

## Original Research Article

# Novel hydroquinone derivatives alleviate algia, inflammation and pyrexia in the absence of gastric ulcerogenicity

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### Abstract

**Purpose:** To synthesize and characterize novel hydroquinone compounds that exhibit an aspirin-like pharmacological profile devoid of ulcerogenic side effects.

**Methods:** Two novel hydroquinone derivatives, viz, 2,5-bis(piperidinomethyl)hydroquinone and 2,5-bis(pyrrolidinomethyl)hydroquinone, were synthesized by refluxing hydroquinone, paraformaldehyde and secondary amines (piperidine or pyrrolidine) in ethanol. The structures were authenticated by infrared (IR) spectroscopy, elemental analysis, mass spectrometry (MS) and <sup>1</sup>H and <sup>13</sup>C nuclear magnetic resonance (NMR) spectroscopic techniques. The synthesized derivatives were evaluated for antinociceptive, anti-inflammatory and antipyretic activities along with gastric-ulcerogenicity using well-known testing paradigms. Aspirin served as reference standard.

**Results:** The newly synthesized hydroquinone derivatives, significantly attenuated tonic visceral chemically-induced nociception at 10 mg/kg ( $p < 0.01$ ,  $p < 0.001$ ), 20 and 40 mg/kg ( $p < 0.001$ ), inhibited the temporal-inflammatory reaction at 50 mg/kg (2 - 5 h,  $p < 0.05$ ,  $p < 0.001$ ), 100 and 150 mg/kg (1 - 5 h,  $p < 0.05$ ,  $p < 0.01$ ,  $p < 0.001$ ) in addition to alleviating the febrile-response at test doses during 0.5 h ( $p < 0.05$ ,  $p < 0.01$ ,  $p < 0.001$ ), 1 and 1.5 h ( $p < 0.001$ ) of the study period. The synthesized compounds exhibited improved gastric tolerability profile since they were devoid of aspirin-associated biochemical and ulcerative changes. The *in silico* studies predicted high binding affinity of the hydroquinone derivatives to the active site of the cyclooxygenase 2 (COX-2) enzyme.

**Conclusion:** The synthesized hydroquinone compounds possess analgesic, antipyretic and anti-inflammatory properties with low gastric-ulcerogenic potential. This may be credited to preferential inhibition of the COX-2 enzyme and the beneficial basic rather than acidic chemical nature of the compounds. However, further molecular studies are required to substantiate these findings.

**Keywords:** 2,5-Bis(piperidinomethyl)hydroquinone], 2,5-Bis(pyrrolidinomethyl)hydroquinone, Anti-inflammatory, Antinociceptive, Antipyretic, Gastric-ulcerogenicity, Algesia

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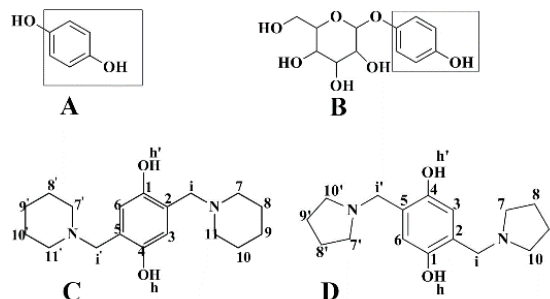
## INTRODUCTION

Non-steroidal anti-inflammatory drugs (NSAIDs) are classic therapeutic agents used predominantly for inflammation, pyrexia and mild to moderate pain [1]. However, their clinical usefulness is limited by accompanying side effects, which include gastric ulceration, bleeding from the gastrointestinal tract, cardiovascular problems and renal complications [2,3]. This is a major restraint on the remedial application of NSAIDs. Therefore, there is a need for therapeutic compounds that are efficacious in alleviating inflammation, pain and pyrexia, while exhibiting fewer gastrointestinal side effects.

Hydroquinone or benzene-1,4-diol may be obtained by hydrolysis of arbutin (Figure 1A) using the enzyme,  $\beta$ -glucosidase [4]. Arbutin is a glycoside of hydroquinone which may be extracted from the leaves of bearberry, *Arctostaphylos uvaursi* [5]. It has anti-inflammatory and anti-tyrosinase activities, and is devoid of any gastric toxicity [6,7]. In this respect, hydroquinone has anti-inflammatory properties which are mediated through inhibition of proinflammatory cytokines (TNF- $\alpha$ , IL-1 $\beta$ , IL-6) or via release of toxic substances like nitric oxide (NO) [8]. The hydroquinone ring structure is very interesting because it lends itself to many pharmacologically active moieties. For example, acetaminophen, a well-known analgesic, has been synthesized by substitution in the hydroquinone structure [9].

The compounds reported in our study, viz, 2,5-bis(piperidinomethyl)hydroquinone (compound **1**) and 2,5-bis(pyrrolidinomethyl)hydroquinone (compound **2**) are derived from hydroquinone ring substitution, via integration of pharmacologically important piperidine and pyrrolidine heterocyclic moieties. It has been reported that chemical compounds containing these moieties possess diverse pharmacological properties including antinociceptive, anti-inflammatory and antipyretic activities [10,11]. Similarly, pyrrolidine ring-containing compounds have been shown to be effective against various disease conditions such as migraine, bipolar disorders, tuberculosis and cancer [12,13]. In this connection, piperidine and pyrrolidine heterocycles are considered as critical nuclei for novel drug design. These moieties associated with diverse pharmacological activities encouraged us to incorporate them into the basic hydroquinone structure. Therefore, hydroquinone derivatives were synthesized and their structures were confirmed by different spectroscopic methods (IR,  $^1\text{H-NMR}$ ,  $^{13}\text{C-NMR}$ , mass spectrometry and elemental analysis) and were then evaluated for antinociceptive, antipyretic

and anti-inflammatory activities as well as any gastro-ulcerogenic proclivity, using *in vivo* testing paradigms. Additionally, the binding affinity of these compounds with cyclooxygenase (COX) enzymes (COX-1 and COX-2) was studied using a molecular docking simulation approach.



