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EVALUATION OF ANTI-INFLAMMATORY, ANTIOXIDANT, AND XANTHINE OXIDASE INHIBITORY POTENTIAL OF N-(2-HYDROXY PHENYL) ACETAMIDE

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Abstract

Nonsteroidal anti-inflammatory drugs have long been used to treat various pathological conditions like arthritis, gout, diabetes, cancer, and cardiovascular diseases. Although exploring the effective anti-inflammatory agents possessing antioxidants along with pain-relieving properties is still challenging. Acetamide-containing compounds are known to possess anti-inflammatory and analgesic activity. The current study evaluated the anti-oxidant, anti-inflammatory, xanthine oxidase, and analgesic activity of an acetamide derivative, N-(2-Hydroxyphenyl) Acetamide. In vitro anti-inflammatory and antioxidant activities were measured by protein denaturation inhibition and DPPH assay, respectively. Xanthine oxidase potential was also evaluated. In vivo anti-inflammatory activity was determined by the Formalin induced paw edema method while acetic acid induced writhing test was used for evaluating analgesic activity. Outcomes obtained from in vitro anti-inflammatory activity shown significant ($p < 0.001$) inhibition of protein denaturation by N-(2-Hydroxyphenyl) Acetamide at both 100 and 300 $\mu\text{g/ml}$. It also exhibited high radical scavenging activity (88.16%) by DPPH at 300 $\mu\text{g/ml}$. In vivo Formalin-induced paw edema method revealed significant ($p < 0.05$) inhibition of paw edema in comparison to the control and formalin groups. Likewise, NA-2 also significantly ($p < 0.001$) reduced the writhing response in the writhing test. Moreover, in both aforementioned in-vivo tests, NA-2 showed comparable results with Indomethacin (10 mg/kg). Our study revealed that N-(2-Hydroxyphenyl) Acetamide had remarkable analgesic and xanthine oxidase inhibition effects via reducing inflammation and preventing oxidative stress. Results of current study suggest that NA-2 may play a role as anti-inflammatory agent and it can be considered as a promising drug candidate for anti-gout or anti-arthritic therapy in future.

Keywords: Analgesic, Anti-inflammatory, Anti-oxidant, Inflammation, N-(2-Hydroxyphenyl) Acetamide, Xanthine oxidase

INTRODUCTION

A defensive response of the immune system to injurious stimuli like pathogens, toxic compounds, irradiation, or trauma, is termed Inflammation. It is protective feedback that acts by eradicating the causative agent and initiating a healing process. Mostly, inflammation involves both kinds of immune responses i.e. innate and adaptive. The innate type of immune response involves cells like macrophages, dendritic, and mast cells for providing primary protection against invading organisms or cancer cells. Whereas, the adaptive immune response involves special cells like B- and T-cells to produce certain receptors and antibodies required to eradicate the pathogens (1).



Inflammation is either acute or chronic. Acute inflammation is a temporary self-limiting process described by enhanced capillary permeability, local vasodilation, and the discharge of inflammatory mediators such as prostaglandins (PGs), serotonin, and histamine. Whereas, the long-term process, lasting over several months or years, is called Chronic Inflammation. It is specified by the infiltration of leukocytes and phagocytic cells, followed by tissue degeneration and fibrosis (2). Hence inflammation has the important participation in the pathological progressions of numerous diseases, for instance, diabetes, arthritis, cancer, and cardiovascular diseases. Inflammatory pathways involve several regulatory pathways and inflammatory mediators. Inflammatory stimuli like microbial agents and cytokines such as interleukin 1 β (IL-1beta), interleukin 6 (IL-6), and tumor necrosis factor α (TNF-alpha) facilitate inflammation via binding with Toll like Receptors (TLRs), IL-6, IL-1, and TNF receptor. Activation of these receptors stimulates intracellular signaling pathways. The three main pathways include; mitogen activated protein kinase (MAPK), nuclear factor kappa B (NF- κ B), and Janus kinase signal transducer and activator of transcription (JAK-STAT) pathways. Dysregulation in any of these pathways may initiate inflammation associated diseases (3).

