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Anti-inflammatory and antioxidant activity of salicylic acid conjugated dihydropyrazoline analogues

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ABSTRACT

Syntheses of substituted salicylic acid appended pyrazoline analogues (**7a-j**) via 1,3-dipolar cycloaddition were reported earlier. In the present investigation we have performed the anti-inflammatory activity by phospholipase A2 (PLA2) inhibition and *in vitro* antioxidant activity by 2,2-diphenyl-1-picrylhydrazyl, nitric oxide, hydroxyl radical scavenging assay and ferrous ion chelating assay wherein compounds **7d**, **7h**, **7i** and **7j** have shown maximum anti-inflammatory activity. Further, compounds **7d**, **7f** and **7h** were proved to be excellent free radical scavengers.

INTRODUCTION

Design and synthesis of nonsteroidal anti-inflammatory drugs (NSAIDs) is the important field in drug design, hence in recent years the newer strategy of synthesizing molecules for the inhibition of enzyme leading to inflammation is of special interest, PLA2 is a low molecular mass enzyme (Moeira *et al.*, 2011) which is responsible for the release of arachidonic acid and lysophospholipid by catalyzing the hydrolysis of Sn2-ester bond of phospholipids. Arachidonic acid is precursor in the biosynthesis of eicosanoids and the lysophospholipid serves as a precursor for platelet activating factor, these products when produced in excess are responsible for chronic diseases such as cancer and autoimmune disorders (Dennis, 1997). Further, supported by extensive research and clinical evidences, it is found that pathophysiological conditions during inflammation are associated with depleting intrinsic antioxidants and generating free radicals causing oxidative stress.

Inspite, of profoundest development new molecules the urge to screen newer compounds for the development of new antioxidants which also specifically inhibit PLA2 remains constant, proenzyme in the inflammatory pathways, but the protecting cycloxygenase enzyme in gastric mucosa unlike the currently available nonsteroidal anti-inflammatory drugs and also bearing antioxidant property.

The broad spectrum of biological application of salicylic acid and its ester (acetyl salicylic acid and methyl salicylate) has fueled up the researchers in synthesizing organic molecules comprising of salicylic acid. Further, the pyrazolines are class of heterocyclic core which is known for its wide range of biological efficacies like anti-microbial (Karthikeyan *et al*., 2007; Hassan, 2013; Zitouni *et al*., 2005), anti-inflammatory, (Reshma and Nevagi, 2014), antidepressant (Palaska *et al*., 1996) and anticancer (Havrylyuk *et al*., 2009) etc. Numerous drugs like phenazone (1), metamizole (2) aminopyrine (3) and celecoxib (4) are available in the present market comprised of pyrazoline derivatives.

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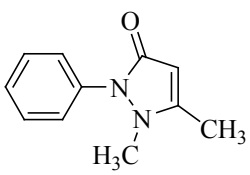
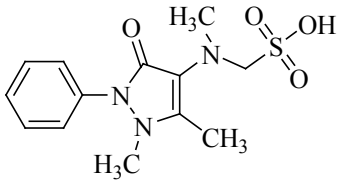
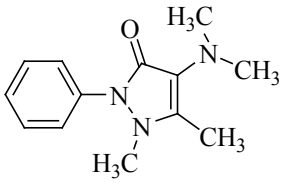


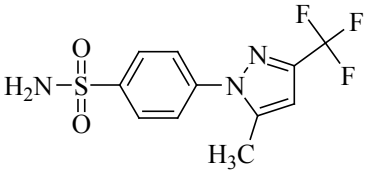
Phenazone
(1)



Metamizole
(2)



Aminophenazone
(3)



Celecoxib
(4)

Bearing the above observations in mind, we have made an emphasis on synthesizing a salicylic acid integrated pyrazoline analogues and screening them for their antioxidant and phospholipase A2.

EXPERIMENTAL PROTOCOLS

Chemistry

Materials and methods

The Materials and methods are clearly discussed in earlier reference (Naveen *et al*., 2017).