**Figure 1:** Chemical structures of hydroquinone (A), arbutin (B), compound **1** (C) and compound **2** (D)

## EXPERIMENTAL

### Instruments and apparatus

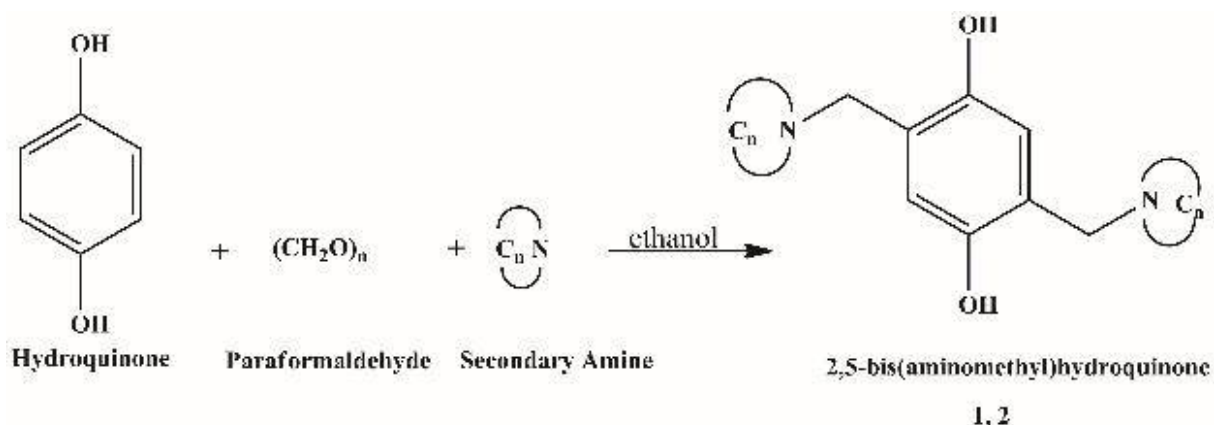
A Nicolet 380 thermoscientific FTIR (USA) instrument was used for recording Infrared (IR) spectra.  $^1\text{H}$  and  $^{13}\text{C-NMR}$  analyses (Bruker AVANCE 400 MHz, Germany) were conducted using DMSO- $d_6$  or  $\text{D}_2\text{O}$  as solvents. Mass spectra (ESI-MS) were obtained on a Bruker micrOTOF II (USA) mass spectrometer. Elemental analyses were performed on an Elementar Analysensysteme (GmbH VarioEL V4.01 20.Aug. 2002 (Germany) instrument operated in CHN mode. Histological slides were prepared with the help of a microtome (SLEE MAINZ, Germany).

### Synthesis of hydroquinone derivatives

Hydroquinone derivatives were prepared by a simple reaction between secondary amine, paraformaldehyde and hydroquinone. While synthesizing these derivatives, quinone was amino methylated according to a known method [14] as given in Scheme 1. The synthesized compounds were obtained in a good yield.

#### **Compound 1 (2,5-bis(piperidinomethyl)hydroquinone)**

Piperidine (20 mmol, 1.96 mL) and paraformaldehyde (33.3 mmol, 1 g) were slowly mixed and then refluxed for 1 h to obtain a clear solution. The resulting solution was cooled to room temperature ( $25\text{ }^\circ\text{C}$ ) and a solution of hydroquinone (10 mmol, 1.1 g) in ethanol (100 mL) was added to the above mixture. The mixture was stirred using a magnetic stirrer and



Where: 1)  $n = 4$ , Compound 1

2)  $n = 5$ , Compound 2

**Scheme 1:** Synthetic route for compounds 1 and 2

was monitored by taking samples for TLC at 1 h interval. The reaction was completed after 8 hours. After removal of solvent at ambient temperature, a brownish solid was obtained which was recrystallized three times with ethanol to obtain pure white crystals (Figure 1C).

#### **Compound 2 (2,5-bis(pyrrolidinomethyl)hydroquinone)**

A mixture of hydroquinone (10 mmol, 1.1 g), pyrrolidine (20 mmol, 1.64 mL) and paraformaldehyde (33.3 mmol, 1 g) was refluxed in ethanol (100 mL) for 20 h. The resulting solution was cooled to room temperature (25 °C) and filtered to obtain a brownish solid. The solid was recrystallized three times with ethanol in order to remove any entrapped impurity and to yield pure white crystals of compound (2) (Figure 1D).

#### **Animals**

BALB/c mice and Sprague–Dawley rats of either sex were used and housed separately as 6 animals per cage, under laboratory conditions of  $22 \pm 2$  °C, 40 - 60 % relative humidity (RH) and 12 h light/12 h dark cycle. The experimental animals were provided free access to food and water and were kept under the standard laboratory conditions in the animal housing facility established at Department of Pharmacy, University of Peshawar, Pakistan. Prior to commencement of the experiments, animals were allowed to habituate for 2 h to the laboratory conditions. The experimental procedures reported below were approved by the Institutional Animals Ethics Committee with registration no. 11/ EC-15/Pharm and performed in compliance with the UK approved guidelines for laboratory animals [15].

#### **Acute toxicity test**

Mice (25-30 g,  $n = 6$ ) were administered with test compounds intraperitoneally (i.p.) at 50, 100, 250 and 500 mg/kg. Morbidity was observed continuously for the first 2 h, while mortality up to 24 h after dose administration. The animals were observed for impulsive activity, cyanosis, convulsions, catalepsy, contortions, tail pinch response, and abnormal behavior [16].

