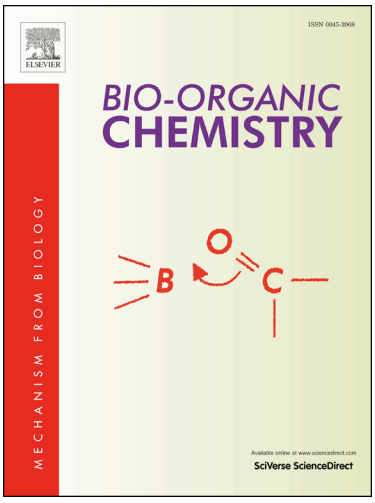

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Multi-targeted dihydrazones as potent biotherapeutics

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Abstract

Hydrazone compounds were considered as a useful moiety in drug design development. Therefore, these studies were aimed at the synthesis of new dihydrazones and were screened for their *in vitro* H^+/K^+-ATPase and anti-inflammatory activities. The results revealed that compounds **9 (**22±0.62 µg/mL**), 10 (**26±0.91 µg/mL**), 15 (**24±0.44 µg/mL**), 16 (**28±0.63 µg/mL**), 17 (**12±0.38 µg/mL**), 18 (**14±0.47 µg/mL**), 19 (**26±0.54 µg/mL**), 20 (**16±0.41 µg/mL**), 25 (**06±0.68 µg/mL) and **26 (**08±0.43 µg/mL) showed excellent H^+/K^+-ATPase activity and their IC_{50} value were lower than the standard drug Omerazole (48±0.12 µg/mL). Compounds **5 (**28±0.65 µg/mL**), 6 (**24±0.61 µg/mL**), 7 (**28±0.64 µg/mL**), 8 (**26±0.45 µg/mL**), 11 (**30±0.74 µg/mL**), 12 (**28±0.40 µg/mL**), 13 (**32±0.24 µg/mL**), 14 (**30±0.55 µg/mL) and **21 (**08±0.47 µg/mL**), 22 (**12±0.47 µg/mL**), 23 (**10±0.51 µg/mL) and **24 (**14±0.84 µg/mL) showed better anti-inflammatory activity compared to standard indomethacin (44±0.15 µg/mL). The structure activity relationship (SAR) showed that, electron donating groups (OH, OCH_3) favored the H^+/K^+-ATPase and antioxidants activity, whereas, electron withdrawing groups (F, Cl, Br and NO_2) favored the anti-inflammatory

activity. Furthermore, molecular docking study was performed to investigate the binding interactions of the most active analogs with the active site of H^+/K^+-ATPase enzyme. Compounds **25 (**G-score = -9.063) and **26 (**G-score = -8.977) showed the highest docking G-scores for H^+/K^+-ATPase inhibition activity.

Key words: Dihydrazones; H^+/K^+-ATPase; anti-inflammatory activity; antioxidants; docking study.

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1. Introduction

The continuous active research in the area of chemistry and biology helps pharmaceuticals to reduce the pain and other associated impairments by medicine, which is reported 90% as statistics of world health organization (WHO). The advanced knowledge of endogenous nociceptive and antinociceptive systems, different pain associated diseases such as rheumatoid arthritis and advanced cancers are away from targeted or particular medicine to cure till date [1]. Currently available nonsteroidal anti-inflammatory drugs (NSAIDs) like ibuprofen, indomethacin and naproxen exhibit gastric toxicity. Long-term use of these drugs has been associated with gastrointestinal (GI) ulceration, bleeding and nephrotoxicity [2]. The pharmacological activity of NSAIDs is related to the suppression of prostaglandin biosynthesis from arachidonic acid by inhibiting cyclooxygenases (COXs) [3,4]. The utilization of NSAIDs drugs without the consequence of GI toxicity can increase the adverse effect in the body. With this knowledge, the safety measures should be taken into account during the chemical approaches being involved in synthesizing NSAIDs.

Gastric and duodenal ulcers are common diseases of the gastrointestinal tract with high clinical incidence rates, which may result in severe superior gastrointestinal bleeding. They may be caused by an imbalance between aggressive and defensive forces in the stomach and duodenum. The reduction of acid secretion has been proven to be a useful means for promoting the healing of ulcers [5,6]. As a result, H^+/K^+ ATPase could be a therapeutic target for various acid-related diseases. Although a number of H^+/K^+ ATPase inhibitors are available on the market, there is always a need for new agents with even better efficacy and safety profiles.

Hydrazones containing azomethine (-NH-N=CH) protons constitute a vital class of compounds for new drug development [7]. Several heterocyclic hydrazones were reported to possess various biological activities viz. antimicrobial [8], anti-inflammatory [9], antioxidants [10], analgesic [11], anti-candida [12], antitubercular [13], anticancer [14], antiproliferative [15], and α-glucosidase [16], activities. Particularly, hydrazone moiety attached to heterocyclic systems was shown to offer enhanced activity [17,18]. Based on this background we planned to design and synthesis of new dihydrazone derivatives affecting aromatic hydrazones as an important pharmacophore to development of new future drugs for anti-inflammatory as well as H^+/K^+ -ATPase agents (**Figure 1**).

Based on the above interpretation and in continuation of our drug development program [19-23], herein we report the synthesis of dihydrazones and were screened for their *in vitro* H^+/K^+ -ATPase, antioxidant and anti-inflammatory activities. Moreover, we carried out in silico molecular docking approaches to validate the H^+/K^+-ATPase inhibitor agents of synthesized compounds.

2. Results and Discussion:

2.1 Chemistry:

Syntheses of the dihydrazones were achieved according to the procedures illustrated in **Scheme 1**. Isophthalic acid (**1**) was methylated using trimethylsilylchloride (TMS-Cl) and

methanol at room temperature to get isophthalic acid dimethyl ester (**2**) which upon reaction with an excess of hydrazine hydrate afforded the corresponding isophthalic hydrazides (**3**). The hydrazones (**4-36**) were obtained by reacting **3** with different aromatic or aliphatic aldehydes in presence of catalytic amount of glacial acetic acid. All the derivatives were obtained in excellent yield and the methods employed are very simple. The structures of all the newly synthesized compounds including intermediates were confirmed by ^{1}HNMR, ^{13}CNMR and mass spectral analysis (Supplementary material). The formation of methyl esters (**2**) was confirmed by the appearance of a singlet at 3.83δ for -OCH_3 and absence of COOH proton peak at 12.10δ in the ^{1}HNMR spectrum. In the ^{1}H NMR spectrum of **3**, two singlet signals displayed at 9.79 ppm and 4.50 ppm corresponding to the NH and NH_2 protons, respectively. Moreover, the absence of one signal at 52.3 ppm of –OCH_3 in ^{13}C NMR spectrum confirms the formation of hydrazide **3** from methylester **2**. The final compounds **4-36** were confirmed by their ^{1}H NMR, ^{13}C NMR and mass spectrum analysis. ^{1}H NMR spectrum of **4** showed a singlet for –NH proton at 12.03 ppm and disappearance of the peak for NH_2 proton confirms its formation. Further, it showed a singlet at 8.48 ppm for the newly formed azomethine proton (-N=CH-) confirms its formation of hydrazones. All final compounds **4-36** showed carbonyl (C=O) signal in the region of 162–164 ppm and azomethine carbon (-N=CH-) signal in the region of 146–148 ppm in their ^{13}C NMR spectra clearly confirms their formation hydrazones. Further, compound **4** showed a M+1 peak at 371.2414 in their HRMS confirms the formation of final products.

