of the recruitment of vasoactive elements, the release of proinflammatory cytokines, and the inhibition of chemoattractant receptors remain hallmarks of the onset of central sensitization and neurogenic inflammation.

An increased response to touch, known as tactile allodynia, was observed in both phases after formalin injection, confirming heightened pain sensitivity and allodynia. Spontaneous neurophysiological behaviours, such as decreased locomotion, and the anxiogenic effect of formalin-mediated pain were also significantly downregulated by Rutin treatment in our present study. Rutin, a plant compound, has been found to reduce pain in several rodent models, including those induced by glutamate, oxaliplatin, formalin, and diabetic neuropathy.[41,42] When inflammation occurs, cells in the area release a series of proteins called cytokines, including IL-1β, IL-6, and TNF-α, which contribute to the development of inflammatory pain.[43]

On this front, Paracetamol is a well-established analgesic drug with strong pain-alleviating efficacy, as shown in our study too. Although very effective, Paracetamol, after being metabolized by hepatic cytochrome P450s, generates a highly reactive electrophilic intermediate, N-acetyl-p-benzoquinoneimine (NAPQI). Elevated levels of NAPQI deplete reduced glutathione and enhance lipid peroxidation, cumulating to oxidative damage to hepatic tissues.[44] This significant effect of Paracetamol on hepatocellular injury is evident from our study of ALT and AST from serum samples (Figure S2). On the bright side, Rutin treatment has not only been shown to effectively reduce pain in a dose-dependent manner, with the most effective dose being 100 mg/kg, but also to avert the significant hepatic injury observed with paracetamol treatment.

At this dose, rutin significantly reduced tail-flick and paw-withdrawal responses in the second phase after formalin injection, both as a preventive and a therapeutic treatment, similar to the pain relief provided by Paracetamol. This suggests that rutin effectively relieved pain in a formalin-induced inflammatory pain model. Additionally, Rutin treatment reduced immediate pain behavior, as evidenced by reduced paw licking.

However, a major intriguing question is how rutin ameliorates pain. Neurobehavioral studies have revealed that pain-evoked symptoms can be alleviated by rutin. From the current set of evidence, it may be inferred that rutin prevents or cannot promote any formalin-initiated cellular mechanisms to prompt further action. Therefore, the presence of rutin mitigated these potential threats. We have the conjecture, based on the chemical characteristics of rutin, that the potential electron-sinking role inherent in its structure has subverted subsequent molecular interactions.[45,46] Moreover, rutin remains effective in a physiological state for a long time because it is slowly absorbed.[47] This facilitates the physiological effects of rutin on membrane damage compared to Paracetamol. This rescue event or protection prepares for the initiation stage of the second phase.

The second phase involves canonical calcium- and second-messenger-driven cell signaling to prepare the cell and associated tissues to mount an alarm response to a threat. Thus, we found that formalin-treated animals exhibited p65 nuclear translocation, increased substance P and CGRP levels, and enhanced NK1R expression. Enhanced TNF-α and IL-6 levels were observed, corroborating the early proinflammatory events. Whether rutin binds to all or only part of the upstream regulators is an interesting and unaddressed question. It is likely that rutin scavenges reactive oxygen species by donating electrons to superoxide and hydroxyl radicals. It also inhibits different ROS-producing enzymes, thereby preventing inflammatory events triggered

by free radicals.[13,46] Instead, Paracetamol lacks the ability to scavenge superoxide free radicals *in-vivo*.

NF-κB, a key mediator of inflammation, is activated by the phosphorylation of IκB, which leads to phosphorylation, followed by the nuclear translocation of the transcription factor.[48] The nuclear level of NF-κB was significantly upregulated by formalin treatment, and this upregulation was significantly inhibited by rutin treatment compared with paracetamol treatment. The binding of transcription factors activates downstream proinflammatory cytokines such as IL-6 and TNF-α. The local tissue levels of TNF-α and IL-6 were upregulated in the inflammatory pain group, and this upregulation was significantly inhibited by rutin treatment, as evidenced by reduced TNF-α and IL-6 levels (Figure S3).

In conclusion, the current study demonstrated that dietary rutin efficiently prevented or alleviated formalin-induced inflammatory pain. The neurogenic inflammatory effects of formalin, which involve neuropeptide-and oxidative stress-induced damage in local paw tissue, were significantly reduced by rutin. This was evident from modulation of inflammation-initiating agents and oxidative stress markers, including substance P, CGRP, NK1R, lipid peroxidation, GSH levels, catalase activity, and SOD1 activity. Although there are several occasions when preventive Rutin treatment than by Paracetamol more effectively demonstrated the anti-inflammatory, antinociceptive, and alterations in physiological spontaneous behavior, there are some events where Paracetamol proved to be better at the given dose. Thus, the present study enhances our understanding of formalin-induced acute pain pathogenesis and highlights the potential of dietary rutin in the development of new treatments for pain conditions, as well as other inflammatory response

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DATA AVAILABILITY

Data supporting the findings of this study are available from the corresponding author upon reasonable request.

