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Hinokitiol, a natural tropolone derivative attenuates inflammoalgesia induced by LPS in experimental animals

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ABSTRACT: Hinokitiol is a natural bioactive compound synthesized as a secondary metabolite in numerous aromatic, medicinal plants and commonly used in food and cosmetic industries. The investigation was carried out to screen the potential activity of hinokitiol against LPS-induced inflammation, algesia, and oxidative stress in experimental animals. The hinokitiol was screened in two doses, i.e. 0.2 mg/kg (H-1), 0.4 mg/kg (H-2), and inflammoalgesia were induced by intraplantar administration of lipopolysaccharides (LPS) at a dose of 1 mg/kg. The assessment was carried out on the 7th, 14th, 21st, and 28th days for the severity of arthritic hyperalgesia score, secondary inflammatory lesions, stair climbing ability, motility, and dorsal flexion-pain score test. Serum analysis was carried out for tumor necrosis factor-alpha (TNF-α), interleukin (IL-2 and IL-6), prostaglandin (PGE-2), nitric oxide (NO), and thymus/spleen index. The joint samples were further subjected to histopathological observations. The results showed that treatment with H-1 and H-2 shows dose-dependent significant decreases ($P<0.05$) in arthritic hyperalgesia and secondary lesions, whereas a significant increase ($P<0.05$) in stair climbing ability, motility, and dorsal flexion pain score test was noted. Treatment with H-1 and H-2 shows significant lowering ($P<0.05$) in serum TNF-α, IL-2, IL-6, PGE-2, NO, and thymus/spleen index. Histopathological observations indicated minimal damage and restored the synovial structure. The present study reveals that hinokitiol shows significant anti-inflammatory, anti-algesic, and antioxidant effects against LPS-induced inflammation in experimental animals.

KEYWORDS: Hinokitiol; Lipopolysaccharide; Arthritis; Inflammation; Hyperalgesia; Inflammatory mediators.

1. INTRODUCTION

Pain and inflammation are among the most common reasons for seeking pharmacotherapy [1]. The perception of pain is generally addressed as algesia, and the pronounced pain threshold is known as hyperalgesia [2]. Pain and inflammation are often associated with joints that are hyperalgesic and need immediate medical attention. It has been demonstrated that the peripheral and central nociceptive systems are significantly more sensitive to inflammatory mediators [3, 4]. Current pharmacotherapy mainly involves non-steroidal anti-inflammatory drugs (NSAIDs) and opioid analgesics to combat inflammation and pain components. However, the chronic consumption of these drugs is associated with hepatotoxicity, nephrotoxicity, gastrointestinal ulceration and bleeding, cardio-respiratory depression, and dependent tendency [5]. Considering these limitations, it is urgent to tap a new potent molecule that has maximum effectiveness without creating negative side effects. Plants-based medicines always attract the researcher for their potential benefits. The Ayurvedic medicinal system has been greatly investigated using scientific methods. The recent studies explored the potential pharmacological effects of a plant-based bioactive metabolite named hinokitiol. Hinokitiol, known as Thujaplicinis, is a natural bioactive compound synthesized as a secondary metabolite in aromatic and medicinal plants of the Cupressaceae family [6]. Chemically, it is a monoterpenoid and is known as 2-hydroxy-4-isopropylcyclohepta-2,4,6-trien-1-one($C_{10}H_{12}O_2$) with a molecular weight of 164.20 [7-10]. It is widely a common ingredient in food products,

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cosmetics, and health care products as an antimicrobial agent. Potential pharmacological activities of hinokitiol include anticancer [7], antioxidant [8], antidiabetic [9,10], antiviral [11], antifungal [12,13], antibacterial [14], antiplasmodial[15], anti-inflammatory [16,17], neuroprotective [18] and hepatoprotective[19] has been reported. The keen review of reported studies addresses that hinokitiol exhibits remarkable beneficial effects against cancer cells. The hinokitiol blocks and inhibits several checkpoints in cancer cell development. In addition, hinokitol possesses potent antioxidant, and anti-inflammatory properties mainly downregulation of inflammatory mediators expression and inhabiting (NF)-κB activation. Considering the broad spectrum of activities [20,21], it was postulated that hinokitiol might be beneficial to combat the inflammation and analgesia induced in experimental animals.

2. RESULTS

2.1. Effect of H-1 and H-2 on severity of arthritic inflammation

On day 0, all the groups of animals shows an absence of edema or swelling (0), whereas animals treated with LPS shows mild arthritis inflammation (0–1) with a median score of 0. No significant difference in the severity of arthritis was observed on the 0th day in all the groups of animals. The median score of 3 on day 7 and 4 on day 14, 21, and 28, respectively, was observed in LPS-treated animals. Animals treated with H-1 show a significant decrease ($P<0.05$) in the severity of arthritic inflammation with a median score of 2 on days 7 and 14 and 0 on days 21 and 28, respectively. The median score for H-2 treated animals was 2 on day 7, 1 on day 14, and 0 on days 21 and 28, respectively. Animals that received standard drugs shows significant inhibition of arthritic severity with a median score of 2 on days 7 and 14 and 0 on days 21 and 28 (Figure1a).

2.2. Effect of H-1 and H-2 on secondary lesions

The presence or absence of secondary inflammatory lesions on the forepaws, hind paws, nose, and tail were observed, and percentage inhibition was calculated. No significant ($P>0.05$) difference in the percent inhibition was observed in all the groups of animals on day 7. Animals treated with H-1 and H-2 show significant ($P<0.05$) percent inhibition of secondary lesions on days 14 (H-1 27 ± 2.0%, H-2 43 ± 2.3%), 21 (H-1 57 ± 2.8%, H-2 31 ± 2.5%), and 28 (H-1 49 ± 3.1%, H-2 65 ± 3.0%) respectively compared against LPS (Figure1b).