2.2 Biology:

2.2.1 H^+/K^+-ATPase activity:

In this study, we aimed to investigate the pharmacological potential of synthesized compounds were synthesized and considering the medicinal use of synthetic chemistry. To find out potential H^+/K^+-ATPase inhibition candidates, all the newly synthesized compounds **4-36** were screened for their *in vitro* H^+/K^+-ATPase inhibition activity and their results were

tabulated in **Table 1** and results were compared to standard Omeprazole. Among the derivatives, compound **4** with no substitution on the phenyl ring showed activity with IC_{50} = 88 µg/mL, which is less potent than omeprazole IC_{50} = 48 µg/mL. It can be inferred that compounds without substitution on the phenyl ring showed less activity. Therefore, the effect of substituent on the phenyl ring was further investigated. Dihydrazones with different electron donating (OH and OCH_3), electron withdrawing (Cl, NO_2, F and Br), groups on the phenyl ring and replacing phenyl ring with some aliphatic and heterocyclic substituents were also evaluated. It is found that, when hydroxy and/or methoxy groups substituted on the phenyl ring, the corresponding derivatives showed potent H^+/K^+-ATPase inhibition activity [24]. According to the old hypothesis, acid secretion was thought to be the sole cause of ulcer formation and reduction in acid secretion was thought to be the major approach towards therapy. However, in the light of recent evidences this concept has changed. With the knowledge of ulcer research, nowadays during targets have been identified and compounds are synthesized to decrease the acid secretion system as potential targets of this condition. In our previous H^+/K^+-ATPase inhibition studies of quinazolinones and benzo[*d*]thiazole derived hydrazones showed potent H^+/K^+-ATPase inhibition activity [6, 20]. But in this paper we thinking on the different way and designed the molecules without any biologically active heterocycles. On this basis we design and synthesis of simple dihydrazones and they screened for *in vitro* antiulcer activity and showed excellent inhibitory activity compared to our previously published quinazolinone and benzo[*d*]thiazole hydrazones [6, 20]. Compounds **9, 10, 15, 16, 17, 18, 19, 20, 25** and **26** showed excellent H^+/K^+-ATPase inhibition activity with IC_{50} values of 22, 26, 24, 28, 12, 14, 26, 16, 06 and 08 µg/mL respectively, which were much better than the standard Omeprazole (IC_{50} = 48 µg/mL). The inhibition activity increased along with increased number of hydroxyl and methoxy groups. With six hydroxyl (**25)** and six methoxy (**26**) groups on the phenyl ring, the derivatives showed potent H^+/K^+-ATPase inhibition activity with IC_{50} values of 06 and 08 µg/mL respectively and the activity trends

were 2 OH or OCH_3 > 4 OH or OCH_3 > 6 OH or OCH_3. Further, we were also interested in designing the molecule, by replacing the aromatic and aliphatic aldehyde (**27-29**) and with some heterocyclic aldehydes (**31-33**) and test for their H^+/K^+-ATPase inhibition activity. Furan (**31**), thiophene (**32**) and pyrazole (**33**) derived analogs showed moderate H^+/K^+-ATPase activity. Derivatives with electron withdrawing groups (Cl, NO_2, F and Br) on phenyl ring and aliphatic analogs (**27-29**) showed least antiulcer activity.

2.2.2 Anti-inflammatory activity:

Inflammation is a response of immune system that activates many enzymatic and cellular processes to protect the body from all kinds of trauma. To address this, the synthesized all compounds were evaluated for their anti-inflammatory activity using *in vitro* human erythrocytes model [25]. A significant number of compounds have been identified and showed excellent to moderate inhibitory activity compared to standard drug indomethacin. IC_{50} was determined for the compounds showing more than 50% inhibition concentration (**Table 1**). The compounds **5, 6, 7, 8, 11, 12, 13, 14, 21, 22, 23** and **24** showed excellent activity with IC_{50} values of 28, 24, 28, 26, 30, 28, 32, 30, 08, 12, 10 and 14 µg/mL respectively, much better than the standards indomethacin (IC_{50} = 44 µg/mL). The electron withdrawing groups were most favorable to show anti-inflammatory activities [26] exhibited by two or more electron withdrawing groups (**21-24**) present in benzene ring which are involved in highly potent anti-inflammatory activity compared to the single electron withdrawing groups (**5-8** and **11-14**). Electron withdrawing (Cl, NO_2, F and Br) group presents in para position (**5-8**) showed more anti-inflammatory activity compared to ortho position (**11-14**). Electron donating groups (OH and OCH_3) shows the least anti-inflammatory activity.

2.2.3 Inhibition of COX-2 enzyme activity

Modulation of the activity of pro-inflammatory enzymes is one of the most important mechanisms of action for synthetic compounds. Pro-inflammatory enzymes, such as cytosolic

phospholipase A2 (cPLA2), cyclooxygenases (COX), lipoxygenases (LOX), and NO synthase (NOS), produce very potent inflammatory mediators, and therefore their inhibition contributes to the overall antiphlogistic potential of synthetic derivatives in the drug discovery programs compared to the natural compounds [27, 28]. Compounds **1-36** were studied for inhibition of COX-2 [29] at a concentration of 30 μM in order to compare the effects of each of the compounds and to select the most promising active substance, using ibuprofen as a positive control. The compounds **5**, **6**, **7**, **8**, **11**, **12**, **13**, **14**, **21**, **22**, **23** and **24** showed significant effects on COX-2 inhibition, comparable with the reference compound. Therefore, further analyses of selected promising **5**, **6**, **7**, **8**, **11**, **12**, **13**, **14**, **21**, **22**, **23** and **24** compounds were carried out to determine the IC_{50} values against both COX-2 to assess the promising effect of selectivity of each compound (**Table 1**). Among them, the compounds **21** (06±0.10), **22** (10±0.75), **23** (08±0.51) and **24** (06±0.73) showed potent COX-2 inhibitors. This indicates that, these inhibitors involved in the decrease in the sign of inflammation accordance with literature reports [30, 31].