SYNTHESIS

Synthesis of the compounds **7a-j** were clearly discussed earlier (Naveen *et al*., 2017), to a stirring solution of compound **5a** (1.8 mmol) in absolute alcohol, compound **6a** (1.9 mmol), was added followed by chloramines-T (2 mmol) the reaction mixture was refluxed on water bath. Further, the completion of the reaction was checked by TLC, the reaction mass was concentrated by evaporating the solvent, the reaction mass was dissolved in dichloromethane and the product was extracted by 10% sodium bicarbonate solution. The sodium bicarbonate extract was neutralizing with 5% hydrochloric acid to achieve compound **7a** as solid, which was further purified by column chromatography on silica gel using petroleum ether and methanol as an eluent, compounds **7b-j** was synthesized by similar method.

BIOLOGY

Antioxidant assays

DPPH radical scavenging assay

The antioxidant potential of the synthesized compound was noted through free radical scavenging assay with slightly modified method of Manzocco *et al.*, various concentrations of the synthesized compounds, ranging from 25 to 100 µmole/ml in methanol was added to 4 ml of 0.004% (w/v) of DPPH, prepared in methanol (Scherer and Godoy, 2009). The resulting mixture was incubated for 20 min at room temperature, and the absorbance was measured at 517 nm against a blank. The effective free radical scavenging activity was measured as the decrease in the absorbance of DPPH and calculated using the following equation

Percentage of scavenging = 1 − (Absorbance sample (517 nm)/Absorbance control (517 nm)) × 100.

Nitric oxide radical scavenging assay

The title compounds were examined for Nitric oxide radical scavenging assay (Marcocci *et al*., 1994), the sodium nitroprusside (5 mM) in aqueous solution at physiological pH, spontaneously produces nitric oxide, thus produced nitric oxide reacts with oxygen to generate nitrite ions that can be measured by Griess reagent. Nitric oxide scavengers compete with oxygen leading reduced production of nitric oxide. Sodium nitroprusside (5 mM) in PBS was mixed with the synthesized compounds and kept at 25°C for 2 hours. The above samples were treated with Griess reagent (1% sulphanilamide, 0.1% napthylethylenediamine dihyrdochlorid and 2% orthophosphoric acid). The diazotization of nitrite with sulphanilamide and subsequent coupling with napthylethylenediamine results in the formation of chromophore with an absorbance at 540 nm and referred to the absorbance of standard solutions of BHT treated in the same way with Griess reagent. The radical scavenging activity was measured using the equation as described for DPPH assay.

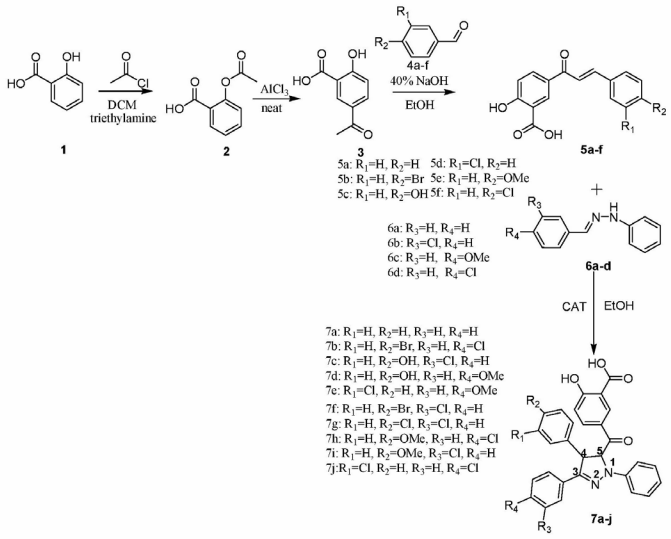
Ferrous ion chelating assay

Ferrous ion chelating activity was recorded according to the method of Suter and Richtes (Gordon *et al*., 1990). Control was prepared by adding $FeCl_3$ (200 mM) and $K_3Fe(CN)_6$ (400 mM) and the volume was made to 1 ml using water. EDTA (40 mM) was used as positive control in the other set of reactions. Title compounds of different concentrations ranging from 2 to 10 µg/mL, $FeCl_3$ (200 µM) and $K_3Fe(CN)_6$ (400 mM) were added. BHT was used as the reference compound. The tubes were kept for 10 min at room temperature and optical density was measured

at 700 nm. The ion chelating activity was found by using formula as described for DPPH radical scavenging assay.