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PEER-REVIEWED CERTIFICATION

During the review of this manuscript, a double-blind peer-review policy has been followed. The author(s) of this manuscript received review comments from a minimum of two peer-reviewers. Author(s) submitted revised manuscript as per the comments of the assigned reviewers. On the basis of revision(s) done by the author(s) and compliance to the Reviewers' comments on the manuscript, Editor(s) has approved the revised manuscript for final publication.

SUPPLEMENTARY DATA FILE

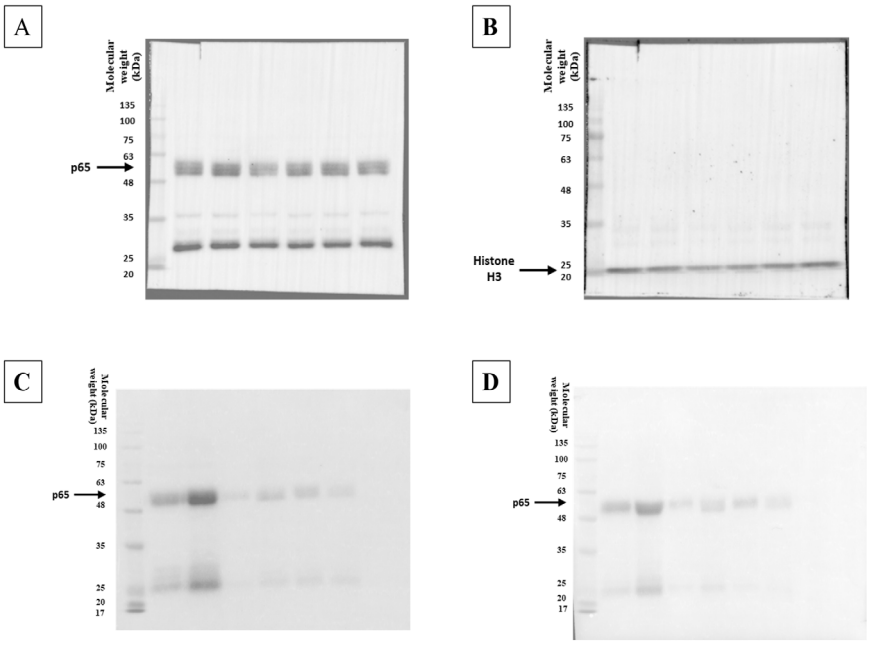

Figure S1: Raw full-length immunoblot of nuclear p65 expression. **(A)** Uncropped full-length Western blot image showing nuclear p65 protein levels (first set). **(B)** The same membrane shown in (A) after stripping and reprobing with Histone H3, which served as the nuclear loading control. **(C–D)** Full-length immunoblot images representing the second and third independent sets of nuclear p65 experiments, respectively. Molecular weight markers (kDa) are shown on the left. No selective image processing was performed other than uniform adjustment of brightness and contrast applied to the entire image. Formalin was injected in the plantar region at a dose of 2.5% v/v bearing a volume of 20µl. Rutin was administered orally at a dose of 100 mg/kg. Paracetamol was orally administered at 100mg/kg dose. Mean ± SD data were represented after One-way ANOVA followed by Dunnett's post hoc analysis. (n=6, $p<0.05$, * indicates significant difference with formalin treated group).