2.2.4 Antioxidant activity

In vitro antioxidant activities profile of the synthesized dihydrazones (**4-36**) were evaluated by (i) 1,1-diphenyl-2-picryl-hydrazyl (DPPH) assay which is a rapid and convenient technique for screening the antioxidant activities of the antioxidants, (ii) 2,2-azinobis-3-ethylbenzothiazoline-6-sufonic acid (ABTS) cation radical assay which is a conventional and excellent model for assessing the antioxidant activities of hydrogen donating and chain breaking antioxidants [32] and (iii) *N*,*N*-dimethyl-*p*-phenylenediamine dihydrochloride (DMPD) cation radical assay which is similar to the DPPH radical scavenging assay. The values of IC_{50}, the effective concentration at which 50% of the radicals were scavenged, were calculated to evaluate the antioxidant activities. A lower IC_{50} value indicated greater antioxidant activity. IC_{50} values of lower than 10 mg/mL usually implied

effective activities in antioxidant properties [33]. The IC_{50} of ascorbic acid (AA) and gallic acid (GA) was also determined for comparison. The results were revealed in **Table 1**.

Most of the synthesized compounds showed potent antioxidant activities. Compounds **9, 10, 15, 16, 17, 18, 20, 25** and **26** showed excellent radical scavenging activities with IC_{50} values 28, 34, 30, 36, 20, 22, 20, 08 and 12 µg/mL respectively in DPPH assay much better than the standards ascorbic acid (IC_{50} = 44 µg/mL) and gallic acid (IC_{50} = 48 µg/mL). In $ABTS^{+}$ radical scavenging assay, the compounds **9, 10, 15, 16, 17, 18, 20, 25** and **26** showed potent antioxidant activity with IC_{50} values 26, 36, 32, 38, 16, 24, 16, 10 and 14 µg/mL respectively which is much better than the commercial standards ascorbic acid (IC_{50} = 48 µg/mL) and gallic acid (IC_{50} = 42 µg/mL). The compounds **9, 10, 15, 16, 17, 18, 20, 25** and **26** also exhibited striking antioxidant activity with IC_{50} values 30, 34, 32, 36, 20, 18, 18, 12 and 10 µg/mL respectively which is better than the standards ascorbic acid (IC_{50}= 42 µg/mL) and gallic acid (IC_{50} = 46 µg/mL) in DMPD assay.

To study the structure activity relationship (SAR), compounds having –OH (phenolic) and $–OCH_3$ (anisole) groups in the phenyl ring (**9, 10, 15, 16, 17, 18, 20, 25** and **26)** were found to be the most potent antioxidants [34]. The number of electron donating groups (OH and OCH_3) increases on the phenyl ring, the activity also increases (**25** and **26**). The compounds **25** and **26** showed most potent antioxidants compared to the other electron donating compounds. Due to the presence of three hydroxy or three methoxy groups presents on the both side of phenyl rings. Compound **32** showed good antioxidant activity in all the tested antioxidants methods due to the presence of sulpur atom in the molecule. The compounds with electron withdrawing Cl, F, NO_2 and Br substituents on the phenyl groups (**5-8, 11-15** and **21-24**) showed least antioxidants activity [34]. Compounds containing aliphatic analogs (**27-29**) showed nil antioxidants activity in all the three tested antioxidants methods.

2.2.5 Docking studies:

In order to determine the possible binding modes for compound **25**, molecular docking studies were performed for ligands **2-36**. The structure of the protein from Daboia russelii (PDB ID: 1E9Y) was employed for docking studies. Analysis of the interaction mode for the lower energy docking pose among the ensemble indicated that compound **25** interacted with Ala365, Gly279, Asp223 and His221 by π-π stacking (**Fig. 2**). Importantly, binding of ligand 25 near the α-helical random coil clearly suggests that it had interaction with His221, which resides near the random coil [35].

Likewise, to rationalize the inhibition seen for Na^+/K^+ ATPase and o gain insights into the possible binding modes, rigid-body docking studies of the compounds was carried out with the receptor structure. Crystal structure of Na^+/K^+-ATPase from Squalus acanthias (Spiny Dogfish) (PDB ID: 2ZXE) was used for molecular docking studies as a prototype instead of the structure of the protein from human for ease of target structure preparation. Molecular docking studies showed an interacting map of Na^+/K^+-ATPase enzyme with compound **25** (**Fig 3**). Based on XP glide score, compounds **25** and **26** showed a promising scoring function, when compared to other structurally related compounds (See supporting information **Table 2**). Additionally, the low E model value indicated that the binding affinity between protein and ligand is energetically favourable [36]. Pharmacodynamics parameters play an important role in determining the success of a lead candidate for further therapeutic development.

3. Conclusion:

In conclusion, in an attempt of developing a new class of anti-inflammatory, antioxidants and H^+/K^+-ATPase agents, novel isophthalic dihydrazone analogs were efficiently synthesized in good yield. Bioassay results revealed that compounds **9, 10, 15-20, 25** and **26** with OH and OCH_3 groups on phenyl ring (electron donating) showed excellent H^+/K^+-ATPase and antioxidants activities than the corresponding standard drugs. Compounds **5-8, 11-14** and **21-25** with Cl, NO_2, F and Br on phenyl ring (electron withdrawing) also

showed excellent anti-inflammatory activity. Furthermore, molecular docking studies were performed for all the synthesized compounds, among which compounds **25** and **26** showed the highest docking G-scores for H^+/K^+-ATPase inhibition activity.

4. Biological activity

4.1 Gastric H^+/K^+-ATPase activity

Isolation of parietal cells from sheep stomach

The fundic stomach portion of sheep was collected soon after sacrificing and was rinsed with Krebs ringer buffer (250 mM sucrose, 2 mM $MgCl_2$, 1 mM EGTA and 2 mM Hepes-Tris of pH 7.4). The upper layer was pinned with the help of needles on the dissection table. Mucosal scrapings were hanged in 10 volumes of Krebs ringer buffer (pH = 7.4) containing sucrose (250 Mm) and homogenized with 20 strokes of a mortar driven Teflon pestle homogenizer. The tissues were discarded and the filterate was subjected to sub cellular fractionation. The pellets obtained were dissolved in 2 mL of sucrose-EGTA buffer and used as enzyme sample.