Hydroxyl radical scavenging assay

Hydroxyl radical scavenging assay of the synthesized compounds (Halliwell *et al*., 1987) was done by incubating the pyrazoline analogues with H_2O_2 (1 mM), deoxyribose (2.8 mM), EDTA, $FeCl_3$ and ascorbic acid in phosphate buffer 0.02 M, pH 7.4 for 1 hour at 37°C. 1% TBA was added to quench the reaction. The tubes were boiled in water bath for 20 min. The optical density was measured at 535 nm using a suitable reagent blank. The % radical scavenging activity was done using the formula as described for DPPH scavenging assay.



Scheme 1: Synthesis of N-phenyl-3,4bis pheny1-5(3-hydroxy-4-carboxybenzoy14,5-dihydro-pyrazoline (**7a-j**).

Anti-inflammatory activity by inhibition of PLA2

Protein concentration in the Russel viper venom was calculated (Lowry *et al*., 1957), using bovine serum albumin fraction (0–75 µg). PLA2 inhibition activity was calculated by (Boman *et al*., 1957). Indirect hemolytic assay, a semi quantitative method was employed. Briefly, egg yolk, packed human erythrocytes and phosphate buffer saline was mixed (1:1:8 V/V). 1 ml of this as substrate was incubated with 60 µg of enzyme which was pretreated with various concentration title compounds for 30 min at room temperature. 9 ml of cold phosphate buffer saline was added to stop the reaction and centrifuged at 4°C for 10 min at 1500 rpm. The released hemoglobin in the supernatant was measured at 540 nm. The assay was also performed in the presence of various concentrations 3, 6, 9, 12 and 15 µg/ml of test compound, and the percent enzyme inhibition was calculated.

RESULTS AND DISCUSSION

Chemistry

The synthesis of 5-(3-phenylacryloyl)-2-hydroxybenzoic acid analogues (**5a-f)** is outlined in Scheme **1**. 5-Acetyl-2-hydroxybenzoic acid **(3)** was obtained by acetylating (Kodela *et al*., 2011) salicylic acid (**1**), followed by Fries rearrangement (Prashanth *et al*., 2013) of 2-acetoxybenzoic acid **(2)** in the presence of anhydrous aluminum chloride. Compound **3** on condensing with corresponding aromatic aldehydes (**4a-f)** in the presence of strong base furnished (Naveen *et al*., 2016) 5-(3-phenylacryloyl)2-hydroxybenzoic acid (**5a-f)** in excellent yield. 2-Benzylidene-1-phenylhydrazine analogues (**6a-d)** were synthesized by reported method (Sun *et al*., 1996). The title compounds **7a-j** were obtained as reported in our previous literature (Naveen *et al*., 2017) as represented in Scheme **1,** by oxidization of compounds **7a-d** to nitrilimines by chloramine T followed by 1,3-dipolar cycloaddition with compounds (**5a-f**).

Pharmacological screening

Anti-inflammatory activity (PLA2 inhabition)