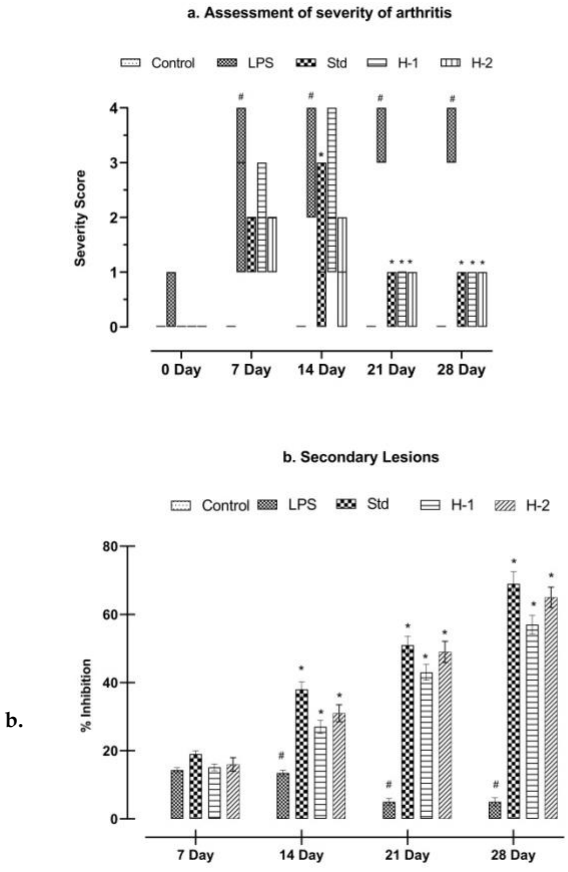

Figure 1.a. Effect of H-1 and H-2 on the severity of arthritic inflammation; b. Effect of H-1 and H-2 on secondary lesions.

a. N=6, Bar are expressed as median valves. The control group was compared against LPS treated group, and standard/H-1/H-2 groups were compared against LPS treated group. P≤0.05 is considered statistically significant and represented with an asterisk (*) and # for the LPS group. **b.** N=6, Bar are expressed as mean ± S. D. Standard/H-1/H-2 groups were compared against LPS treated, P≤0.05 is considered as statistically significant and represented with an asterisk (*) and # for LPS group.

2.3. Effect of H-1 and H-2 on arthritic hyperalgesia

In all the treatment groups, animals were able to walk easily with a median motility score of 2, and there was no statistically significant (P>0.05) difference in the motility score on day 0. LPS treatment significantly lowers motility and shows a median score of 1 on day 7 and 0 on days 14, 21, and 28 compared to control group animals. H-1 and H-2 treated animals reduce arthritic pain and significantly (P<0.05) improve their walking abilities. The median motility score was found to be 1 on days 7, 14, 21, and 2 on days 28 in H-1 treated animals. The H-2 treated animals showed significant (P<0.05) improved walking abilities, and the median motility score was found to be 1 on days 7, 14, and 2 on days 21 and 28 (Figure2a). The stair-climbing ability was assessed, and found a no statistical difference in median score on day 0 in all treatment groups. LPS-treated animals show significant (P<0.05) diminished stair climbing ability, and a median score of 0 was found on days 7, 14, 21, and 28. Animals treated with H-1 and H-2 showed significantly (P<0.05) improved stair-climbing ability. The median score of 2 on day 7, 1 on day 14, 2 on day 21 and 3 on day 28 was found in H-1 treated animals, whereas H-2 treated animals showed a median score of 2 on day 7, 14, 21, and 3 on day 28 (Figure2b). On day 0 all the groups showed a median DFP score of 0, which represents no significant changes. A significant (P<0.05) decrease in pain-bearing threshold was observed in LPS-treated animals with median DFP scores of 1 on day 7 and 2 on days 14, 21, and 28 compared to the control group (0). Treatment with H-1 and H-2 increased the pain-bearing threshold significantly (P<0.05) compared to LPS-treated animals. The median DFP score for H-1 was 2 on day 7, 1 on day 14, and 0 on days 21 and 28. For H-2, the median DFP score was 1 on days 7, 14, and 0 on days 21, 28. The median score was almost zero on day 28 for hinokitiol-treated animals indicating a significant antinociceptive effect against LPS-induced hyperalgesia (Figure2c).

