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2-[5-(2-fluorophenyl)-3-(trifluoromethyl)-1H-pyrazol-1-yl] benzoic acid: Synthesis, Characterization and Pharmacological Evaluation

K. E. Manojkumar[1], S. Sreenivasa[1]*, N. R. Mohan[1], P. A. Suchetan[1], C. G. Darshan Raj[2] and H. Raja Naika[3]

1. Department of Studies and Research in Chemistry, Tumkur University, Tumkur-572 103, Karnataka, **INDIA**
2. Department of Bio-Medicinal Research, Vidya Herbs Pvt. Ltd, Anekal, Bangalore - 562 106, Karnataka, **INDIA**
3. Department of Studies and Research in Environmental Science, Tumkur University, Tumkur-572 103, Karnataka, **INDIA**

Email: drsreenivasa@yahoo.co.in

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ABSTRACT

2-[5-(2-fluorophenyl)-3-(trifluoromethyl)-1H-pyrazol-1-yl]benzoic acid was synthesized by 2-fluoroacetophenone, which was converted into diketone which was cyclized with 2-hydrazinylbenzoic acid in the presence of catalytic amount of acetic acid in dry ethanol. Compound characterization was done by LCMS, IR, ^{1}H-NMR, CHN and XRD analysis. The newly synthesized compound was screened its antibacterial activity with four bacterial strains of Gram positive S. aureus (NCIM-5022) and Gram negative E. coli (NCIM-5051), using cup plate method, anthelmintic activity against P. posthuma, anti-inflammatory activity carried on carrageenan induced paw edema. Further antioxidant and anti-proliferative studies were done. It was found that fluorinated pyrazole nucleus exhibited significant antibacterial, anthelmintic and anti-inflammatory activity and moderate activity in anti-oxidant and anti-proliferative studies.

Keywords: 2-[5-(2-fluorophenyl)-3-(trifluoromethyl)-1H-pyrazol-1-yl]benzoic acid, 2-hydrazinyl benzoic acid, acetic acid, antibacterial activity, anthelmintic activity, anti-inflammatory activity, anti-oxidant activity, anti-proliferative studies.

INTRODUCTION

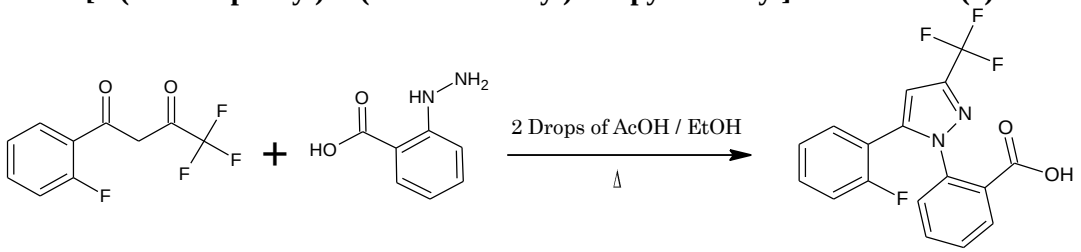
Pyrazoles have been considered as important pharmacophores in the past decades, because of their wide variety of pharmacological applications. Compounds bearing a trifluoromethyl substituent show a broad spectrum of pharmacological properties, including antitumor [1], anti-inflammatory [2], antioxidant [3], analgesic [4] and antiviral activity [5]. It was also reported that substituted pyrazoles show significant activities such as antimicrobial, anticancer [6], antitumor [7], analgesic [8], antitumor, antimalarial, anticonvulsant and leishmanicidal activity [9]. In addition to this some condensed pyrazoles have wide spectrum of biological effects including anti-inflammatory [10-12] and antiviral activity [13]. Compounds including 1,2-diazole nucleus and their N-substituted derivatives are known to possess corrosion inhibitor tendency [14-16]. It has been observed that Pyrazole preparation was well documented [17-19]. By view

of these positive pharmacophore reports, we have synthesized a new compound, 2-[5-(2-fluorophenyl)-3-(trifluoromethyl)-1H-pyrazol-1-yl] benzoic acid **(3)** using 2-fluroacetophenone as a starting material and it was fully characterized and evaluated for its pharmacological potency.

MATERIALS AND METHODS

All the reagents were purchased from commercial sources and used without further purification. Melting point was determined in one end open capillary tube on a liquid paraffin bath and was not corrected. Reaction was carried out under an inert nitrogen atmosphere. LCMS and ^{1}H-NMR spectra were recorded for the compound on Agilent Mass spectrometer, Bruker Avance II (300.65 MHz, ^{1}H-NMR) instruments respectively. Chemical shifts were reported in parts per million (ppm) using tetramethylsilane (TMS) as an internal standard and Elemental (C, H and N) analysis was performed on an Elementarvario MICRO cube. X-Ray diffraction study was carried out in Bruker Smart X2S diffractometer.