#### **Determination of antinociceptive activity**

##### **Acetic acid-induced abdominal constriction assay**

BALB/c mice (18-22 g,  $n = 6$ ) were randomly divided into ten groups. Group I was administered normal saline (i.p.). Group II-IV received standard aspirin at 10, 20 and 40 mg/kg (i.p.), respectively. Similarly, groups V-VII received compound (1) and groups VIII-X received compound (2) at 10, 20 and 40 mg/kg (i.p.), respectively. Thirty min after treatment, 1 % acetic acid (i.p.) was injected and after a further 5 min, abdominal constrictions (writhes) for each group were determined and the behavior was observed for 20 min [17]. Percentage protection (PP) was calculated as in Eq 1.

$$PP = \{(1 - Mt/Mc)\}100 \dots\dots\dots (1)$$

where *Mt* and *Mc* represented mean number of abdominal constrictions of the treated drug and the control group, respectively.

##### **Hot plate assay**

BALB/c mice (18-22 g,  $n = 6$ ) exhibiting a latency < 15 s signified by a hind limb flick, licking or jumping, when placed on the hot plate ( $54.0 \pm$

0.10 °C, Harvard apparatus, USA) were included in the study. Animals were administered compounds (1) or (2) (10, 20 and 40 mg/kg, i.p.) or morphine (5 mg/kg, i.p.) and 30 min after each treatment, response latencies were noted at 30, 60 and 90 min. To avoid tissue injury, a 30 s cut-off time was imposed [18]. The percent antinociception (PA) was calculated as in Eq 2.

$$PA = \{(TI - CI/Ct - CI)\}100 \dots \dots \dots (2)$$

where *TI*, *CI* and *Ct* represented test latency, control latency and cut-off time, respectively.

### Carrageenan induced inflammatory assay

BALB/c mice (25 - 30 g, *n* = 6) were divided into ten groups in carrageenan-induced inflammatory assay. Group I received normal saline (i.p.). Groups II - IV received standard aspirin at 50, 100 and 150 mg/kg (i.p.), respectively. Similarly, groups V - VII received compound (1) and groups VIII-X received compound (2) at 50, 100 and 150 mg/kg (i.p.), respectively. One hour after treatment, each group received 1 % carrageenan suspension, injected subcutaneously (s.c) into the planter region of the rear paw. Mean paw swelling was measured using a digital plethysmometer (Model LE 7500 Plan lab S.L, Italy) before carrageenan administration and thereafter at 1 h intervals for 5 h [19]. Inhibition of edema (H) was calculated as in Eq 3.

$$H (\%) = \{(A - B)/A\}100 \dots \dots \dots (3)$$

where A is the mean paw volume of control and B represented the mean paw edema volume of the test group.

### Brewer's yeast induced pyrexia assay

BALB/c mice (25 - 30 g) were divided into ten different groups of six animals per group. All animals were deprived of food with free access to water. The baseline rectal temperature of all mice was noted by inserting a lubricated digital thermometer probe (model CA92121, ACON Laboratories, USA) into the rectum (to a depth of 2 cm) of each animal and then pyrexia was induced using a suspension of 20 % Brewer's yeast (Merck, Germany, 10 mL/kg) by the subcutaneous route.

Any change in the rectal temperature of each animal was measured 24 h after Brewer's yeast administration and those animals which expressed a rise of 0.3 to 0.5 °C were selected for the study [20]. Group I was treated with normal saline (i.p.). Groups II - IV received aspirin (50, 100 and 150 mg/kg, i.p, respectively),

groups V-VII received compound (1) while the remaining groups (VIII - X) received compound (2) at doses of 50, 100 and 150 mg/kg, i.p., respectively. Following drug administration, the rectal temperature of each mouse was measured at 0.5, 1 and 1.5 h. The mean decrease in rectal temperature across treated groups was compared relative to untreated controls.

### Assessment of gastric ulcerogenicity

Sprague-Dawley rats (150 - 200 g, *n* = 6) were randomly divide to treatment groups and each animal was administered the following treatment once a day orally for 6 days as follows:

- Group I – normal saline
- Group II – aspirin (150 mg/kg)
- Groups III – compound (1) (100 mg/kg)
- Groups IV – compound (1) (150 mg/kg)
- Groups V – compound (2) (100 mg/kg)
- Group VI – compound (2) (150 mg/kg).

The last dose for each animal group was administered 1 h prior to pyloric ligation. At the end of the protocol, each rat was killed and the stomach mucosa was removed. The macroscopic injury was observed with 10x lens and ulcer severity was scored [21]. The gastric content was also collected and centrifuged for 10 min at 1,000 rpm and the supernatant was taken apart. The volume (ml), pH, free acidity, total acidity and pepsin concentration of the gastric juice were determined using the procedures reported previously in our laboratory [22]. After centrifugation, the gastric juice volume was measured with a pipette and pH was determined with a digital pH meter. For determination of free acidity, 1 mL of supernatant was mixed with 2 – 3 drops of Toper's reagent and was titrated against 0.01 N NaOH.

The changes in the color of solution from light red to canary yellow determined the free acidity. To this solution, two drops of phenolphthalein indicator were added and the titration process was continued until the appearance of permanent a pink color. The volume (mL) of alkali utilized was noted and this represented the total acidity. For pepsin activity determination, the absorbance of protein was measured at 700 nm and the results were presented as micromoles of tyrosine liberated per mL.

### Assessment of histopathological changes

At the end of each experiment, the stomach specimens were fixed immediately in formalin (10 % neutrally buffered) for 48 h, dehydrated in variant ethanol (50–100 %) solutions, cleared in

100 % xylene and immersed in paraffin. Sections (5  $\mu\text{m}$  thickness) were cleared, hydrated and then stained (hematoxylin and eosin) [23]. The prepared slides were evaluated for any histopathological changes.