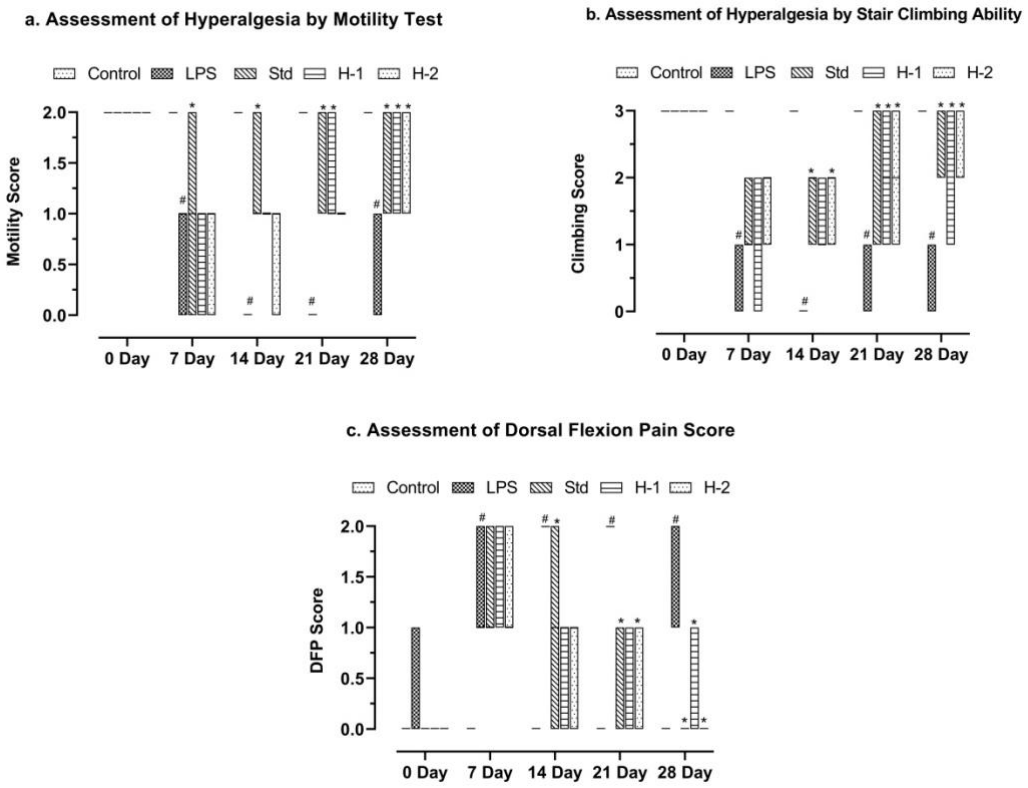

Figure 2.Effect of H-1 and H-2 on arthritic hyperalgesia.

N=6, Bar are expressed as median valves. The control group was compared against LPS treated group, and standard/H-1/H-2 groups were compared against LPS treated group. P≤0.05 is considered statistically significant and represented with an asterisk (*) and # for the LPS group.

2.4. Effect of H-1 and H-2 on behavioral responses

No statistical difference was found in the Paw withdrawal latency (PWL) for cold allodynia on day 0. A significant (P<0.05) increase in PWL was observed in LPS-treated animals on days 7 (8.5 ± 0.9), 14 (7 ± 0.5), 21 (7 ± 0.8), and 28 (7.5 ± 0.9). H-1 and H-2 treated animals show significant (P>0.05) decreases in PWL on days 7 (H-2 9 ± 0.75), 14 (H-1 8 ± 0.5, H-2 9.5 ± 0.9), 21 (H-1 9.3 ± 0.7, H-2 10.1 ± 0.8) and 28 (H-1 11 ± 0.9, H-2 11.6 ± 0.8). The result indicates that hinokitiol antinociceptive effect against noxious cold stimuli (Figure3a). No statistical difference was observed in PWL for hot stimuli in all the treatment groups on day 0. Administration of LPS significantly results in the development of hyperalgesia and decreases PWL on days 7 (11.3 ± 0.9), 14 (8.5 ± 0.8), 21 (8.4 ± 0.8), and 28 (8.9 ± 0.7). Significant increases (P<0.05) in PWL were observed in H-1 and H2 treated animals on days 14 (H-1 11.8 ± 0.8, H-2 12.4 ± 0.8), 21 (H-1 12.2 ± 0.9, H-2 12.7 ± 0.8) and 28 (H-1 12.5 ± 0.8, H-2 13.1 ± 0.6). This indicated a significant antinociceptive response to noxious thermal stimuli compared to LPS-treated animals (Figure3b). LPS administration significantly (P<0.05) decreases the tail withdrawal latency on days 7 (14.3 ± 0.7), 14 (13.5 ± 0.8,), 21 (13.0 ± 0.8), and 28 (13.1 ± 0.7) as compared to the control group. A significant increase in tail withdrawal latency was noted in the H-1 and H-2 treated animals on day 14 (H-1 13.6 ± 0.5, H-2 15.2 ± 0.7), 21 (H-1 14.9 ± 0.7, H-2 16.7 ± 0.7) and 28 (15.8 ± 0.6, H-2 16.9 ± 0.5) compared to LPS treated animals. This indicates that hinokitiol treatment significantly increases the pain-bearing threshold and addresses its antihyperalgesic response against LPS-induced algesia (Figure3c).