Drugs used for treating inflammation include steroidal drugs and non-steroidal anti-inflammatory drugs (NSAIDs). NSAIDs are mainly prescribed drugs as they also exhibit analgesic properties along with the anti-inflammatory activity. NSAIDs produce effect by inhibition of cyclooxygenase enzymes; COX 1 and COX 2, and thus prostaglandins (PGs) production is inhibited. Primarily, COX-2 inhibition is responsible for producing desired anti-inflammatory and analgesic effects while, COX-1 produces PGs and thromboxane A2 (TXA2) which control physiological functions like renal homeostasis, and mucosal barrier in the GI tract. Therefore, common side effects of NSAIDs are mediated via COX-1 inhibition (4).

Free radicals play a prime role in the oxidative stress development leading to inflammation. Free radicals are the molecules that contain an unpaired electron in the valence shell which makes them highly unstable and reactive (5). Two kinds of free radicals include reactive nitrogen species (RNS) and reactive oxygen species (ROS). Both RNS and ROS have a dual role as both useful and toxic agents. Within moderate or low concentrations, they have beneficial roles such as protection against microbial pathogens. However, the overproduction of ROS molecules gives rise to a damaging process named oxidative stress. Oxidative stress is demonstrated as the imbalance between the formation of free radicals and their clearance from the cells through anti-oxidants in the body. It stimulates inflammatory processes that stimulate the production and secretion of pro-inflammatory cytokines. This leads to irreversible cellular damage and progression of various chronic diseases (6).

Xanthine oxidase enzyme is capable of generating reactive oxygen species by accelerating the hypoxanthine oxidation to xanthine and then oxidation to the uric acid. Uric acid, along with produced ROS, has detrimental effects on the body. Increased production of uric acid results in hyperuricemia (increased serum uric acid levels). Furthermore, hyperuricemia leads to the development of gouty arthritis (7). Gout is a metabolic disease described as the monosodium urate (MSU) crystals deposition in tissues, particularly joints. Concurrently, oxidative stress produced by ROS damages the organ function, which is a great challenge in gout therapy. Treatment of gout includes NSAIDs like indomethacin, corticosteroids, and colchicine. Uricosuric agents such as probenecid and xanthine oxidase inhibitors like Allopurinol are used to maintain uric acid serum levels. However, these drugs are known to have serious adverse effects affecting the renal, hepatic, and gastrointestinal systems. Hence, comparatively safer alternatives are required in the treatment of gout and related conditions (8).

Acetamide-containing compounds are known to have analgesic and anti-inflammatory activity such as Paracetamol, the most widely used drug that belongs to this group. A study investigated the analgesic potential of some acetamide derivatives containing benzothiazole and tetrazole rings, against chemical, mechanical and thermal stimuli. They conclude that the tested acetamide derivatives possess anti-nociceptive potential (9). Perveen K (2013), in their study investigated the protective effectiveness of NA-2 against adjuvant-induced arthritis (AIA) in rats. The results suggested that NA-2 significantly suppressed inflammation and also decelerated progression of arthritis in the AIA model. Therefore acetamide derivatives can be considered to have a promising role in managing gouty arthritis by relieving pain and

inflammation (10). In the light of above mentioned properties we aimed to assess the effect NA-2 on in vitro antioxidant and xanthine oxidase activity as well as its in vivo analgesic and anti-inflammatory potential in mice models of acetic acid induced writhing activity and formalin induced paw edema respectively.

MATERIALS AND METHODS

IN-VITRO

ANTI-INFLAMMATORY ACTIVITY

This activity was measured by *protein denaturation inhibition* with slight variations. One millilitre of Bovine serum (5%) was added to the test compound (NA-2) and diclofenac sodium in various concentrations (10, 50, 100 and 300 µg/ml), followed by incubated at 27°C for 15 min. Mineral water and BSA mixture were taken as control. Then mixture was kept for 10 minutes at 70°C for albumin denaturation. After cooling down the mixtures at room temperature, absorbance was taken at 660 nm. The assay was performed thrice. The percent inhibition can be obtained with this formula,

$$\% \text{ Inhibition of Denaturation} = \frac{\text{Absorbance of control} - \text{Absorbance of test sample}}{\text{Absorbance of control}} \times 100$$