### In silico studies

The structures of cyclooxygenase-1 (COX-1) with bound Flurbiprofen (PDB ID 1CQE) and cyclooxygenase-2 (COX-2) enzyme with bound selective inhibitor (PDB ID 1CX2) were downloaded from the website ([www.rcsb.org/pdb](http://www.rcsb.org/pdb)) of the Protein Data Bank (PDB) and the binding site residues of the receptors were identified. Bound ligands were then removed from the receptor binding site using discovery studio 4.0 (Dassault Systèmes BIOVIA, Discovery Studio Modeling Environment, Release 2017, San Diego: Dassault Systèmes, 2016).

The enzyme and ligand structures were then prepared for docking using Autodock Tools. The study ligands namely 2,5-bis(piperidinomethyl) hydroquinone (compound 1) and 2,5-bis(pyrrolidinomethyl)hydroquinone (compound 2) were then docked into the binding site of COX active sites using Autodock Vina [24]. Nine binding modes were proposed by the program and the binding mode with lowest binding energy and suitable interactions was selected and studied.

### Statistical analysis

Statistical significance of pharmacological data was determined using one-way ANOVA followed by *post hoc* Dunnett's test.  $P < 0.05$  level was considered as significant. Data are expressed as mean  $\pm$  SEM ( $n = 6$ ).

## RESULTS

### Spectral characteristics of synthesized molecules

#### Compound 1 (2,5-bis(piperidinomethyl) hydroquinone)

White crystals; Yield: 56.0 %, m.p. 189.0  $^{\circ}\text{C}$ . IR (KBr)  $\nu_{\text{max}}$ : 3853, 2945, 1452, 1377, 1307, 1107, 988, 879, 787  $\text{cm}^{-1}$ .  $^1\text{H}$  NMR (400 MHz, DMSO- $d_6$ )  $\delta$  2.39 (t, 8H,  $\text{H}^{7, 7', 11, 11'}$ ), 1.42-1.51 (m, 12H,  $\text{H}^{8, 8', 9, 9', 10, 10'}$ ), 3.49 (s, 4H,  $\text{H}^{i, i'}$ ), 10.16 (s, 2H,  $\text{H}^{\text{h}, \text{h}'}$ ), 6.41 (s, 2H,  $\text{H}^{3, 6}$ ).  $^{13}\text{C}$  NMR (75 MHz,  $\text{D}_2\text{O}$ )  $\delta$  24.08 (2C,  $\text{C}^{9, 9'}$ ), 25.94 (4C,  $\text{C}^{8, 8', 10, 10'}$ ), 53.74 (4C,  $\text{C}^{7, 7', 11, 11'}$ ), 60.43 (2C,  $\text{C}^{i, i'}$ ), 115.92 (2C,  $\text{C}^{3, 6}$ ), 121.82 (2C,  $\text{C}^{2, 5}$ ), 149.61 (2C,  $\text{C}^{1, 4}$ ). HRMS (ESI): calcd for  $\text{C}_{18}\text{H}_{28}\text{N}_2\text{O}_2$  305.221:  $[\text{M} + \text{H}]^+$ ,

found: 305.224. Anal. Calcd. for  $\text{C}_{18}\text{H}_{28}\text{N}_2\text{O}_2$ : C, 71.02; H, 9.27; N, 9.20. Found: C, 70.90; H, 10.37; N, 9.38.

#### Compound 2 (2,5-bis(pyrrolidinomethyl) hydroquinone)

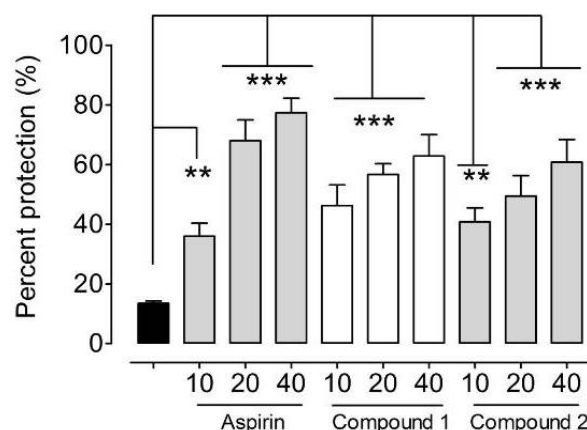
White crystals; Yield: 55.5 %, m.p. 153.8  $^{\circ}\text{C}$ . IR (KBr)  $\nu_{\text{max}}$ : 3848, 2801, 1480, 1389, 1307, 1198, 888, 833  $\text{cm}^{-1}$ .  $^1\text{H}$  NMR (400 MHz, DMSO- $d_6$ )  $\delta$  2.48-2.49 (t, 8H,  $\text{H}^{7, 7', 10, 10'}$ ), 1.72-1.76 (m, 8H,  $\text{H}^{8, 8', 9, 9'}$ ), 3.61 (s, 4H,  $\text{H}^{i, i'}$ ), 9.93 (s, 2H,  $\text{H}^{\text{h}, \text{h}'}$ ), 6.46 (s, 2H,  $\text{H}^{3, 6}$ ).  $^{13}\text{C}$  NMR (75 MHz,  $\text{D}_2\text{O}$ )  $\delta$  23.60 (4C,  $\text{C}^{8, 8', 9, 9'}$ ), 53.54 (4C,  $\text{C}^{7, 7', 10, 10'}$ ), 56.78 (2C,  $\text{C}^{i, i'}$ ), 115.47 (2C,  $\text{C}^{3, 6}$ ), 122.88 (2C,  $\text{C}^{2, 5}$ ), 149.23 (2C,  $\text{C}^{1, 4}$ ). HRMS (ESI): calcd for  $\text{C}_{16}\text{H}_{24}\text{N}_2\text{O}_2$  277.188:  $[\text{M} + \text{H}]^+$ , found: 277.191. Anal. Calcd. for  $\text{C}_{16}\text{H}_{24}\text{N}_2\text{O}_2$ : C, 69.53; H, 8.75; N, 10.14. Found: C, 69.17; H, 9.16; N, 9.88.