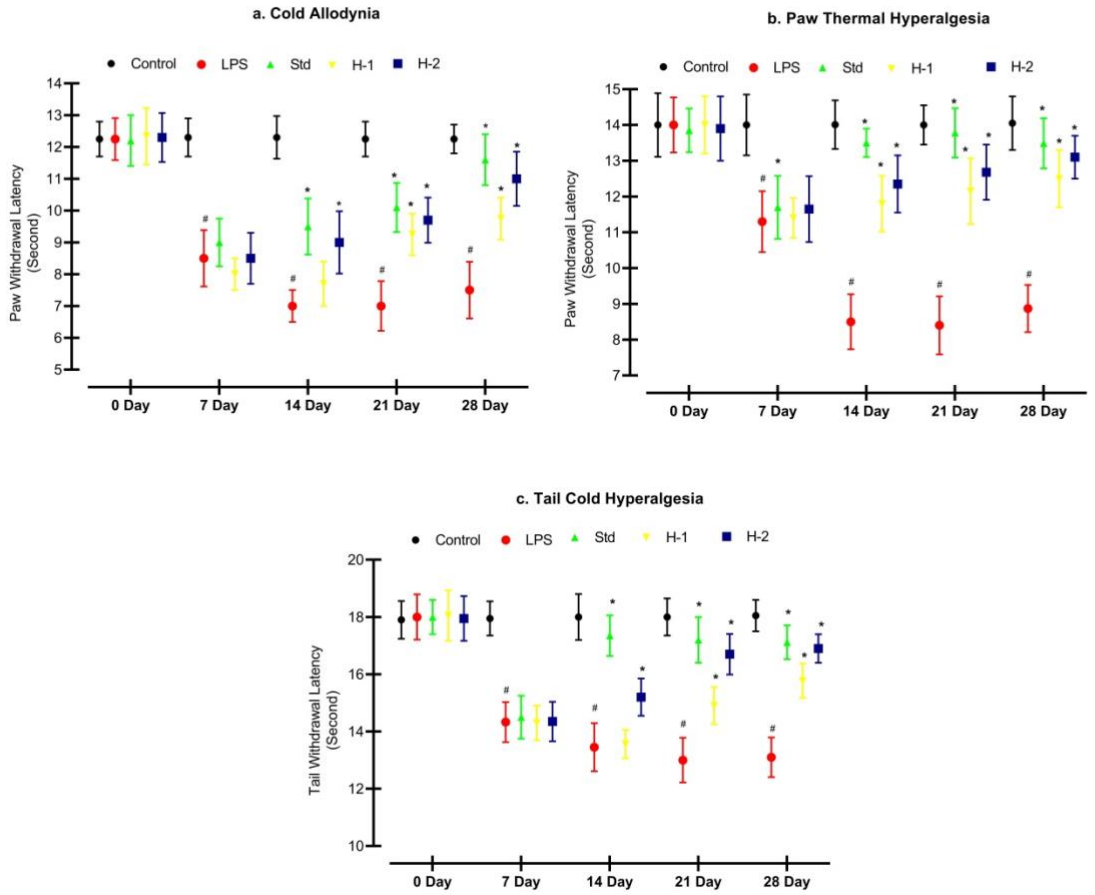

Figure 3. Effect of H-1 and H-2 on behavioral responses.

N=6, Barare expressed as mean ± S. D., The control group was compared against LPS treated group, and standard/H-1/H-2 groups were compared against LPS treated group., P≤0.05 is considered statistically significant and represented with an asterisk (*) and # for the LPS group.

2.5. Effect of H-1 and H-2 on serum cytokine levels and NO

LPS administration leads to the activation of several inflammatory pathways and elaborated synthesis of pro-inflammatory mediators. LPS-treated animals shows significantly increased (P<0.05) serum levels of TNF-α (227 ± 10.7), IL-2 (57.6 ± 6.3), and IL-6 (265 ± 12.7) than in control group animals. Animals treated with H-1 and H-2 show significant (P<0.05) decreases in cytokine TNF-α (H-1 167 ± 8.5, H-2 137 ± 7.9), IL-2 (H-1 43 ± 3.6, H-2 39 ± 4.5) and IL-6 (H-1 189 ± 10.5, H-2 166 ± 9.5) serum levels as compared to LPS treated animals. This indicates the significant anti-cytokine activity of hinokitiol in a dose-dependent manner (Figure4a,b,c). Administration of LPS significantly increases the level of PGE-2 (90 ± 9) as compared to the control group. Standard-treated animals show a significant decrease (P<0.05) in TNF-α (110 ± 8.7), IL-2 (32 ± 4.5), and IL-6 (150 ± 8.9) levels which confirm the anti-cytokine effect against LPS induced elaborated cytokine synthesis. Administration of H-1 and H-2 shows a dose-dependent significant decrease (P<0.05) in PGE-2 (H-1 59 ± 7.2, H-2 53 ± 6.7) levels (Figure4d). Treatment with standard drug significantly PGE-2 (39 ± 4.5) levels and shows an anti-cytokine effect. The LPS induces oxidative stress and leads to a significant (P<0.05) increase in NO (64.05 ± 4.19) levels. Animals that received H-1 and H-2 showed a significant (P<0.05) decrease in NO (H-1 48.15± 4.23, H-2 42.99 ± 5.01) levels, which confirms antioxidant potential against LPS-induced oxidative stress (Table 1).

Table 1. Effect of H-1 and H-2 on Thymus/spleen index and NO

Group	Control	LPS	Std	H-1	H-2
Thymus	0.32 ± 0.04	0.76 ± 0.06#	0.40 ± 0.04*	0.52 ± 0.05*	0.46 ± 0.04*
Spleen	2.12 ± 0.10	4.15 ± 0.29#	2.68 ± 0.19*	3.14 ± 0.20*	2.99 ± 0.15*
NO(nmol/g)	22.12 ± 2.22	64.05 ±4.19#	35.68 ±5.14*	48.15±4.23*	42.99 ± .01*

N=6, valves are expressed as mean ± S. D., The control group was compared against LPS treated group, and standard/H-1/H-2 groups were compared against LPS treated group. P≤0.05 is considered statistically significant and represented with an asterisk (*) and # for LPS group.

2.6. Effect of H-1 and H-2 on thymus and spleen index

LPS activates the host immune system and shows a significant increase (P<0.05) in the thymus and spleen index (0.76 ± 0.06 and 4.15 ± 0.29), respectively. Animals treated with H-1 and H-2 showed significantly decreased thymus index (H-1 0.52 ± 0.05, H-2 0.46 ± 0.04) and spleen index (H-1 3.14 ± 0.20, H-2 2.99 ± 0.15) respectively compared to LPS treated animals. Standard-treated animals show significant decreases in thymus and spleen index of (0.40 ± 0.04, 2.68 ± 0.19) compared to LPS-treated animals (Table 1).

2.7. Effect of H-1 and H-2 on histopathology

Control group animals show normal histology of joints as articular cartilage (Ac), synovial membrane (Sm), synovial folds (Sf), spongy bone (Sb), bone marrow cells (Bm), and joint cavity (Jc) (Figure5a). Animals injected with LPS showed substantial enlarged Sm, hyperplastic synovium (Sh), augmented synovial vascularity (Sv), inflammation (In), cartilage erosion (Ce), and bone erosion (Be) (Figure5b). Standard drug-treated animals have few enlarged Sm and Ce (Figure5c).H-1 treated animals show enlarged Sm increased Sv, In, Ce, and Be (Fig 5d). Sh and increased Sv were observed in H-2-treated animals (Figure5e). The histological observation indicates that hinokitiol in H-2 doses shows minimum cartilage damage as compared to LPS-treated animals.