Protein estimation

Protein was calculated using the Lowry method [37] and bovine serum albumin as standard (0-75 µg). Eight clean and dry test tubes were taken and aliquots of various concentrations of the synthesized derivatives were made. To the 7^{th} and 8^{th} test tube, the unknown sample (5 µL and 10 µL of the cells isolated from the sheep's stomach) for which the protein content was added. In every test tube, the solution was made up to 1 mL by the addition of distilled water, followed by the addition of 5 mL of Lowry's reagent. All the test tubes are incubated at rt for 8-10 min and 0.5 mL of Folin-Ciocalteu reagent was added and again incubation at rt for 30 min. Absorbance of each solution was recorded at 670 nm against the blank solution. A graph was plotted by using concentration of protein on X-axis and OD on Y-axis. From the standard graph obtained, the unknown concentration of protein sample was calculated and found to contain 21 mg of protein per 8 g of tissue homogenate.

Inorganic phosphorus estimation

Inorganic phosphorus was estimated according to Fiske Subbarow's method [38]. Aliquots of working standard solution (40µg/mL) were added into 8 fresh and dry test tubes in the volume of 0 to 1mL; 5 µL and 10 µL of the enzyme sample were taken in test tube 7th and 8th respectively. The volumes of all test tubes were made up to 8.6 mL using 10% TCA. Ammonium molybdate (1mL) and ANSA reagent (0.4 mL) were added to all test tubes. All test tubes were allowed to stand for 10 min at rt. Then the color was developed and the λ_{max} at 660 nm were recorded.

4.2 ATPase activity

ATPase activity was determined using reported method [39, 40]. Basal Mg^{2+} dependent ATPase activity was calculated in 1 mL reaction mixture comprising of 2 mmol/L ATP and 50 mmol/L Tris-HCl buffer (pH=7.5). K^+ stimulated and HCO_3^- stimulated ATPase activity were tested in the basal medium. The ATPase reaction was started by the addition of the substrate (ATP), carried out at 37 °C for 10-15 min and the terminated by the addition of 1 mL cold 20% TCA. Liberated inorganic phosphate from ATP was estimated using literature method [40].

Activity of the enzyme sample in the presence of ATP is 0.066 µmoles.

Activity of the enzyme sample in the absence of ATP is 0.042 µmoles.

100% activity of the enzyme is 0.066 µmoles-0.042 µmoles = 0.024 µmoles.

4.3 Anti-inflammatory activity:

Human erythrocyte suspension

The human blood was purchased from public hospital, Mysore, India and collected in heparinzed vacutainer. The collected healthy human blood was washed with 0.9% saline and centrifuged for 10 minutes at 3000 rpm. The packed cells were washed with 0.9% saline and 40% v/v suspension was made by isotonic phosphate buffer of 154 mM NaCl in 10 mM Sodium Phosphate Buffer at pH 7.4 and used as stock erythrocyte or RBC suspension.

Hypotonic solution-induced haemolysis

The activity of the synthesized compounds was tested according to the reported method [25]. The tested sample 0.5mL consisted of stock erythrocyte (RBC) suspension. 5 mL of hypotonic solution (50 mM NaCl in 10 mM Sodium Phosphate buffered saline at pH 7.4) and different concentrations of sample (20, 40, 60, 80 and 100 µg/mL) was prepared. The blank control consisted of 0.5 mL RBC suspension mixed and 5 mL hypotonic buffered solution alone. The mixtures were incubated for 10 minutes at room temperature, centrifuged for 10 minutes at 3000 rpm and supernatant was measured by spectrophotometrically at 540 nm. The % inhibition of haemolysis was calculated according to the following formula.

$$\% \text{ Inhibition of haemolysis} = \left[\frac{A_1 - A_2}{A_1}\right] \times 100$$

Where:

A_1 = Absorbance of hypotonic buffered solution alone

A_2 = Absorbance of test /standard sample in hypotonic solution

4.4 Anti-inflammatory activity by COX-2 as model enzyme

The enzymatic *in vitro* assays using human recombinant COX-2 (Sigma-Aldrich) were performed to test the inhibitory activities of the test compounds [41]. COX-2 (0.5 unit/ reaction) was added to 180 µL of the incubation mixture consisting of 100 mM Tris buffer (pH 8.0), 5 µM porcine hematin, 18 mM L-epinephrine, and 50 µM Na_2EDTA. The test substances were then dissolved in DMSO and added (10 µL), with the mixture incubated for 5 min at room temperature. Pure DMSO was used as a blank and (S)-(+)-ibuprofen (Sigma-Aldrich) as a reference inhibitor. Then, 5 µL of 10 µM arachidonic acid was added, and the reaction was incubated for 20 min at 37 °C. The reaction was terminated by adding 10 µL of 10% (v/v) formic acid. Prostaglandin E2 (PGE2), the main product of the reaction, was quantified using a Prostaglandin E2 ELISA kit (Enzo Life Sciences, NY, USA). The samples were diluted 1:15 in assay buffer (provided in the kit), and then the incubation was performed according to the manufacturer's instructions. The absorbance at 405 nm was measured using

a Tecan Infinite M200 microplate reader (Tecan Group, Switzerland). The inhibitory activity was expressed as the percent inhibition compared to the blank. Experiments were repeated at least three times with two technical replicates for screening as well as to determine the IC_{50} values

4.5 Antioxidants activity

4.5.1 DPPH (1,1-diphenyl-2-picryl-hydrazyl) assay

The radical scavenging activities of DPPH free radicals by synthesized compounds were determined according to the reported method [42]. Briefly, 50 µL of test compounds was mixed at different concentrations (20, 40, 60, 80 and 100 µg/mL) with 1 mL of 0.1 mM DPPH in methanol solution and 450 µL of 50 mM Tris HCl buffer (pH 7.4). Methanol (50 µL) only was used as the experimental control. After 30 min of incubation at room temperature, the reduction in the number of DPPH free radicals was measured by reading the absorbance at 517 nm. BHA, AA and GA were used as standards similar to test concentrations. Percent inhibition was calculated from the following equation:

$$\% \text{ Inhibition} = \left[\frac{\text{Absorbance of control - Absorbance of test sample}}{\text{Absorbance of control}} \right] \times 100$$

4.5.2 ABTS (2,2-azinobis-(3-ethylbenzothiazoline-6-sufonic acid) assay

The ability of the test sample to scavenge $ABTS^{\cdot+}$ radical cation was determined according to the literature method [43] with slight modifications. The $ABTS^{\cdot+}$ radical cation was pregenerated by mixing 7 mM $ABTS^{\cdot+}$ stock solution with 2.45 mM potassium persulfate (final concentration) and incubating for 12–16 hrs in the dark at room temperature until the reaction was complete and the absorbance was stable. The absorbance of the $ABTS^{\cdot+}$ solution was equilibrated to 0.70 (± 0.02) by diluting with distilled water at room temperature, then 2 mL was mixed with different concentration of the test sample (20, 40, 60, 80, and 100 µg/mL) and the absorbance was measured at 734 nm after 6 min. The scavenging capability of $ABTS^{\cdot+}$ radical was calculated using the following equation:

$$ABTS^{\cdot+} \text{ scavenging effect (\%)} = [(A_c - A_s) / A_c] \times 100$$

Where, Ac is the initial concentration of the ABTS.+ and As is the absorbance of the remaining concentration of ABTS.+ in the presence of compounds.