### Acute toxicity

Both compounds did not reveal any behavioral changes or mortality at doses up to 500 mg/kg.

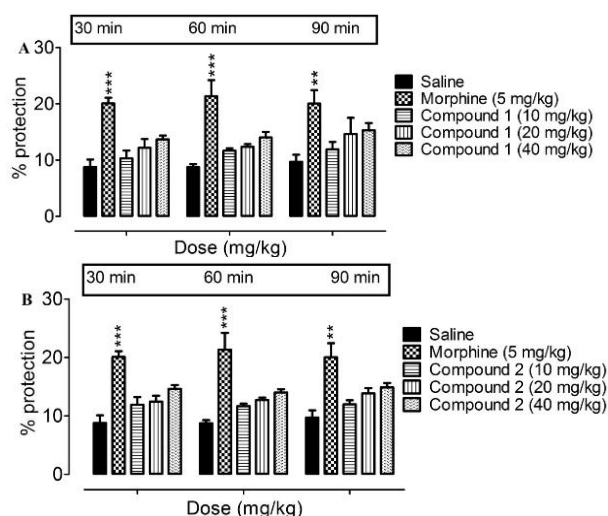
### Compounds (1) and (2) attenuated tonic visceral nociception

As shown in Figure 2, a significant attenuation of tonic visceral nociception was produced by aspirin at doses of 10 mg/kg ( $p < 0.01$ ), 20 and 40 mg/kg ( $p < 0.001$ ) and also the head to head doses of compound (1) ( $p < 0.001$ ) in the acetic acid-induced abdominal constrictions assay. Likewise, compound (2) also afforded maximum protection against nociceptive behavior at the tested doses of 10 mg/kg ( $p < 0.01$ ), 20 and 40 mg/kg ( $p < 0.001$ ). In the hot plate test, both compounds (1) and (2) were inactive at all tested



**Figure 2:** Antinociceptive effect of aspirin, compounds 1 and 2, at 10, 20 and 40 mg/kg (i.p.). Values are percent inhibition of abdominal constrictions  $\pm$  SEM ( $n = 6$ ); \*\* $p < 0.01$ , \*\*\* $p < 0.001$  as compared to saline treated control;  $n = 6$

doses against acute phasic nociception, however, in comparison morphine (5 mg/kg) provided significant protection against thermal hyperalgesia after 30, 60 min ( $p < 0.001$ ) and 90 min ( $p < 0.01$ ) as shown in Figure 3.



**Figure 3:** Antinociceptive effect of (A) compound 1, and (B) compound 2, at 10, 20, 40 mg/kg (i.p.) in the hot plate assay. Values are percent response latencies  $\pm$  SEM of 6 mice per group; \*\* $p < 0.01$ , \*\*\* $p < 0.001$  as compared to saline treated control

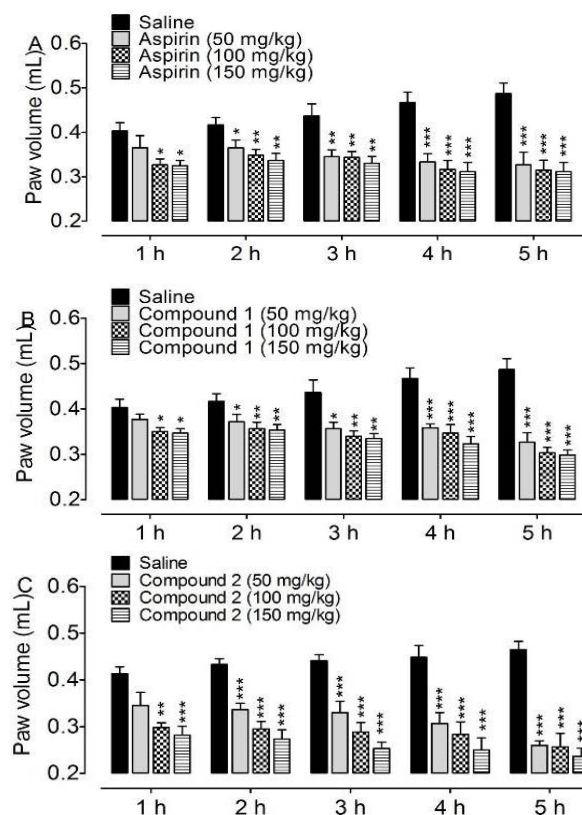
### Compounds (1) and (2) alleviated temporal inflammatory response

After 1 h, a significant reduction in paw swelling was observed in response to doses of 100 and 150 mg/kg of aspirin ( $p < 0.05$ ), compound (1) ( $p < 0.05$ ) and compound (2) ( $p < 0.01$ ,  $p < 0.001$ ). After 2 h, all the test doses (50 mg/kg,  $p < 0.05$ ; 100 and 150 mg/kg;  $p < 0.01$ ) of aspirin and compound (1) downgraded the inflammatory response. These doses of aspirin as well as compound (1) were more effective during the late phase of inflammation, where a significant diminution of the swelled paw was observed at 3 h ( $p < 0.05$ ,  $p < 0.01$ ) and 4-5 h ( $p < 0.001$ ). Compound (2) was more effective ( $p < 0.001$ ) in decreasing the edema induced by the phlogistic agent and was able to counteract both early and late phases of inflammation (Figure 4).