3. DISCUSSION

The current investigation deals with the assessment of hinokitiol effects against LPS-induced inflammation and hyperalgesia in experimental animals. Bacterial endotoxins (LPS) induced inflammation has been reported for cartilage [22], pulmonary [23], neuronal [24], kidney [25], pancreas [26], liver [27], cardiac [28], etc. LPS activates the endogenous pain and hyperalgesia component. Animals and cell line-based experimental models explored LPS-induced inflammatory responses. The underlying mechanism involves the activation of the LPS-induced NF-κB signaling pathway causes enhanced transcription for pro-inflammatory cytokines such as NO, iNOS, IL-6, and TNF-α [46,48,29]. ILs are primarily responsible for monocyte growth and differentiation, B cell proliferation, and T cell activation [29, 30], while TNF-α causes cell death, inflammation, and pain. Further, the effect leads to increased endothelial membrane permeability,

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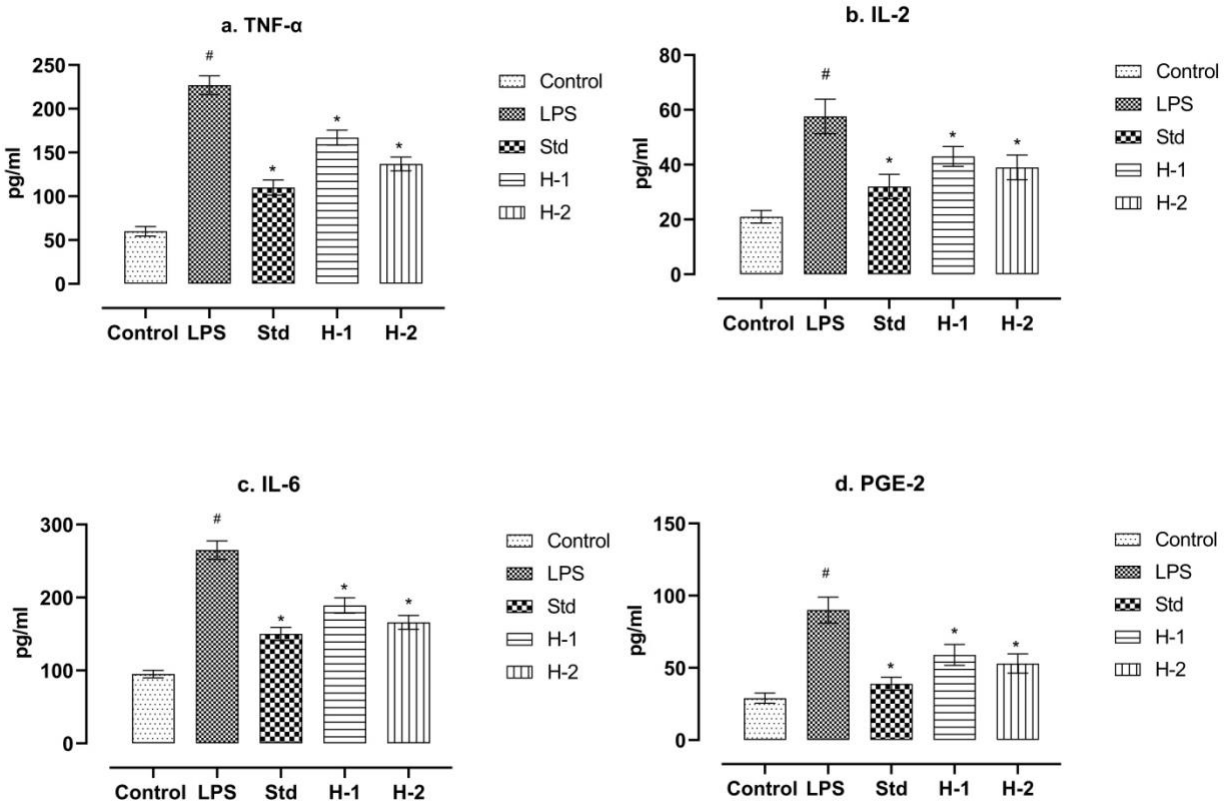

Figure 4. Effect of H-1 and H-2 on serum cytokine levels.

N=6, Bars are expressed as mean± S. D., The control group was compared against LPS treated group, and standard/H-1/H-2 groups were compared against LPS treated group. P≤0.05 is considered statistically significant and represented with an asterisk (*) and # for the LPS group.

fluid accumulation, and cell recruitment at the site of inflammation/infection [48,30]. The presence or absence of edema, swelling, and secondary lesions confirms the severity of inflammation [48]. Treatment with hinokitiol significantly limits the severity of arthritic inflammation due to its anti-cytokine effect. Pro-inflammatory cytokines are also involved in the modulation of pain perception. Altered neuronal activity in different classes of peripheral and central nervous system neurons is reported in inflammatory conditions [31]. Application of TNF-α to peripheral neuronal axons or somata of dorsal root ganglion neurons generates abnormal spontaneous activity within nociceptive nerve fibers as well as large myelinated conducting Aβ neurons [32].TNF-α and PG (PGE-2) have the potential to enhance the sensitivity of pain-carrying sensory neurons. In addition, IL-6 also has a profound role in enhanced pain behavior in the DRG [33]. These hyperalgesic changes can be assessed using a motility test, stair climbing ability, and dorsal flexion pain test [46]. As discussed earlier, LPS administration enhances the synthesis and activity of pro-inflammatory cytokines, leading to the activation of nociceptive receptors [46,48]. Administration of H-1 and H-2 improves the motility and climbing ability while minimizing DFP and shows its anti-nociceptive effect against LPS-induced hyperalgesia. Physical activities are greatly reduced due to severe pain. Hinokitiol shows a significant decrease in algesia and improves overall physical activities [10]. The anti-nociceptive effect can be correlated with the hinokitiol anti-cytokine property. Behavioral responses to noxious stimuli can be evaluated using cold allodynia and hot stimuli to assess the withdrawal latency. Administration of LPS, to a great extent, alters the behavioral pattern against noxious stimuli and decreases the pain-bearing ability for cold and hot stimuli [46]. LPS-induced hyperalgesia is due to enhanced synthesis and activation of pro-inflammatory mediators, glial cell activation, and the release of PGs [34]. Administration of hinokitiol significantly increases the pain-bearing threshold against noxious cold and hot stimuli. This anti-hyperalgesic effect may be attributed due to limiting the activity of pro-inflammatory mediators. As