ANTIOXIDANT ASSAY

An upgraded *DPPH* assay (1,1-diphenyl-2-picrylhydrazyl) was selected for evaluation of antioxidant or radical scavenging activity of our tested compounds. If the tested compounds have antioxidant capacity the DPPH radicals vanish at an absorbance of 517nm. The test compound NA-2 (200µliter) at different concentrations (10, 50, 100, and 300µg/ml) was added to 0.1mM DPPH (3ml) and allowed to mix and react. The mixture was vortexed and incubated in the dark at room temperature for 30 minutes. A microplate reader (RE, ver. 5.0.0.42 with SkanIt Software 5.0) was used to determine the absorbance at 517 nm. The reducing power of our test compound was equated with ascorbic acid absorbance served as control. The radical scavenging activity was determined in percentage via equation.

$$\% \text{ RSA} = ((A_c - A_t) / A_c) \times 100$$

Here A_c and A_t represent control (including all reagents, without the test compound) and test compounds, respectively.

The IC50 values (concentration of the sample that can inhibit 50% of DPPH free radicals) were calculated through regression equation. IC50 values are inversely proportional to the free radical inhibitory activity, therefore, lower IC50 values indicate higher antioxidant potential of the sample.

XANTHINE OXIDASE INHIBITION ASSAY

The formation of uric acid can be used to estimate xanthine oxidase inhibitory response in the xanthine oxidase system (11). The assay mixture for XO system was prepared by mixing 0.1 ml XO (0.2U/ml), 0.6 ml phosphate buffer (100Mm; pH7.4), and 0.1 ml of NA-2 at various concentrations (10, 30, 100 and 300µg/ml). Then pre-incubate for 15 minutes at 25°C. Afterward, the reaction began with the addition of xanthine (1 mM; solubilized in 0.1 N NaOH) and then allowed to incubate again at 25°C for 15 minutes. Afterward, the enzymatic reaction was stopped with the addition of 0.2 ml HCl (1 N) and the inhibitory potential was measured at 550 nm by means of a spectrophotometer. The standard drug taken for positive control was Allopurinol.

The xanthine oxidase percent inhibition was calculated by the formula;

$$\% \text{ Inhibition} = \frac{(A - B) - (C - D)}{(A - B)} \times 100$$

Where, A was enzyme activity without test material, B was Control without test material, C was enzyme activity of test solution with XO and D was activity of test solution without XO.

IN-VIVO

ANTI-INFLAMMATORY ACTIVITY

Formalin induced paw edema technique is utilized for estimation of anti-inflammatory activity which was developed formerly. Wistar albino rats were distributed into five groups (5 animals per group).



0.2 ml of Formalin (2% v/v) was prepared using distilled water and injected into the right hind paw of all animals.

Group I (Normal control group): was given 500 μ L of saline only (p.o).

Group II (Formalin group): Distilled water was given p.o followed by formalin injection.

Group III (Standard group): pre-treated with an aqueous solution of indomethacin 10mg/kg (p.o) for 30 minutes followed by formalin injection.

Group IV and V (NA-2 treated groups): pre-treated with 10 and 100 mg/kg (p.o) of NA-2 for 30 minutes, respectively followed by formalin injection.

MEASURING PAW VOLUME

After injecting the test compound and formalin, the volume of the paw was assessed with the help of a vernier caliper from day 1 to day 5 on daily basis. The average paw edema volume was calculated for the standard drug as well as test compounds and comparison were made with the normal control. Afterward, the percentage inhibition was evaluated by using the below formula.

$$\% \text{ Inhibition} = [(F_1 - F_2) / (F_1)] \times 100$$

F1= paw volume prior to formalin injection

F2= paw volume after treatment and formalin injection on a daily basis

ANALGESIC ACTIVITY

Acetic acid induced writhing test was used for evaluation of analgesic activity. Intraperitoneal injection of 0.1ml/10g acetic acid (10ml /kg) was used to induce nociception. For positive control, animals were initially treated with indomethacin (i.p, 10 mg/kg) then 25 minutes later group was treated with acetic acid. Each test group was pre-treated with various concentrations (5, 10, and 100mg/kg) of NA-2 i.p for 25 minutes before injecting acetic acid. The stretching and no. of writhing were estimated after five minutes of intraperitoneal injection of acetic acid.