4.5.3 DMPD (N, N-dimethyl-p-phenylenediamine) assay

The DMPD radical scavenging ability of synthesized compounds was determined by the method [44] with slight modifications. This assay is based on the capacity of the extract to inhibit $DMPD^{\cdot+}$ cation radical formation. Briefly, 105 mg of DMPD was dissolved in 5 mL of distilled water. Then, 1 mL of this solution was added to 100 mL of 0.1 M acetate buffer (pH 5.3). $DMPD^{\cdot+}$ was produced by adding 0.3 mL ferric chloride (0.05 M) to this solution. Different concentrations of standard antioxidants or synthesized compounds (20, 40, 60, 80 and 100 µg/mL) were added, and the total volume was adjusted to 1 mL with distilled water. One millilitre of the $DMPD^{\cdot+}$ solution was directly added to the reaction mixture. The reaction mixtures were incubated in the dark for 15 min. The absorbance was measured at 505 nm.

$$\% \text{ Inhibition} = \left[\frac{\text{Absorbance of control - Absorbance of test sample}}{\text{Absorbance of control}} \right] \times 100$$

4.6 Molecular docking studies

The co-ordinates of H^+ /K^+ -ATPase were obtained from the Brookhaven Protein Data Bank, whose PDB ids are 1E9Y and 2ZXE respectively. Ligands were drawn using Maestro 2D sketcher and energy minimize was computed by OPLS 2005. Proteins were prepared by retrieving into Maestro platform (Schrödinger, Inc.). Protein structures were corrected, by using a Prime software module of Schrödinger to correct the missing loops and in the protein. Water molecules from H^+ /K^+ -ATPase were removed beyond 5Å from the hetero atom respectively. Water molecules which are through to be important in aiding the interaction between the receptor were optimized during protein pepwizard. Automated, necessary bonds, bond orders, hybridization, explicit hydrogens and charges were assigned. OPLS 2005 force

field was applied to the protein to restrained minimization and RMSD of 0.30Å was set to converge heavy atoms during the preprocessing of protein before starting docking. Using Extra-precision (XP) docking and scoring each compound were docking into the receptor grid of radii 20Å × 20Å × 20Å and the docking calculation were judged based on the Glide score of all the ligands and molecular visualization was done under Maestro workspace [45].

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References:

1. C. Vergelli, M. P. Giovannoni, S. Pieretti, A. D. Giannuario, V. Dal Piaz, Bioorg. Med. Chem. 15 (2007) 5563-5575.
2. T. K. Motawi, H. M. Abd Elgawad, N. N. Shahin, J. Biochem. Mol Toxicol. 21 (2007) 280-288.
3. Y. Nakano, E. Kuroda, T. Kito, S. Uematsu, S. Akira, A. Yokota, S. Nishizawa, U. Yamashita, J. Neurosurg. 108 (2008) 311-319.
4. P. Singh, A. Mittal, Mini, Rev. Med. Chem. 8 (2008) 73-90.
5. S. Gustavsson, H. O. Adam, L. Loof, Lancet. 8342 (1983) 124-125.
6. K. P. Rakesh, C. S. Shantharam, H. M. Manukumar, Bioorg. Chem. 68 (2016) 1- 8.
7. S. Rollas, S. G. Kucukguzel, Molecules. 12 (2007) 1910-1939.
8. D. H. Soliman, M. A. Abdelrahman, M. S. Gomaa, M. M. Elasser, M. M. Abdel-Aziz, I. Salama, Eur. J. Med. Chem. 138 (2017) 698-714.
9. C. M. Moldovan, O. Oniga, A. Pârvu, Brîndus¸ Tiperciuc, P. Verite, A. Pirnau, O. Crisane, M. Bojita, R. Pop, Eur. J. Med. Chem. 46 (2011) 526-534
10. H. S. Kareem, A. Ariffin, N. Nordin, T. Heidelberg, A. Abdul-Aziz, K. W. Kong, W. A. Yehye, Eur. J. Med. Chem. 103 (2015) 497-505.

11. P. Hernandez, M. Cabrera, M. L. Lavaggi, L. Celano, I. Tiscornia, T. R. D. Costa, L. Thomson, M. B. Fogolin, A. L. P. Miranda, L. M. Lima, E. J. Barreiro, M. Gonzalez, H. Cerecetto, Bioorg. Med. Chem. 320 (2012) 2158-2171.

12. S. Carradori, D. Secci, A. Bolasco, D. Rivanera, E. Mari, A. Zicari, L.V. Lotti, B. Bizzarri, Eur. J. Med. Chem. 65 (2013) 102-111.

13. E. Vavrikova, S. Polanc, M. Kocevar, K. Horvati, S. Bosze, J. Stolarikova, K. Vavrova, J. Vinsova, Eur. J. Med. Chem. 46 (2011) 4937-4945.

14. P. Nagender, R. Naresh Kumar, G. Malla Reddy, D. Krishna Swaroop, Y. Poornachandra, C. Ganesh Kumar, B. Narsaiah, Bioorg. Med. Chem. Lett. 26 (2016) 4427-4432.

15. Zhong-Hua Li, Dong-Xiao Yang, Peng-Fei Geng, Ji Zhang, Hao-Ming Wei et al., Eur. J. Med. Chem. 124 (2016) 967-980.

16. Guangcheng Wang, Ming Chen, Jing Wang, Yaping Peng, Luyao Li, ZhenZhen Xie, Bing Deng, Shan Chen, Wenbiao Li, Bioorg. Med. Chem. Lett. 27 (2017) 2957-2961.

17. A. R. Todeschini, A. L. P. Miranda, K. C. Silva, S. C. Parrini, E. J. Barreiro, Eur. J. Med. Chem. 33 (1998) 189-199.

18. J. R. Dimmock, S. C. Vashishtha, J. P. Stables, Eur. J. Med. Chem. 35 (2000) 241-248.

19. Gao-Feng Zha, Jing Leng, N. Darshini, T. Shubhavathi, H. K. Vivek, A. M. Asiri, H. M. Marwani , K. P. Rakesh, N. Mallesha, Hua-Li Qin, Bioorg. Med. Chem. Lett. 27 (2017) 3148-3155.