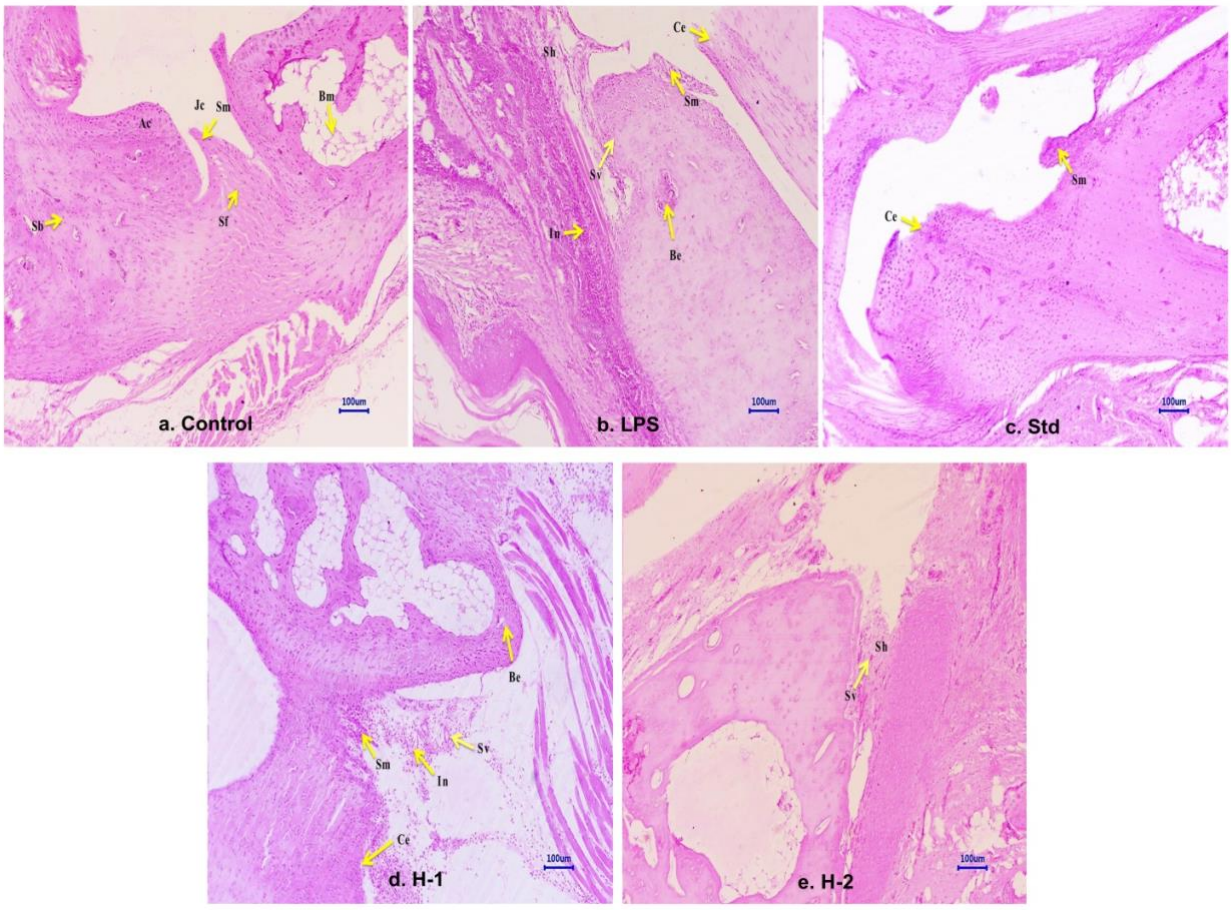

Figure 5. Effect of H-1 and H-2 on Histopathology.

Articular cartilage (Ac), synovial membrane (Sm), synovial folds (Sf), spongy bone (Sb), bone marrow cells (Bm), joint cavity (Jc), substantial enlarged Sm, hyperplastic synovium (Sh), augmented synovial vascularity (Sv), inflammation (In), cartilage erosion (Ce), and bone erosion (Be).