RESULTS

IN-VITRO

INHIBITION OF PROTEIN DENATURATION

In vitro anti-inflammatory response of NA-2 against the protein denaturation was evaluated. The effects of the NA-2 compound on the inhibition of protein denaturation are summarized in **Error! Reference source not found.** NA-2 exhibited significant inhibition ($p < 0.001$) of 58.66% and 69.11% at 100 and 300 μ g/ml respectively, while diclofenac produced 88.91% inhibition at 300 μ g/ml.

Table I. Effect of NA-2 on inhibition of protein denaturation

| Compounds | Concentration (μ g/ml) | Protein denaturation | Protein denaturation (Inhibition %) |
|------------|-----------------------------|----------------------|-------------------------------------|
| Control | - | 0.616 \pm 0.010 | - |
| Diclofenac | 10 | 0.124 \pm 0.001* | 79.87 |
| | 50 | 0.152 \pm 0.0008* | 75.27 |
| | 100 | 0.062 \pm 0.001* | 89.88 |
| | 300 | 0.068 \pm 0.001* | 88.91 |
| NA-2 | 10 | 0.408 \pm 0.021* | 33.65 |
| | 50 | 0.397 \pm 0.015* | 35.45 |
| | 100 | 0.254 \pm 0.015* | 58.66 |
| | 300 | 0.190 \pm 0.003* | 69.11 |

ANTI-OXIDANT ACTIVITY

NA-2 was examined for radical scavenging potential by DPPH and Fig. 1 shows the DPPH radical scavenging activity of NA-2 and standard Ascorbic acid. Among different concentrations, NA-2 exhibited



the highest activity at 100 and 300 µg/ml i.e. 80.54% and 81.35%, respectively. Whereas Ascorbic acid displayed stronger scavenging activity of 81.81% at 100 µg/ml and 88.16% and 300 µg/ml. IC50 values were computed by linear regression analysis of plot by % inhibition against the concentration. As shown in Fig. 2, IC50 of NA-2 and Ascorbic acid was found to be 2.28 and 2.08 µg/ml, respectively.

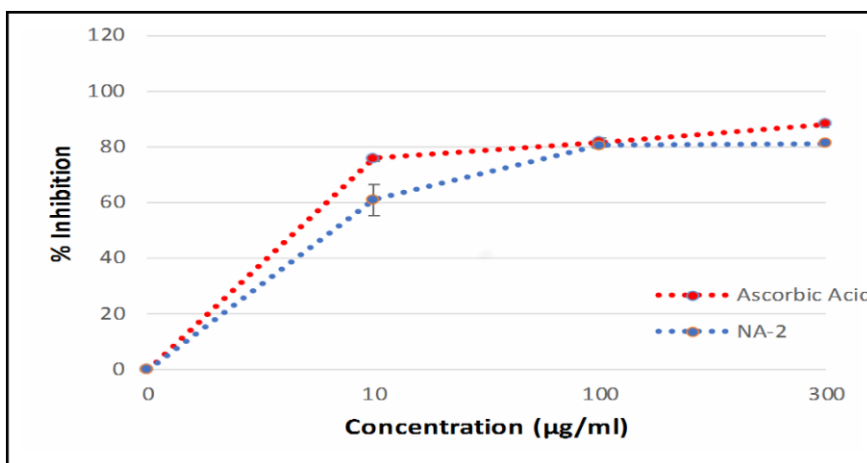


Fig. 1. DPPH radical Scavenging response. DPPH Scavenging activity of NA-2 and ascorbic acid at different concentrations. Values are expressed as Mean±SD