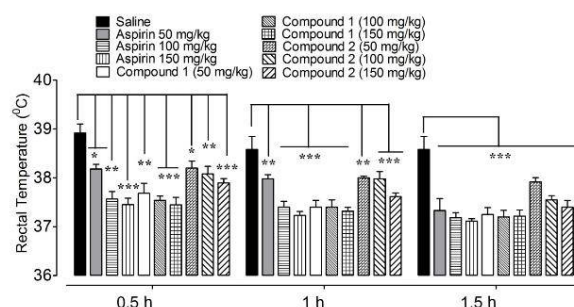
### Compounds (1) and (2) suppressed yeast-induced pyrexia

As shown in Figure 5, after 0.5 h, a significant decrease in the Brewer's yeast induced febrile response was observed in response to aspirin, compounds (1) and (2) at the tested doses of 50 mg/kg ( $p < 0.05$ ,  $p < 0.01$ ), 100 mg/kg ( $p < 0.01$ ,  $p < 0.001$ ) and 150 mg/kg ( $p < 0.001$ ). As time progressed, the reduction in body temperature become even more highly significant and the antipyretic effect was even more marked after 1

and 1.5 h for aspirin, compounds (1) and (2), when evaluated at doses of 50, 100 mg/kg ( $p < 0.01$ ,  $p < 0.001$ ) and 150 mg/kg ( $p < 0.001$ ).



**Figure 4:** Anti-inflammatory effect of (A) aspirin, (B) compound 1 and (C) compound 2, at 50, 100 and 150 mg/kg (i.p.). Values are mean paw volume in mL  $\pm$  SEM ( $n = 6$ ); \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  as compared to saline treated control



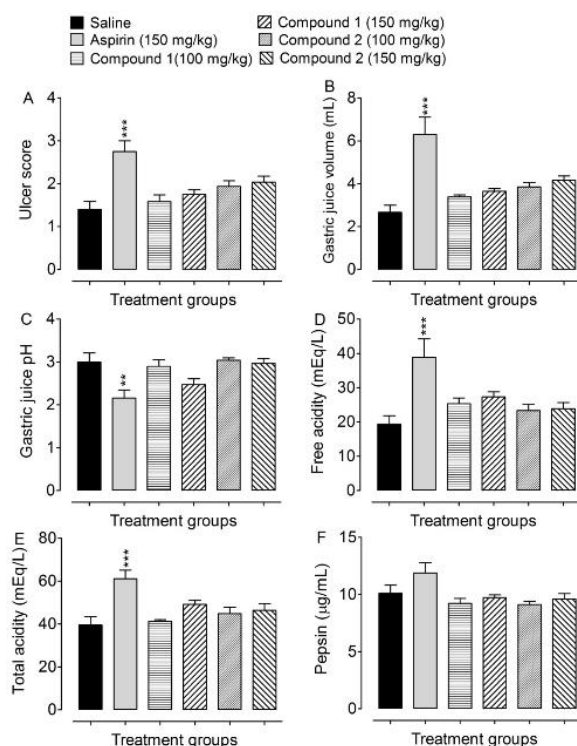
**Figure 5:** Antipyretic effect of aspirin, compounds 1 and 2, at 50, 100 and 150 mg/kg (i.p.). Values are mean rectal temperature in  $^{\circ}\text{C}$ ,  $\pm$  SEM ( $n = 6$ ); \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  as compared to saline treated control

### Compounds (1) and (2) were devoid of gastric-ulcerogenicity

Figure 6A depicted the dose of 150 mg/kg of aspirin per day for a period of 6 consecutive days and it significantly increased the ulcer score ( $F_{5,30} = 7.79$ ,  $p < 0.001$ ), the volume of gastric juice ( $F_{5,30} = 10.64$ ,  $p < 0.001$ , Figure 6B), gastric pH

( $F_{5,30} = 5.47$ ,  $p < 0.01$ , Figure 6C), free acidity ( $F_{5,30} = 5.75$ ,  $p < 0.001$ , Figure 6D) and total acidity ( $F_{5,30} = 6.56$ ,  $p < 0.001$ , Figure 6E), when compared to control. However, these parameters were unchanged with the tested compounds at 100 and 150 mg/kg, and they were comparable to that of control animals. Moreover, the pepsin concentration was not affected in any of the treatment groups (Figure 6F).

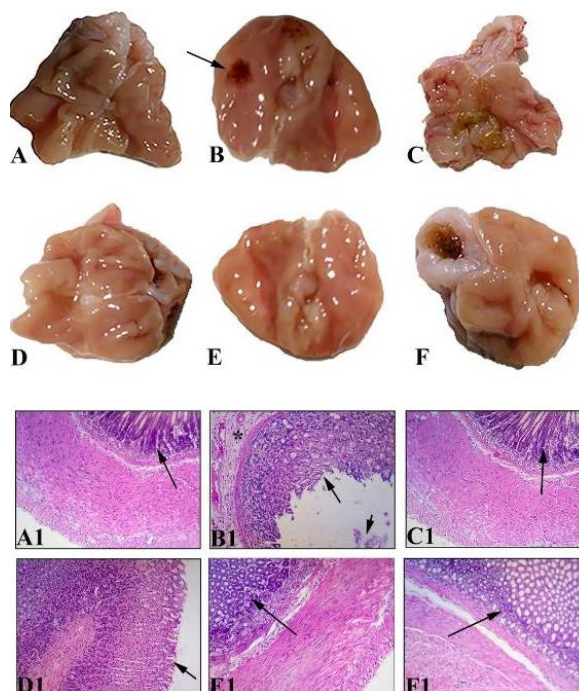
Examination of gastric mucosa (photomicrographs) revealed an absence of morphological changes (hemorrhagic streaks or spot ulcers) in the stomach mucosa of animal groups treated with compounds (1) and (2) and saline. Nonetheless, visually noticeable changes were perceived in the mucosa of aspirin-treated group (Figure 7A - F).