discussed earlier, pro-inflammatory TNF-α is involved in vasodilatation, fluid accumulation, edema, cell recruitment, and increased expression of leukocyte adhesion, all of which contribute to oxidative stress and associated cellular damage, blood coagulation, and fever induction [35]. Targeting TNF-α and encountering its pathological role is a significant attempt to limit the inflammatory cascades and associated painful responses. Hinokitiol treatment reduces TNF- α activity and has significant anti-inflammatory and anti-algesic effects, according to our findings. IL-1 is mainly involved in the destruction of joint cartilage by induction of metalloprotease secretion. IL-1 is pyrogenic and is responsible for elaborating the synthesis of IL-6, collagenase, proteoglycans, COX, and PGs. IL-1 is expressed on host immune cells and enhances their activity and recruitment at the site of inflammation. Limiting IL-1 activity manages inflammation and noxious stimuli significantly [36]. IL-6 is involved in microglial cell activation and associated neuropathic pain responses. Down-regulation of NF-κB and MAPK signaling and inhibition of expression and synthesis of inflammatory cytokines would be possible mechanisms of hinokitiol action [37].The recent finding suggests that inflammatory actions are interconnected with PGE2. During the inflammatory cascades, PGE2 causes vasodilatation and promotes the migration of neutrophils, macrophages, and mast cells at the site of inflammation/injury, causing swelling and edema [38]. Moreover, it stimulates the sensory nerve to enhance pain responses by activating nociceptive receptors. Studies suggested that targeting PGE2 would result in an effective and promising therapeutic strategy to combat inflammation and pain [38, 39]. We observed that hinokitiol significantly reduces PGE2 levels and limits LPS-induced inflammation and algesic behavior. Over-production of NO in unusual conditions induces the synthesis of pro-inflammatory mediators. NO is thought to stimulate vasodilatation and activate macrophages. It also enhances peripheral and neuronal pain perception through the activation of microglial cells and sensory nerve fibers [40,41]. The results of the current study showed that hinokitiol significantly reduces the PGE2 and NO levels induced by LPS intoxication. As a consequence of LPS administration, the host immune system is activated, initiating its

defense mechanism through antibodies and cell-mediated immunity [42]. As a primary lymphoid organ of the immune system, the thymus gland is essential for the maturation and function of T cells. To combat foreign invaders, T cells are essential for activating the adaptive or acquired immune system. As a result of LPS administration, the mass of the thymus increases as an adaptive immune response is activated upon antigen invasion and the activation of T cells [43]. It has been found that WBC turnover increases in inflammatory cascades to fight against the antigen. In severe inflammatory conditions, the spleen is found to be enlarged as a result of storing and recruiting WBSs [44]. Administration of hinokitiol demonstrates the least activation of the host immune system and WBCs. Due to its anti-inflammatory effects, hinokitiol treatment significantly reduced the spleen thymus index in animals. We observed that treatment with hinokitiol causes minimum damage to cartilage, limits inflammation, and protects the normal joint structure. During the experimental procedures, we did not note any side effects of hinokitiol in the experimental doses in the animals. However few studies have reported hinokitiol toxicity in pregnant rats like embryonic/fetal survival and growth in doses of 15, 45, or 135 mg/kg on days 6-15 of pregnancy [45]. Hinokitiol chronic toxicity study was carried out at doses of 0, 0.005% (50mg/kg), 0.015% (150mg/kg) and 0.05% (500mg/kg) for 52 weeks. No significant changes in survival rate, general condition, body weights, food consumption, urinalysis, hematology, and clinical chemistry were noted. However, 0.05% hinokitiol showed a significant increase in the weight of the spleen, liver, and hemosiderin with slight centrilobular hypertrophy of male hepatocytes. However, these alterations were negligible and not toxicologically significant [46]. Considering the translation perspectives hinokitiol pharmacokinetics and pharmacodynamics need to be understood in more detail and require further validation and safety profile in human beings.

4. CONCLUSION

In conclusion, it can be stated that hinokitiol has significant anti-inflammatory and anti-algesic potential against LPS-induced inflammation in experimental animals. The underlying mechanism involves the inhibition of pro-inflammatory and inflammatory mediators as well as the reduction of oxidative stress, which contributes to inflammatory and algesic cascades. Further studies are needed to pinpoint the hinokitiol signaling mechanism at a molecular level for its protective effect.

5. MATERIALS AND METHODS

5.1. Chemicals

The test sample of hinokitiol and LPS was obtained from Sigma Aldrich, for the assay, Shimadzu UV-1800 spectrophotometer was used, and cytokines were estimated using commercial cytokine ELISA assay kits. Rat TNF α ELISA (Sigma Aldrich), IL-2 and IL-6 (rat) ELISA (Biovision), and Prostaglandin E2 (PGE2) ELISA Kit (Millipore) were procured from institutional suppliers. The indomethacin drug was procured from the institute's central store. The required chemicals used were of analytical grade.

5.2. Preparation of hinokitiol, LPS, and standard drug

10mg of hinokitiol was dissolved in dimethylsulfoxide (DMSO) to obtain a concentration of 1mg/ml. based on previously reported studies two doses of hinokitiol were selected as 0.2mg/kg and 0.4mg/kg and identified as H-1 and H-2 [47]. LPS was prepared in phosphate buffer (pH 7.4) and 1mg/kg was used to induce the inflammation [48]. Indomethacin 30mg/kg was used as a standard drug and prepared in 1% acacia [48].

5.3. Experimental animals

Animal experimentation was carried out as per the guidelines of the committee for the purpose of control and supervision of experiments on animals (CPCSEA) with the utmost care. Prior permission was taken from the Institutional Animal Ethics Committee (IAEC) (Ref No. CCOPR/IAEC./2021-51/2021 dated 07.09.2021) before animal experimentation. 30 adult male Wistar rats weighing between 150-200 g were procured from the approved animal supplier. Animals were kept for acclimation for 15 days. All animals were routinely checked for any illness or infection before following the treatment protocol. The standard animal house conditions were maintained throughout the experimentation.

5.4. Treatment protocol

6 animals were randomly divided into 5 groups. The control group received vehicle p.o.; LPS-treated animals were injected intraplantar with LPS (1mg/kg) on day 0; standard-treated animals were injected with

LPS (1mg/kg) on day 0 and p. o. indomethacin (30mg/kg); H-1 treated animals were injected intraplantar with LPS (1mg/kg) on day 0 and i. p. hinokitiol (0.2mg/kg) for 28 days; H-2 treated animals were injected intraplantar with LPS (1mg/kg) on day 0 and i. p. hinokitiol (0.4mg/kg) for 28 days. The dosing of all animals is carried out in the morning between 9:00 a.m. to 10:00 a.m. Animals had free access to food and water during the treatment protocol.