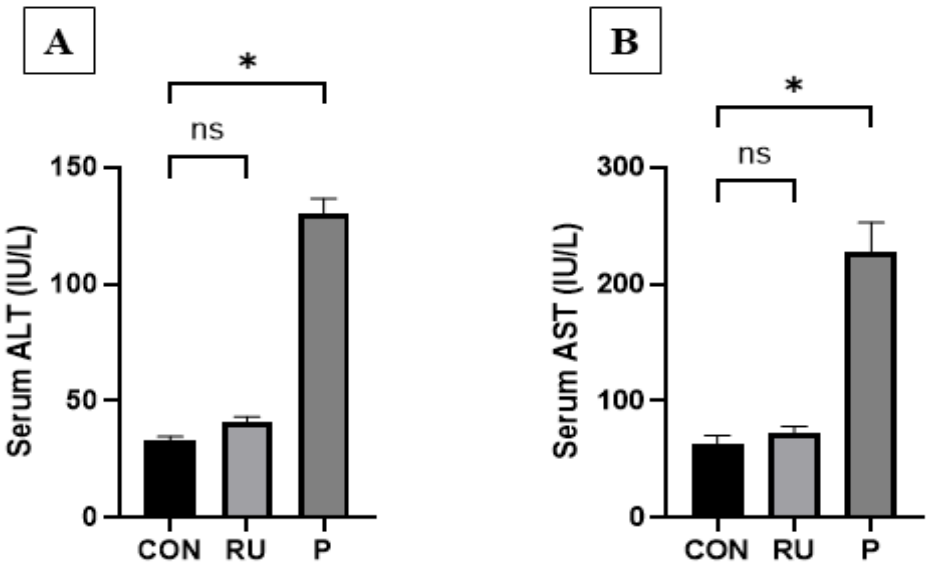

Figure S2: Representative graphs of serum ALT and AST levels **(A)** Representative graphical presentation of serum alanine aminotransferase (ALT) levels. **(B)** Representative graphical presentation of serum aspartate aminotransferase (AST) levels. Rutin and Paracetamol were administered orally at a dose of 100 mg/kg. Mean ± SD data were represented after One-way ANOVA followed by Dunnett's post hoc analysis. (n=3, $p<0.05$, * indicates significant difference with formalin treated group).

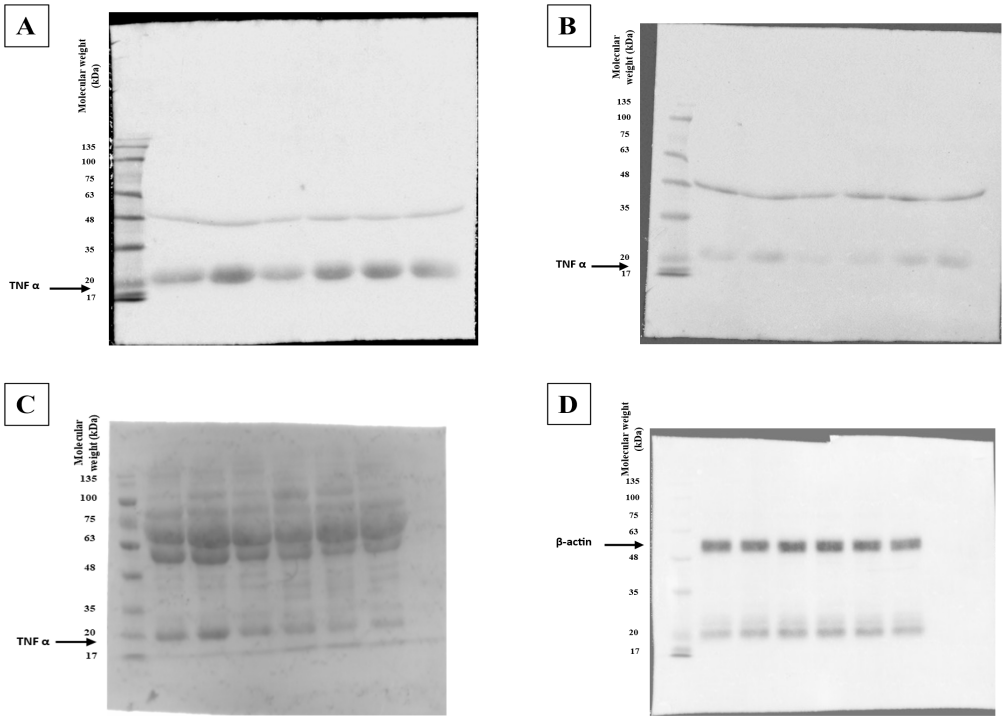

Figure S3: Raw full-length immunoblots of TNF-α expression. (A–C) Uncropped and unprocessed full-length Western blot images representing three independent experimental sets of TNF-α protein expression. (D) Corresponding β-actin immunoblot serving as the loading control. Molecular weight markers (kDa) are shown on the left. No selective image processing was performed other than uniform adjustment of brightness and contrast applied to the entire image. Formalin was injected in the plantar region at a dose of 2.5% v/v bearing a volume of 20 µL. Rutin was administered orally at a dose of 100 mg/kg. Paracetamol was orally administered at 100 mg/kg dose. Mean ± SD data were represented after One-way ANOVA followed by Dunnett's post hoc analysis. (n=6, $p<0.05$, * indicates significant difference with formalin treated group).