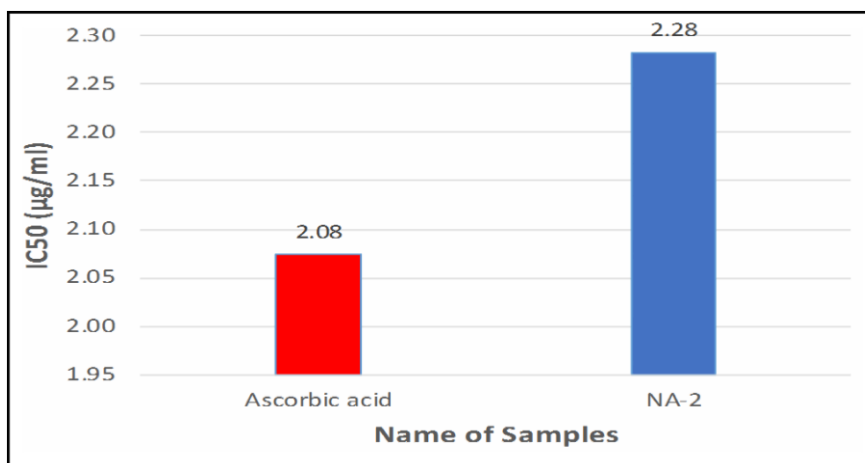


Fig. 2. IC50 of NA-2 and standard ascorbic acid

XANTHINE OXIDASE POTENTIAL

Xanthine oxidase activity of NA-2 was assessed by xanthine oxidase inhibition assay. The compound was assayed for inhibition activity at 10, 30, 100, and 300 µg/ml. The results of NA-2 and Allopurinol were described in *Error! Reference source not found.* Dose-dependent inhibition of xanthine oxidase potential was observed for both NA-2 and Allopurinol. NA-2 showed 5.69%, 7.77% and 15.19% inhibition at 10, 30 and 100 µg/ml, respectively. A stronger inhibition of 21.78% was observed at 300 µg/ml whereas, the positive control Allopurinol showed 29.69% inhibition at 25 µg/ml.

Table II. Xanthine oxidase activity of NA-2 compared to control

| Compound | Concentration (µg/ml) | Inhibition % (Mean±SD) |
|-------------|-----------------------|------------------------|
| Allopurinol | 10 | 18.27±0.27 |
| | 25 | 29.69±0.77 |
| | 50 | 97.62±1.61 |
| | 100 | 97.63±0.83 |
| NA-2 | 10 | 5.69±0.38 |
| | 30 | 7.77±0.46 |
| | 100 | 15.19±0.54 |
| | 300 | 21.78±1.07 |

IN-VIVO**ANTI-INFLAMMATORY ACTIVITY**

Anti-inflammatory activity of NA-2 was estimated by the formalin induced paw edema. As shown in the Table 3, GP-4 and GP-5 (NA-2 10 and 100 mg/kg) significantly ($p < 0.05$) inhibited formalin induced rat paw edema in comparison to GP-1 (NC) and GP-2 (Formalin). Also, it was shown that GP-3 significantly ($p < 0.05$) inhibited formalin-induced paw edema when compared with GP-4 (NA-2 10 mg/kg), however, there was no significant difference when compared with GP-5 (NA-2 100 mg/kg). Moreover, the Formalin group (GP-2) showed significant ($p < 0.05$) difference when compared with GP-3 (Indomethacin 10 mg/kg). As shown in **Error! Reference source not found.**, NA-2 at 100 mg/kg displayed comparable effects to Indomethacin (10 mg/kg) for inhibiting paw edema.

Table III. Effect of NA-2 treatments on the paw edema thickness (cm) and the percentage of inhibition recorded on day 1 till day 5