20. Shi-Meng Wang, Gao-Feng Zha, K. P. Rakesh, N. Darshini, T. Shubhavathi, H. K. Vivek, N. Mallesha, Hua-Li Qin, Med. Chem. Commun. 8 (2017) 1173-1189

21. K. P. Rakesh, A. B. Ramesha, C. S. Shantharam, K, Mantelingu, N. Mallesha, RSC Adv. 6 (2016) 108315-108318.

22. K. P. Rakesh, N. Darshini, S. L. Vidhya, Rajesha, N. Mallesha, Med. Chem. Res. 26 (2017) 1675-1681.

23. Xing Chen, Jing Leng, K.P. Rakesh, N. Darshini, T. Shubhavathi, H.K. Vivek, N. Mallesha, Hua-Li Qin, Med. Chem. Comm. 8 (2017) 1706-1719.

24. A. Sharma, R. Suhas, K. V. Chandana, S. H. Banu, D. C. Gowda, Bioorg. Med. Chem. Lett. 14 (2013) 4096-4098.

25. U. A. Shinde, A. S. Phadke, A. M. Nair, A. A. Mungantiwar, V. J. Dikshit, M. N. Saraf, Fitoterapia. 70 (1999) 251-257.

26. D. M. Lokeshwari, D. K. Achutha, B. Srinivasan, S. Naveen, N. K. Lokanath, K. Ajay Kumar, Bioorg. Med. Chem. Lett. 27 (2017) 3806-3811.

27. A. G. Lafuente, E. Guillamon, A. Villares, M. A. Rostagno, J. A. Martinez, Inflamma. Res. 58 (2009) 537-552.

28. H. P. Kim, K. H. Son, H. W. Chang, S. S. Kang, S. S. J. Pharmaco. Sci. 96 (2004) 229-245.

29. A. L. Blobaum, L. J. Marnett, J. Med. Chem. 50 (2007) 1425-1441.

30. K. Seibert, Y. Zhang, K. Leahy et al., Proc. Natl. Acad. Sci. U S A. 91 (1994) 12013-12017.

31. H. L. Willingale, N. J. Gardiner, N. McLymont, S. Giblett, B. D. Grubb, Br. J. Pharmacol. 122 (1997) 1593-1604.

32. L. P. Leong, G. Shui, Food. Chem. 76 (2002) 69-75.

33. Y. L. Lee, M. T. Yen, J. L. Mau, Food. Chem. 104 (2007) 1-9.

34. K. P. Rakesh, H. M. Manukumar, D. C. Gowda, Bioorg. Med. Chem. Lett. 25 (2015) 1072-1077.

35. A. Husain, M. Ajmal, Acta. Pharm. 59 (2009) 223-233.

36. S. P. Sharma, N. Kumar, R. Dudhe, Der. Pharm. Chem. 2 (2010) 253-263.

37. O. H. Lowry, N. J. Rosebrough, A. L. Farr, R. J. Randall, J. Biol. Chem. 193 (1951) 265-275.

38. C. H. Fiske, Y. Subbarow, J. Biol. Chem. 66 (1952) 375-400.

39. W. B. Im, J. C. Sih, D. P. Blakeman, J. P. McGrath, J. Biol. Chem. 260 (1985) 4591-4597.

40. D. M. Lokeshwari, N. D. Rekha, B. Srinivasan, H. K. Vivek, K. Ajay Kumar, Bioorg. Med. Chem. Lett, 27 (2017) 3048-3054.

41. C. Fernandes, A. Palmeira, I. I. Ramos, C. Carneiro, C. Afonso, M. E. Tiritan, H. et al. Pharmaceuticals. 10 (2017) 50.

42. M. S. Blois, Nature. 181 (1958) 1199-1200.

43. R. Re, N. Pellegrini, A. Proteggente, A. Pannala, M. Yang, C. Rice-Evans, Free. Radical. Bio. Med. 26 (1999) 1231-1237.

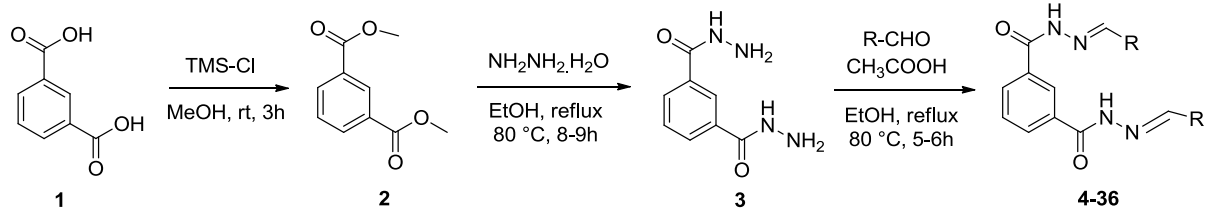
44. V. Fogliano, V. Verde, G. Randazzo, A. Ritieni, J. Agric. Food Chem. 47 (1999) 1035-1040.

45. R. A. Friesner, J. L. Banks, R. B. Murphy, T. A. Halgren, J. J. Klicic, D. T. Mainz, M. P. Repasky et al., J. Med. Chem. 47 (2004) 1739-1749.