**Figure 6:** Ulcerogenicity of aspirin (150 mg/kg), compound 1 and compound 2 (100, 150 mg/kg). Each bar represents (A) ulcer score, (B) gastric juice volume, (C) gastric pH, (D) free acidity, (E) total acidity, and (F) pepsin concentration  $\pm$  SEM ( $n = 6$ ); \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , compared to saline control

Using light microscopy, the gastric mucosa of control rats revealed normal histo-architecture with undamaged structures and normal epithelial lining (Figure 7A1). The gastric mucosa of aspirin treated rats showed damaged mucosal epithelium, submucosal edema and inflammation at the ulcer site (Figure 7B1); whereas, rats administered compound 1 (100, 150 mg/kg, Figure 7C1 and D1) and compound 2 (100, 150 mg/kg, Figure 7E1 and F1) exhibited no

ulceration, and the histological appearance was similar to that of control animals.



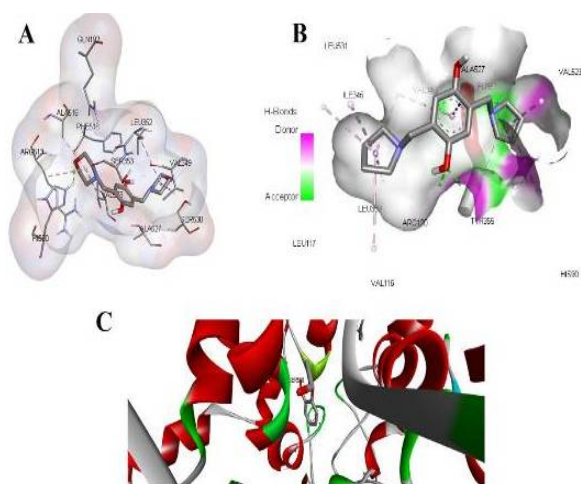
**Figure 7:** Gross appearance of stomach mucosa from groups of animals treated with (A) saline control rats; (B) aspirin treated rats showing ulcer spots and bleeding; (C) compound 1 (100 mg/kg) treated rats; (D) compound 1 (150 mg/kg) treated rats; (E) compound 2 (100 mg/kg) treated rats, and (F) compound 2 (150 mg/kg) treated rat, which were essentially devoid of ulcers. Sections of gastric mucosa of control and experimental animals were stained by employing hematoxylin and eosin and were observed under light microscopy. (A1) The control group exhibited normal structure of the epithelial lining (arrowed). (B1) The aspirin (150 mg/kg) treated group displayed disruption and erosion of mucosa (large arrow), infiltration of red blood cells and lymphocytes in the submucosa (asterisk) along with necrotic debris visible in the lumen (small arrow) of the ulcer, while a normal gastric epithelial lining (arrows) was observed for (C1) compound 1 (100 mg/kg), (D1) compound 1 (150 mg/kg), (E1) compound 2 (100 mg/kg) and (F1) compound 2 (150 mg/kg) which are similar to that of control. *Magnification: 10x*

### Compounds (1) and (2) exhibited an antagonistic tendency against COX enzymes

The active sites of both isoforms of COX were predominantly hydrophobic except for the Arg120 residue. The active site binding mechanism of NSAIDs to the COX isoforms involved extensive non-covalent  $\pi$ -interactions, in addition to the charged interactions of the ionized carboxylic group of the NSAID with the protonated arginine side chain.

The results of our docking study show that aspirin (2-Acetoxy Benzoic Acid) binds both of the cyclooxygenase isoforms. Aspirin was present near a Serine residue (SER 530) of the cyclooxygenase-2 enzyme, which is located outside the active site of the enzyme (Figure 8C). Aspirin is known to act through acetylation of this serine residue by the transfer of its own acetyl group. Our docking results predict binding of aspirin to SER 530 of both cyclooxygenase isoforms through the same mechanism. On other hand, compounds (1) and (2) showed higher affinity to the COX-2 isoform mainly due to H-bonding with this enzyme. The most favourable binding modes of compounds (1) (Figure 8A) and compound (2) (Figure 8B) showed H-bonding of the hydroxyl groups on the hydroquinone nucleus with the histidine residue (His 90 in compound 1) and tyrosine and arginine residues (Tyr 355 and ARG 120 in compound 2).

Compounds (1) and (2) were docked in the cavity of COX-1 enzyme. The binding to COX-1 is mainly of hydrophobic nature and binding energy to this enzyme is considerably higher as compared to the COX-2 isoform. Predictive in-silico modelling divulged an absence of charged interactions between the molecules and the enzyme. However, an extensive hydrophobic interaction could be seen.