| Group | Dose mg/kg | Mean \pm SD (% Inhibition) | | | | |
|---------------|------------|------------------------------------|-----------------------------------|------------------------------------|------------------------------------|------------------------------------|
| | | Day 1 | Day 2 | Day 3 | Day 4 | Day 5 |
| GP-1 | - | 3.18 \pm 0.52 | 3.57 \pm 0.47 | 3.9 \pm 0.43 | 3.71 \pm 0.57 | 3.82 \pm 0.48 |
| NC | - | 6.02 \pm 0.25 ^{a,c,d,e} | 5.81 \pm 0.2 ^{a,c,d,e} | 5.34 \pm 0.32 ^{a,c,d,e} | 5.12 \pm 0.26 ^{a,c,d,e} | 4.95 \pm 0.29 ^{a,c,d,e} |
| GP-2 Formalin | - | (2.59) | (11.57) | (9.49) | (10.33) | (14.86) |
| GP-3 | - | 5.61 \pm 0.54 ^{a,b,d} | 4.67 \pm 0.56 ^{a,b,d} | 4.33 \pm 0.62 ^{a,b,d} | 3.92 \pm 0.55 ^{a,b,d,e} | 4.23 \pm 0.49 ^{a,b,d} |
| Indomethacin | 10 | (9.22) | (28.92) | (26.61) | (31.35) | (27.32) |
| GP-4 (NA-2) | 10 | 5.81 \pm 0.26 ^{a,b} | 5.42 \pm 0.35 ^{a,b} | 4.92 \pm 0.42 ^{a,b} | 4.85 \pm 0.22 ^{a,b} | 4.37 \pm 0.27 ^{a,b} |
| | | (5.99) | (17.50) | (16.61) | (15.06) | (24.91) |
| GP-5 (NA-2) | 100 | 5.67 \pm 0.35 ^{a,b} | 4.81 \pm 0.42 ^{a,b} | 4.55 \pm 0.25 ^{a,b} | 4.32 \pm 0.24 ^{a,b,c} | 4.36 \pm 0.21 ^{a,b} |
| | | (8.25) | (26.79) | (22.88) | (24.34) | (25.09) |

Values are mentioned as Mean \pm SD where n = 5 in each group. Significance values were expressed by Tukey-'s post hoc analysis.
 a. Express a significant difference in paw volume when compared to Group 1, Normal control group receives only saline ($p < 0.05$).
 b. Showed significance versus Group 2, Formalin group ($p < 0.05$)
 c. is significant in contrast with Group 3, 10mg/kg pretreated with Indomethacin ($p < 0.05$).
 d. and e. are significant when compared to Group 4 and 5 pretreated with 10mg/kg and 100mg/kg of NA-2 ($p < 0.05$) respectively.

ANALGESIC ACTIVITY

The peripheral analgesic action of NA-2 was investigated in vivo by an acetic acid-induced writhing test in mice as represented in **Error! Reference source not found.** The oral administration of NA-2 at 5, 10, and 100 mg/kg significantly reduced writhing response in comparison with the control group ($p < 0.001$). As shown in **Error! Reference source not found.**IV, NA-2 inhibited the writhing response by 40.91%, 24.24%, and 25.76% at 100, 5, and 10mg/kg, respectively. However, strong analgesic activity of NA-2 was shown at a dose of 100mg/kg. The magnitude of the effect of NA-2 was comparable to the positive control drug Indomethacin exhibited an inhibitory response of 48.48%.

Table IV. Acetic acid induced writhing response (% Inhibition)

| Compound | Dose (mg/kg) | Writhing count (Mean \pm SD) | % Inhibition |
|--------------|--------------|--------------------------------|--------------|
| Control | - | 44 \pm 1 | - |
| Indomethacin | 10 | 22.7 \pm 2.1 | 48.48 |
| NA-2 | 5 | 33.3 \pm 1.53 | 24.24 |
| | 10 | 32.6 \pm 1.53 | 25.76 |
| | 100 | 26 \pm 1 | 40.91 |