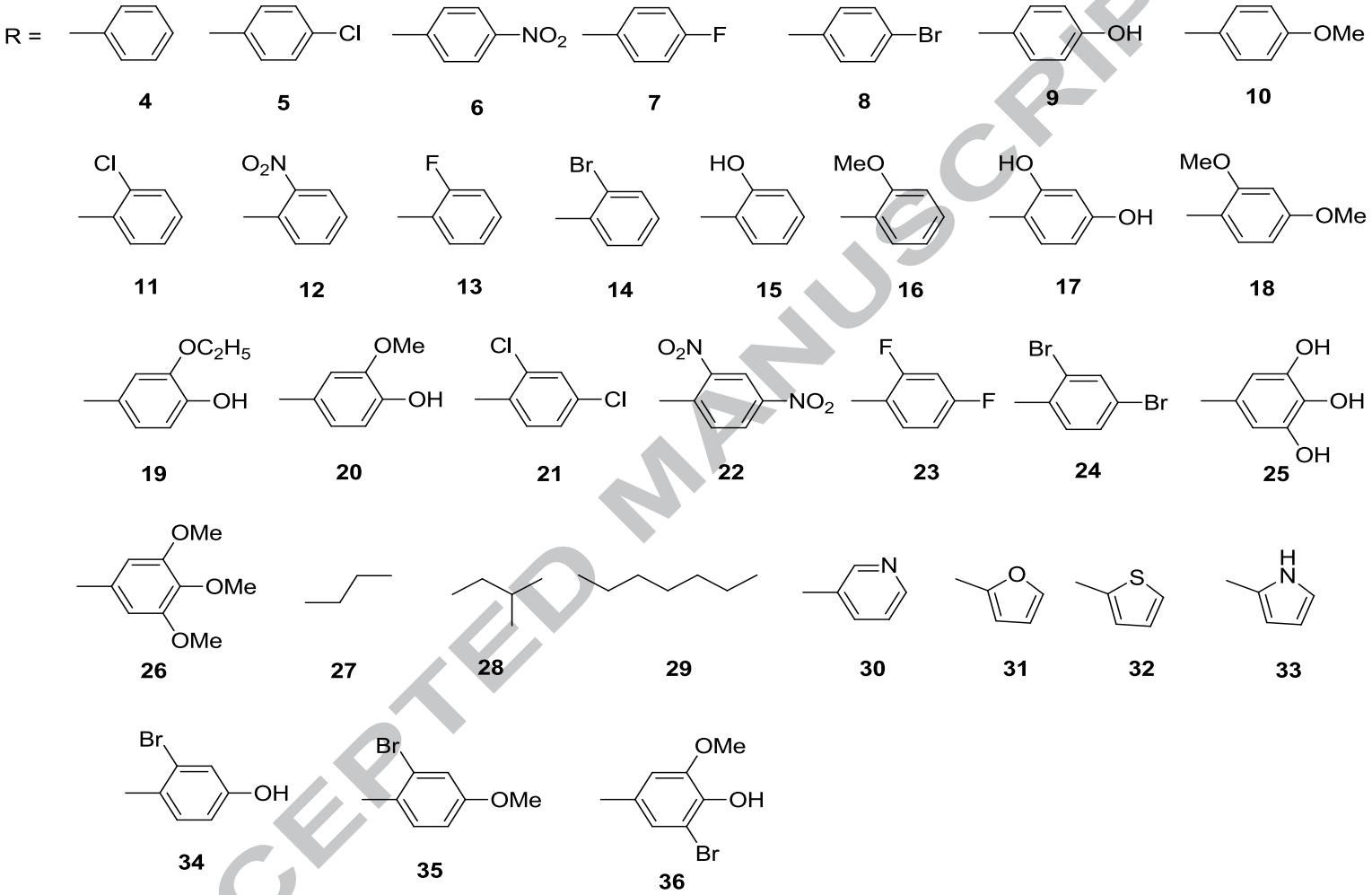
Table 1: Biological activities of the synthesized hydrazones (**1-36**)

Entry	H^+/K^+ ATPase activity[a] IC_{50} μg/mL	Anti-inflammatory activity[a] IC_{50} µg/mL	COX-2 IC_{50} [µM][a]	Antioxidant activity[a] (IC_{50} μg/mL)		
				DPPH	ABTS	DMPD
1	-	-	-	-	92±0.26	-
2	-	-	-	-	-	86±0.93
3	-	-	-	90±0.63	88±0.95	90±0.75
4	88±1.02	90±0.46	-	80±0.16	82±0.93	78±0.18
5	68±1.04	28±0.65	16±0.72	78±0.21	74±0.36	80±0.32
6	72±0.95	24±0.61	20±0.72	70±0.36	68±0.28	66±0.34
7	66±0.40	28±0.64	18±0.31	72±0.38	66±0.31	70±0.18
8	70±0.68	26±0.45	15±0.88	76±0.26	72±0.21	74±0.36
9	22±0.62	72±0.42	31±0.99	28±0.32	26±0.15	30±0.59
10	26±0.91	78±0.12	-	34±0.56	36±0.49	34±0.28
11	72±0.65	30±0.74	13±0.17	80±0.32	78±0.55	78±0.30
12	74±0.63	28±0.40	22±0.79	74±0.30	72±0.20	70±0.14
13	70±0.92	32±0.24	17±0.33	76±0.31	68±0.11	74±0.19
14	74±0.43	30±0.55	12±0.29	80±0.27	74±0.55	78±0.52
15	24±0.44	80±0.23	30±0.77	30±0.17	32±0.22	32±0.51
16	28±0.63	78±0.36	-	36±0.74	38±0.21	36±0.11
17	12±0.38	60±0.23	-	20±0.36	16±0.33	20±0.11
18	14±0.47	66±0.86	-	22±0.27	24±0.36	18±0.18
19	26±0.54	70±0.257	-	42±0.21	38±0.33	40±0.33
20	16±0.41	62±0.27	19±0.67	20±0.17	16±0.37	18±0.32
21	80±0.36	08±0.47	06±0.10	82±0.23	86±0.30	78±0.33
22	84±0.63	12±0.47	10±0.75	78±0.64	84±0.31	82±0.14
23	78±0.56	10±0.51	08±0.51	72±0.28	76±0.31	80±0.33
24	80±0.49	14±0.84	06±0.73	70±0.21	78±0.31	76±0.21
25	06±0.68	56±0.74	17±0.37	08±0.26	10±0.23	12±0.29
26	08±0.43	60±0.48	21±0.88	12±0.29	14±0.29	10±0.39
27	-	-	-	-	-	-
28	-	-	-	-	-	-
29	-	-	-	-	-	-
30	52±0.36	40±0.52	27±0.18	48±0.21	44±0.31	52±0.21
31	50±0.69	48±0.62	-	40±0.21	48±0.31	46±0.18
32	42±0.24	38±0.54	17±0.37	38±0.36	42±0.36	40±0.36
33	48±0.62	42±0.61	22±0.82	56±0.31	60±0.47	56±0.61
34	52±0.85	50±0.62	20±0.81	54±031	58±0.30	62±0.33
35	54±0.62	48±0.41	-	58±0.31	54±0.11	60±0.33
36	44±0.19	56±0.72	25±0.52	52±0.21	58±0.21	50±0.91
Omerazole	**48±0.12**		-	-	-	-
Indomethacin	-	**44±0.15**	-	-	-	-
Ibuprofen	-	-	**4±0.10**			
Ascorbic acid	-	-	-	**44±0.19**	**48±0.26**	**42±0.69**
Gallic acid	-	-	-	**48±0.26**	**42±0.36**	**46±0.28**

[a] Values are mean of three determinations, the ranges of which are <5% of the mean in all cases.