5.5. Evaluation of the severity of arthritic inflammation

An arthritic score method was used to assess the severity of arthritic inflammation on the 0–4 scales as per previously reported methods and is as follows [49,50]. Scale 0: Absolute absence of edema or swelling, Scale 1: Existence of mild edema or swelling and limited erythema, Scale 2: Existence of mild edema or swelling and erythema from the ankle to tarsal, Scale 3: Existence of moderate edema or swelling and erythema from the ankle to tarsal. Scale 4: Severe edema or swelling, as well as erythema over the entire leg. The severity of arthritic inflammation was assessed on days 0, 7, 14, 21, and 28. 16 is the maximum allowed scale considering the sum of all scores.

5.6. Evaluation of secondary lesions

Inflammatory secondary lesion assessment was confirmed by the absence or presence of lesions on the non-injected viz. forepaws, hind paws, nose, and tail. Based on the overall number of animals in each group and the presence of a lesion in the number of animals, percentage inhibition was determined. The evaluation of inflammatory secondary lesions was assessed on days 7, 14, 21, and 28 [50].

5.7. Evaluation of arthritic hyperalgesia

The evaluation of arthritic hyperalgesia was assessed by motility test, stair climbing ability, and dorsal flexion pain on days 0, 7, 14, 21, and 28. Animal motility was recorded for 5 minutes using a score of 0–2. Score 0: The animal walks without making contact with the inflamed paw on the floor; Score 1: The animal walks with difficulty; Score 2: The animal walks easily [51]. The stair-climbing ability was assessed using 3 staircases with a step height of 5, 10, and 15 cm. Step two is restored with water, while the third is with food. The climbing ability was scored using a 0–3 scale as follows. 0: The animal did not ascend any steps. 1: an animal capable of climbing the first step; 2: an animal capable of climbing the second step; and 3: an animal capable of climbing all steps [25, 26]. Dorsal flexion pain (DFP) was performed 5 times at an interval of 5 seconds. The inflamed paw joint flexed dorsally until the toe touched the anterior leg part. The scoring was done on a scale of 0–2 as follows, score 0: No squeaking and leg withdrawal response, 1: Existence of squeaking or leg withdrawal response, 2: Existence of squeaking and leg withdrawal response [50,51].

5.8. Evaluation of behavioral responses

The behavioral assessment was carried out using cold allodynia, paw thermal hyperalgesia, and tail cold hyperalgesia on days 0, 7, 14, 21, and 28 of a treatment protocol. Cold allodynia deals with paw withdrawal latency for cold noxious stimuli. The inflamed paw was dipped in the water bath (10 ± 0.5 °C) and withdrawal latency was measured every 3 minutes. Cold allodynia is confirmed if there is a significant reduction in paw withdrawal latency. The Paw thermal hyperalgesia-associated nociceptive response was assessed using Eddy's hotplate maintained at 55 °C ± 0.5 °C. Animals were placed on a hot plate, and their pain threshold was measured. Animals were kept on a hot plate for no more than 15 seconds. Tail cold hyperalgesia was assessed by dipping the terminal tail end in the cold water maintained at (0–4 °C). The tail withdrawal latency for cold noxious stimuli was measured with a maximum cut-off of 20 seconds [48].

5.9. Assay of serum cytokine levels

After completion of the treatment protocol, blood from all the group animals was collected from the retro-orbital plexus. The blood sample was allowed to clot and subjected to centrifugation at 600rpm for 15 minutes. The assay for TNF-α, IL-1, IL-6 (rat cytokine ELISA assay kits), and PGE2 (Prostaglandin EIA Kit) was carried out as per the supplier's instructions, and values are expressed as pg/ml.

5.10. Assay of nitric oxide

The joint sample was cut into small pieces and homogenized with Tris-HCl buffer (50 mM), pH 7.4. Further, it was triturated with NaCl (0.1 M), Triton X-100 (0.1%) and kept for sonication for 5 minutes. The nitric oxide (NO) level was assayed by the Griess reaction according to the earlier reported methods. This consisted of an equal quantity of tissue homogenate and Griess reagents (equal volumes of N-(1-naphthyl)

ethylenediamine and sulfuric acid) were mixed. The resulting mixture was incubated at room temperature for 5 min. The NO activity was measured at 545 nm using a UV spectrophotometer and expressed as µmole/g tissue [48].

5.11. Thymus and spleen index

All the animals were weighed and sacrificed on the 28th day using a high dose of anesthetic. The animal was dissected and identified with their thymus and spleen. These are gently removed and weighed, respectively. Thymus/spleen index was calculated using the formula weight of thymus (or spleen)/body weight × 100% [50, 52].

5.12. Histopathology

After isolation of the thymus and spleen, the tibiotarsal portion of the joint was cut using bone-cutting scissors. The joint sample was kept in 10% buffered formalin, and decalcification was done using 10% EDTA (pH 7–8) for 30 days and embedded in paraffin. The basic histological staining of hematoxylin-eosin (HE) stain was performed for cartilage matrix observations (Pink/bluish) [53].

5.13. Statistical analysis

Statistical analysis was carried out using Graph Pad Prism software (Version 8.4). The data were analyzed by applying a one-way analysis of variance (ANOVA) followed by Dunnet's multiple comparison tests. Data are expressed as mean ± standard deviation (S.D.) where n=6. The Kruskal-Wallis test was used to assess the statistical significance of arthritic severity, hyperalgesia, and behavioral responses. The valves for the same are expressed as a median. $P<0.05$ is considered significant and represented with an asterisk (*) and # for the LPS group.

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