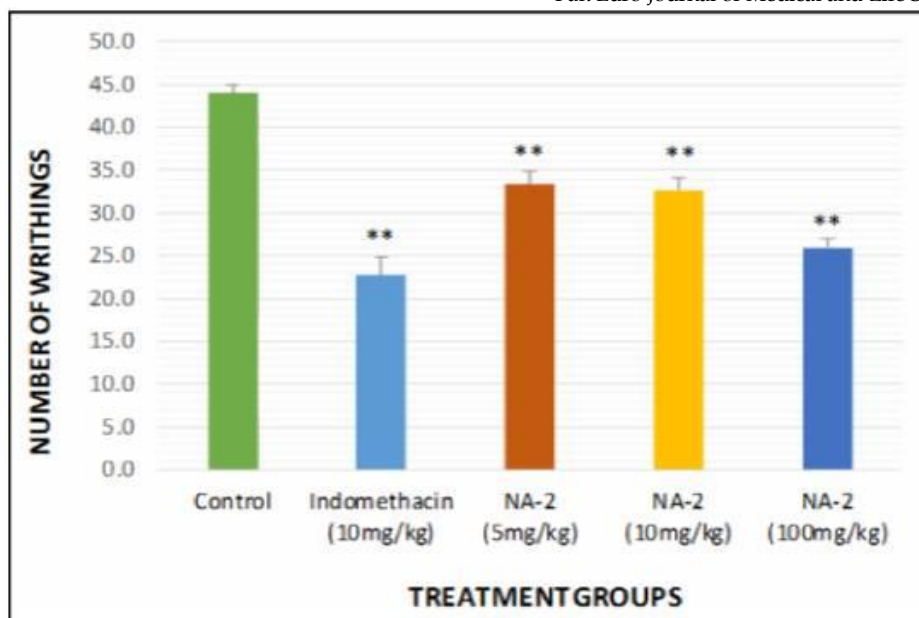


Fig. 3. Effect of NA-2 on writhing response in acetic acid induced writhing test. Results are presented as Mean±SD, **p < 0.001: significant difference compared to the control group done by ANOVA followed by Dunnett's post hoc test

DISCUSSION

Inflammatory responses serve as key player behind the various pathologies in human body like rheumatoid arthritis, asthma, gout and different cancers. In this study N-(2-hydroxy phenyl) acetamide (NA-2) was tested for its *in vitro* and *in vivo* anti-inflammatory, analgesic, and antioxidant efficacy.

In vitro anti-inflammatory effect of NA-2 was evaluated by the means of protein denaturation assay. It is a well-documented method in literature and it provides sensitive and reliable results swiftly. Protein denaturation is a process in which proteins structure is destroyed due to an externally applied stress like heat, any strong acid, or base (12). This leads to activation of type III hypersensitivity reaction which is a major cause of inflammation. Therefore, compounds that show the ability to inhibit protein denaturation indicate that they possess anti-inflammatory activity. A higher degree of inhibition reveals higher anti-inflammatory potential. Results of this study showed significant concentration-dependent inhibition of protein denaturation by NA-2. Whereas the standard diclofenac, a widely used NSAID, also showed marked protein denaturation inhibition at all concentrations, as anticipated (13). Ranaweera *et al.* (2014) states that bioassay of protein denaturation inhibition also acts as an index for evaluation of the anti-arthritis activity. Because in arthritic conditions autoantigens are produced due to protein denaturation, thus NSAIDs used in such conditions impair heat-induced denaturation of proteins. Therefore, the outcomes of this study confirmed the anti-arthritis potential of NA-2 formerly reported by Perveen *et al.* (2014) (14). However, its effects on protein denaturation were not studied.

The onset of inflammatory response is closely associated with the high availability of Free radicals or reactive oxygen species that are capable of damaging DNA and triggering inflammatory mediators. Xanthine oxidase (XO) is also a source of free radicals and is involved in various pathological conditions like inflammation, ischemia, aging, etc (15). Osman *et al.* (2016) mention that XO plays a crucial role in the pathogenesis of gout because it facilitates the formation of uric acid. The potential for xanthine oxidase inhibition is required for the treatment and prevention of inflammatory conditions like gouty arthritis (16).

In this study, a xanthine oxidase assay was performed for *in-vitro* evaluation of the antioxidant property. According to the results, NA-2 showed significant XO inhibition at higher concentrations in comparison to allopurinol, a well-known xanthine oxidase inhibitor also showed a marked XO inhibitory effect. Thus, our results suggest the potential of NA-2 be used as a treatment for gouty arthritis and other inflammatory diseases. However, further *in-vivo* studies are required to determine the therapeutic and toxic levels for consumption (10).