Scheme 1: Synthesis of target compounds **4-36**



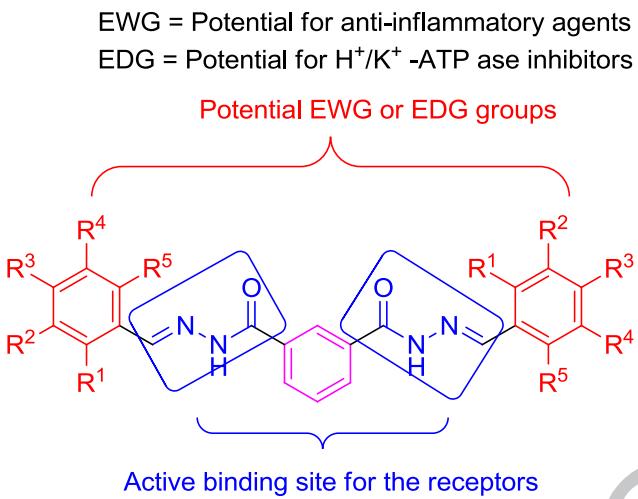


Figure 1: Synthetic strategy for the target compounds **4-36**

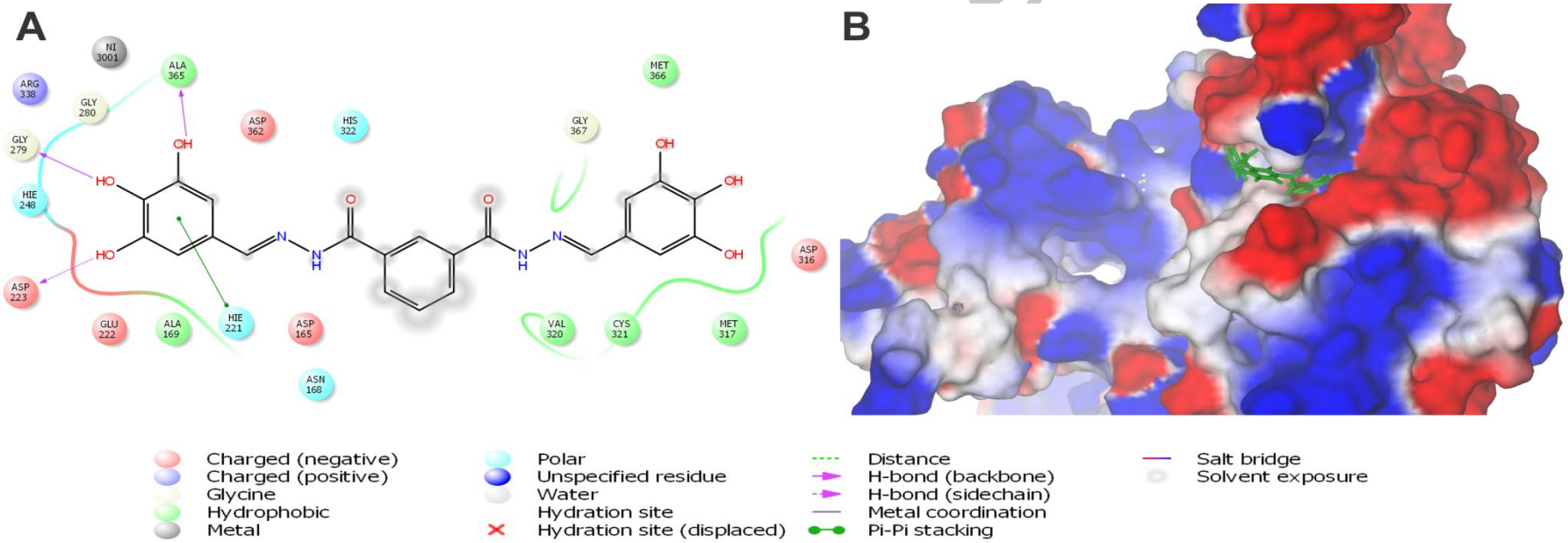


Figure 2: (A) Molecular interaction of H^+/K^+ ATPase enzyme (1E9Y) with Ligand 25 (B) Electrostatic surface representation of the protein depicting the best-docked pose for Ligand 25.

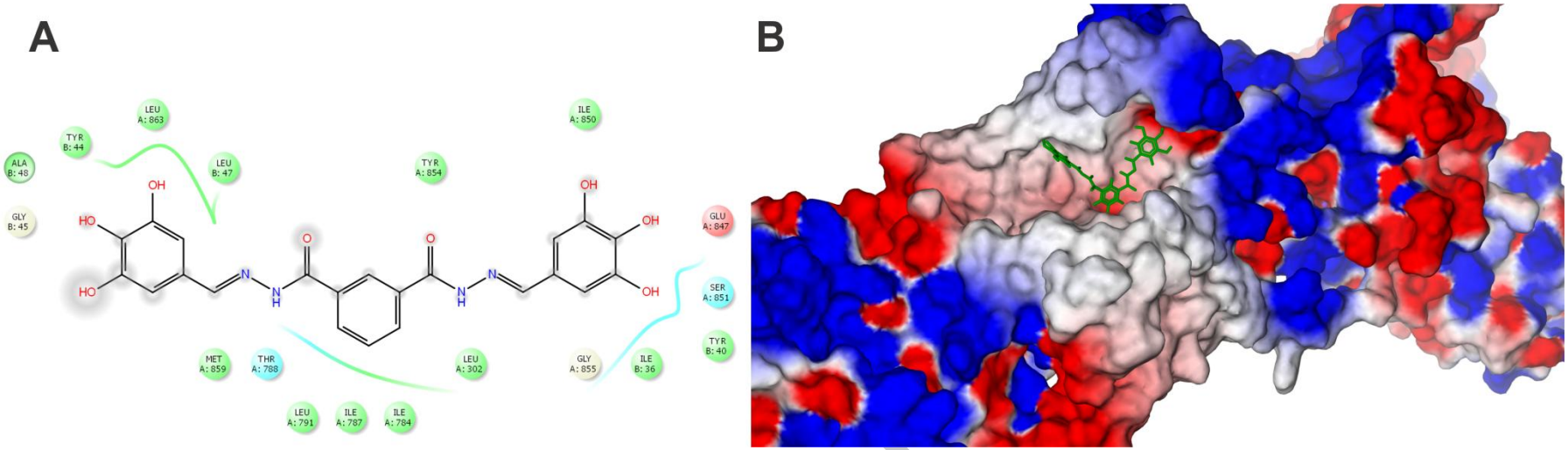


Figure 3: (A) Molecular interaction of H^+/K^+ ATPase enzyme (2ZXE) with Ligand 25 (B) Electrostatic surface representation of the protein depicting the best-docked pose for Ligand 25.

Research Highlights

1. Synthesis of **32** dihydrazones and evaluated for *in vitro* biological activities.
2. Compound **25** was found to be most potent H^+/K^+-ATPase and antioxidants activity.
3. Compound **21** showed excellent anti-inflammatory and COX-2 activities.
4. The SAR showed that, electron donating groups (OH, OCH_3) favored the H^+/K^+-ATPase and antioxidants activity, whereas, electron withdrawing groups (F, Cl, Br and NO_2) favored the anti-inflammatory and COX-2 activity.

Graphical abstract

Multi-targeted dihydrazones as potent biotherapeutics

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