**Figure 8:** (A) Compound 1 in the cavity of COX-2. H-bond is represented by a green discontinuous line. Pi-alkyl and Pi-Pi interactions are represented by grey discontinuous lines. (B) Compound 2 shows both H-bonding and hydrophobic interactions with the active site of COX-2. Some of the surface is removed to show the binding mode of the compound clearly. H-bond is represented by green discontinuous line. Pi-alkyl and Pi-Pi interactions are represented by grey discontinuous lines). (C) Aspirin in the vicinity of SER 530 of COX-2. The acetyl group of Aspirin can be seen close to the serine residue in the model of cyclooxygenase enzyme

## DISCUSSION

The therapeutic utility of non-steroidal anti-inflammatory drugs (NSAIDs), especially such a well-established agent as aspirin is greatly hampered by a propensity to induce gastrointestinal tract (GIT) insult, particularly gastric ulceration [25]. Consequently, over the years, extensive research studies have been conducted in order to discover synthetic substitutes for NSAIDs free from ulcerogenic potential. The pharmacological activities of NSAIDs mainly derive from inhibition of cyclooxygenase (COX) enzymes (COX-1 and COX-2) [26]. Thus, nonselective blockade of COX enzymes by NSAIDs results in inhibition of not only prostaglandins that are induced in response to different inflammatory mediators, but also those which play an important role in normal physiological functions such as mucosal protection. Moreover, due to the acidic nature of classical NSAIDs, they produce localized erosion of the stomach mucosa [27]. Arising from these reasons, the excessive use of NSAIDs is linked with GIT adverse events and is mostly restricted.

To overcome the problems of NSAIDs, therefore we need better alternatives that not only have anti-inflammatory, antipyretic and antinociceptive properties but are also free from the side-effects that are commonly encountered with chronic use of NSAIDs. In this study, novel hydroquinone derivatives containing piperidine and pyrrolidine motifs were synthesized and assessed for antinociceptive, anti-inflammatory and antipyretic activities as well as their potential for ulcerogenic toxicity. The rationale for incorporating piperidine and pyrrolidine heterocycles into the hydroquinone structure was based on observations that these moiety containing compounds, when studied previously for ulcerogenic activity, they lacked the potential for instigating detectable ulcerogenicity [22,28].

NSAIDs in general, but aspirin in particular, at one time, formed a basis for the management of moderate pain. In this respect, we compared the analgesic effect of our synthesized compounds with that of aspirin in the acetic acid induced abdominal constriction model of pain, which is commonly used for evaluating peripheral antinociceptive activity [29]. Pain is induced peripherally by the release of a variety of endogenous substances particularly mediators such as prostaglandins via the action of COX-1 and its isoform, COX-2 [30]. Additionally, local peritoneal nociceptive receptors are very much implicated in the abdominal constriction response [31]. Consequently, the current test compounds exhibited marked inhibitory activity on this



nociceptive reaction suggesting the involvement of a peripheral analgesic effect which may well have been mediated via COX enzymes inhibition and/or local peritoneal nociceptors. In contrast, the hot plate test involves a spinal reflex as well as a more centrally located component which when inhibited, reflects central analgesic activity [32]. In our study however, the synthetic compounds did not produce a selective inhibition of thermal nociception. The findings therefore, lend support to the participation only of a peripheral antinociceptive effect in both cases.

Inflammation is the body's complex protective response involving tissue impairment caused by pathogens, mediators and noxious stimuli (chemical or physical) [33]. Therapy of inflammatory diseases is mainly based on interfering with different mediators that direct the host's response to injury [34]. In the current study, the hydroquinone derivatives were tested in the carrageenan-induced mouse paw edema assay. It is known that carrageenan induced paw edema is a tri-phasic event, ascribed to the release of different mediators including histamine and serotonin (1<sup>st</sup> phase), bradykinin and prostaglandins (2<sup>nd</sup> and 3<sup>rd</sup> phase) [35]. The extended edema mitigating action (up to 5 h) displayed by our novel compounds, might conceivably entail an inhibitory element on at least these four inflammatory mediators.

Pyrexia occurs due to an elevation of prostaglandin levels in the hypothalamus as an upshot of increased synthesis of this substance mediated by the release of pyrogenic cytokines in response to exogenous pyrogens [36]. Subcutaneous injection of Brewer's yeast imparts febrile effect and is considered as a classical method for the screening of potential antipyretics. Inhibition of prostaglandin synthesis via blockade of COX enzyme activity can be regarded as a possible mechanism of antipyretic action similar to that of NSAIDs [37]. Accordingly, the antipyretic outcomes are very promising as both compounds displayed strong antipyretic activity comparable to that of aspirin probably implicating prostaglandin inhibition.

The results of the ulcerogenicity evaluation revealed that the synthesized compounds possessed no propensity to incite gastric ulceration as compared to aspirin. Predictably, the ulcerative effects, as confirmed from the biochemical and histological parameters, were exacerbated in aspirin-treated animals. The non-selective COX enzyme inhibition, as well as acidic properties possessed by aspirin, are largely responsible for local gastric mucosal damage [27,38]. However, no significant

changes in ulcer conducive conditions were observed in the case of the novel compounds indicating safe gastric profiles. In addition, the molecular docking study signified that both compounds disclosed a preferential COX-2 enzyme inhibitory capability which could possibly underlie their lack of ulcerogenic activity. Moreover, pH analysis of the test compounds revealed that they were basic in nature. This property might also play a key role in gastric safety because as acidic drugs like aspirin are present in an unionized state in the stomach. Consequently aspirin readily penetrates the gastric mucosa to cause gastric erosion and ulceration. Furthermore, the test compounds were generally well tolerated in the acute toxicity test since no mortality or behavioral discomfort was observed in doses up to 500 mg/kg and this manifested a favourable safety profile.

## CONCLUSION

Novel hydroquinone derivatives functionalized with piperidine and pyrrolidine have been successfully synthesized. These compounds possess potent aspirin-like anti-inflammatory, analgesic and antipyretic activities, but they are devoid of gastric-ulcerogenicity. The absence of gastric toxicity may be due, in part, to preferential COX-2 enzyme inhibitory activity and basic rather than acidic chemical nature. Thus, the synthesized compounds may be superior therapeutic candidates for the management of pain, pyrexia and inflammation. However, further studies at the molecular level are required to establish these findings.

## DECLARATIONS

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### *Conflict of interest*

No conflict of interest is associated with this work.

### *Contribution of authors*

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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