The synthesized compound **7d**, with para hydroxy and methoxy group (Alam *et al*., 2016) substitution to the phenyl ring at 3rd and 4th position of the heyerocyclic ring, compound **7h,** with chloro and methoxy groups (Geronikaki and Gavalas, 2006) at para position of the phenyl ring at 3rd and 4th position, **7i** with chloro at meta and methoxy at para position at the phenyl ring at 3rd and 4th position respectively and **7a** compound with no substitution showed maximum inhibition with IC_{50} value of 0.020, 0.019, 0.018 and 0.015 µmole/mL respectively which is followed by compound **7j,** having chloro group at para position of the phenyl ring attached to 3rd and 4th position, compound **7b** bearing chloro and bromo group at para to the phenyl ring attached to 3rd and 4th position and compound **7f** with chloro at meta and hydroxy to para position of phenyl ring attached to 3rd and 4th position of heterocyclic moiety showed moderate inhibition with an IC_{50} values of 0.022, 0.025 and 0.024 µmole/mL respectively. Compounds **7c**, **7e** and **7g** showed least potency with IC_{50} values of 0.026, 0.027 and 0.027 µmole/mL respectively.

Antioxidant assays

The title compounds **7a-j** are screened for their *in vitro* antioxidant activity by DPPH (Di(phenyl)-(2,4,6-trinitrophenyl) iminoazanium) radical scavenging, nitric oxide radical scavenging, ferrous ion chelating and hydroxyl radical scavenging assays, which are represented in Table 1. Compound **7d** with para hydroxy and methoxy groups substitution (Burguete *et al*., 2007) at phenyl ring attached to 3rd and 4th position of heterocyclic ring, **7f** bearing chloro group at meta position and hydroxyl substitutions at the para position of phenyl ring attached to 3rd and 4th position of heterocyclic ring respectively and compound **7h** with chloro and methoxy groups both para to the phenyl ring at 3rd and 4th position of heterocyclic ring has shown significant antioxidant activity Compound **7a** with no substitution, **7b** with chloro and bromo group at para position of phenyl ring attached to 3rd and 4th position of heterocyclic ring respectively, **7c** bearing chloro at meta position of phenyl ring attached to 3rd position and bromo group substituted to para position of phenyl ring of 4th position**,** **7e** bearing chloro group at meta and para position of phenyl ring at 3rd and 4th position respectively, **7g** with chloro substitution at para and meta position of phenyl ring at 3rd and 4th position of the heterocyclic ring, **7i** with chloro substitution at meta position and methoxy at para position of the phenyl ring attached at 3rd and 4th position of the heterocyclic ring and **7j** bearing chloro substitutions at the para position of phenyl ring attached to 3rd and 4th position of heterocyclic ring showed moderate activity in comparison with the standard BHT (2,6-di-tert-butyl-4-methyl phenol).

Table 1: Antioxidant and Anti-inflammatory activity of synthesized compounds.

Compound	Antioxidant				Anti-inflammatory
	IC_{50} value (µmole/mL)				
	DPPH	NO	FIC	HO	PLA2 Inhabition
7a	NI	0.017	0.045	0.0016	0.025
7b	0.104	NC	0.089	0.0021	0.022
7c	0.079	0.018	0.023	0.0017	0.026
7d	0.072	0.012	0.012	0.0015	0.02
7e	NI	0.018	0.034	0.0013	0.027
7f	0.071	0.012	0.013	0.0017	0.024
7g	0.091	0.018	0.018	0.0027	0.027
7h	0.075	0.011	0.017	0.0023	0.019
7i	NI	0.013	0.018	0.0021	0.018
7j	NI	0.017	0.018	0.0018	0.015
BHT	0.163	0.043	0.045	0.0045	—

DPPH: DPPH radical scavenging assay; NO: Nitric oxide radical scavenging assay; FIC: Ferrous ion chelating assay; HO: Hydroxyl radical scavenging assay; NC: Not chelating; NI: No inhibition.

CONCLUSION

In this present study we have the synthesized the series of salicylic acid integrated dihydropyrazoline analogous (**7a-j**), which were then evaluated for PLA2 inhibition as well as anti-oxidant activity. All the molecules have exhibited good anti-oxidant activity and in addition the study reveals that the compounds with donating substitution have shown excellent anti-oxidant activity, whereas the compounds with hydroxy and chloro groups have shown good PLA2 enzyme inhibition, which opens up for the further study of the synergetic effect of the two biologically potent molecules for the discovery of new bioactive molecules.

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