In former studies it has been conveyed that the compounds having anti-inflammatory potential might produce their effects by antioxidant action or scavenging free radicals. To further assess the antioxidant property, the DPPH scavenging method was selected to evaluate the capacity of NA-2 to

scavenge free radical formation. In the presence of an antioxidant molecule, DPPH free radical is reduced and becomes stable due to their hydrogen donating ability. The results of this assay are also expressed as IC₅₀ values, which is defined as the concentration of the compound that can scavenge 50% of DPPH free radical (17). This study revealed the anti-oxidant effect of NA-2, which showed incredible DPPH scavenging activity at higher concentrations. Moreover, when compared with standard Ascorbic acid, NA-2 showed almost similar free radical scavenging activity. Outcomes of both of these assays draw a conclusion that NA-2 should be considered as a potential antioxidant compound.

Formalin induced paw edema method is the mainly used *in vivo* method for evaluating the anti-inflammatory activity of the compounds. This method involves a two phase response; the initial part involves neurogenic pain; and the later phase involves inflammatory reactions due to the release of inflammatory mediators; serotonin, PGs, bradykinin, histamine, cytokines like IL 1 β , IL 6 TNF α , and Nitric oxide (18). After Formalin injection, an increase in paw volume, weight, or thickness is considered as a marker for inflammatory response. In the present study, NA-2 showed a strong inhibitory effect on the development of paw edema. This effect can be attributed to the inhibition of vasoactive substances and prostaglandins however, further studies are required to elucidate underlying mechanisms (19). Perveen K *et al.*, (2013) reviewed the role of NA-2 on the development of arthritis and observed a visible reduction in the severity of the disease. On the basis of the results, they suggested NA-2 as a potential therapeutic agent for arthritis (10).

By Acetic acid induced writhing test, *in vivo* analgesic effects of NA-2 were evaluated. It is a well-known method for the determination of peripheral anti-nociceptive activity. Acetic acid produces peritoneal inflammation. Its intraperitoneal (IP) injection leads to increased prostaglandins (PGs) in peritoneal exudates which causes abdominal constrictions (20).

Karim N *et al.* (2019) suggested that pain is produced by inflammatory mediators PGE₂, serotonin, histamine, bradykinin, and cytokines like TNF- α , IL 6, and IL 1 β . In the present study, NA-2 and the standard Indomethacin considerably reduced the number of writhes. Additionally, NA-2 showed a dose-dependent inhibitory response implying that it possesses a notable peripheral analgesic effect (21). The anti-inflammatory and XO inhibitory promising results of NA-2 suggest its future perspective as a serum urate-lowering drug and a better treatment option for complications associated with various inflammatory pathologies.

CONCLUSION

The existing results showed that NA-2 has significant *in vitro* and *in vivo* Anti-inflammatory, antioxidant, and XO inhibitory activities. This study showed that NA-2 had the potential to serve as an anti-inflammatory, analgesic, and anti-gout drug with lower adverse effects, However, further *in vivo*, clinical and mechanistic investigations are required to validate that NA-2 can serve as a promising drug candidate for various diseases associated with inflammation and xanthine oxidase.

Conflict of Interest:

The authors declare no conflict of interest.

Acknowledgement:

The financial support for this research is given by the internal faculty research grant of Ziauddin University. SM and SUS participated in research design. AA¹, MS conducted experiments. SM and AA¹, AA¹, SSK, HKM performed data analysis. AA¹, ML, NK and MS wrote the manuscript. SM, SUS, AA³, SSK and other authors proofread and edited the final manuscript.

Authors Contribution:

SM and SUS participated in research design. AA¹, MI conducted experiments. SM and AA¹, AA³, SSK, HKM performed data analysis. AA¹, ML, NK and MI wrote the manuscript. SM, SUS, AA³, SSK and other authors proofread and edited the final manuscript.



Ethical Approval:

The animal procedures were performed according to standard ethical guidelines approved by Animal ethics committee of Ziauddin University, Karachi, Pakistan.

Human Subjects:

This study does not involve any human participants.

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