Synthesis of 2-[5-(2-fluorophenyl)-3-(trifluoromethyl)-1H-pyrazol-1-yl] benzoic acid (3)



1 **2** **3**

Scheme 1

Title compound (**3**) was synthesized by a known procedure reported for the synthesis of isomeric compound 2-[5-(2-fluorophenyl)-3-isobutyl-1H-pyrazole-1-yl]benzoic acid [20] (**Scheme 1**). 4,4,4-trifluoro-1-(2-fluorophenyl) butane-1,3-dione **(1)** (1.00 g, 4.16 mmol) was taken in absolute alcohol (10 mL) to this added 2-hydrazinylbenzoic acid **(2)** (0.69 g, 4.57 mmol) in one lot at room temperature under nitrogen atmosphere. Reaction mixture was refluxed in the presence of catalytic amount of acetic acid (2 drops) for about 6 h. After completion of the reaction, the reaction mixture was poured into ice-cold water, compound was extracted with ethyl acetate and organic layer was washed with water and brine solution. Organic phase was dried over anhydrous $MgSO_4$ and concentrated to get the crude product. Further purification was done by column chromatography using MDC/MeOH (9:1) as eluent to get the title compound **(3)** as a white solid with R_f= 0.24

Crystal Structure Determination of compound 3: Colorless prisms of the compound **3** were obtained from slow evaporation of the solution of the compound in a mixture of pet ether and ethyl acetate (1:2). A Colorless prism of the title compound with dimensions 0.32 x 0.27 x 0.24 mm was chosen for X-ray diffraction study. The data were collected on a Bruker Smart X2S diffractometer equipped with a fine focus, 3 kW sealed X-ray source (graphite monochromated Mo Kα). The crystal to detector distance was fixed at 120 mm with a detector area of 422 x 221 mm^2. Thirty six frames of data were collected at room temperature by the oscillation method. Each exposure of the image plate was set to a period of 300s. Successive frames were scanned in steps of 5^o/min with an oscillation range of 5^o. Image processing and data reduction were done using SAINT-Plus and XPREP [21]. All the frames could be indexed using a primitive triclinic lattice. The structure was solved by direct methods using SHELXS-97 [22]. All the Q peaks of non-hydrogen atoms were located in the first Fourier map itself. In the second stage, non-hydrogen atoms were refined anisotropically. All H atoms were positioned geometrically, with C-H =

0.93Å and O-H = 0.85Åand refined using a riding model with *U*iso(H) = 1.2Ueq(C) for C-H atoms. The details of the crystal data are given in **Table 1**. The structure of the molecule with thermal ellipsoids [23] drawn at 50% probability is shown in **Fig. 1**.

Table 1: Crystal data of 2-[5-(2-fluorophenyl)-3-(trifluoromethyl)-1H-pyrazol-1-yl] benzoic acid **(3)**

Empirical formula	$C_{17}\ H_{10}\ F_4\ N_2\ O_2$
Formula weight	350.27
Temperature/K	272(2)
Crystal system	Triclinic
Space group	P-1
a/Å	7.2052(1)
b/Å	13.903(3)
c/Å	18.579(3)
α/°	71.758(9)
β/°	110.674(6)
γ/°	89.357(9)
Volume/Å^3	1753.2(6)
Z, Z'	4, 2
ρ_{calc}mg/mm^3	1.317
Absorption coefficient (mm^{-1})	0.116
F(000)	706.7
Crystal size/mm^3	0.32 × 0.27 × 0.24
2Θ range for data collection	4.66 to 50.00°
Index ranges	-8 ≤ h ≤ 8, -16 ≤ k ≤ 16, -22 ≤ l ≤ 22

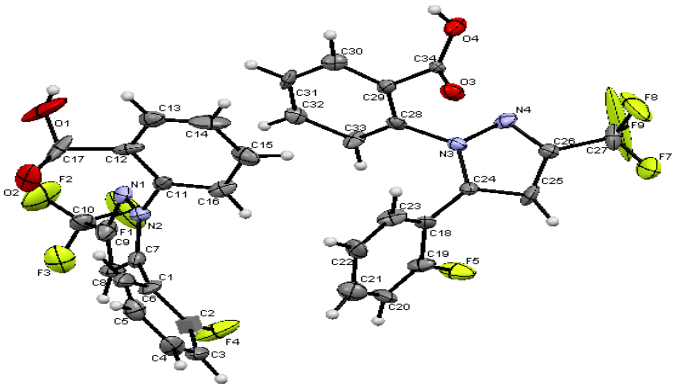


Fig. 1: Molecular structure of the title compound, showing displacement ellipsoids drawn at the 50% probability level.

***In vitro* Antibacterial Activity:** 2-[5-(2-fluorophenyl)-3-(trifluoromethyl)-1H-pyrazol-1-yl]benzoic acid was studied against Gram positive *Staphylococcus aureus* (NCIM-5022) and Gram negative *Escherichia coli* (NCIM-5051bacterial strains. All the bacterial strains were procured form CSIR-National Chemical Laboratory (NCL) Pune. The antibacterial property of the compound was determined through agar well diffusion method, [24, 25] broth cultures of bacterial strains were incubated for 24 h and were uniformly smeared on sterile nutrient agar medium in each petri plates using sterile L-Shaped glass rod. Five uniform wells with 6 mm diameter were bored using cork borer to accommodate 50 µL of solution in each well. Sample was dissolved in dimethylsulfoxide (DMSO) a negative control which showed no zone of inhibition and Ciprofloxacin (5µg/50mL) was taken as standard drug (positive control), purchased from Himedia, Mumbai, India. Concentrations of 200 and 400 µg/well were used to assess the dose dependent activity. Sterile micropipette tips were used to load the wells with appropriate amount of sample, control and standard. Then the plates were incubated at 37 °C for 36 h. After the incubation period, the diameter of the zone of inhibition of each well was measured in mm; the experiment was performed in triplicates the average values were calculated and are given in **Table 3.**

Anthelmintic Activity: Anthelmintic activity of 2-[5-(2-fluorophenyl)-3-(trifluoro methyl)-1H-pyrazol-1-yl]benzoic acid was determined using *P. posthuma* (Indian Earthworm), worms were maintained under normal vermicomposting medium with adequate supply of nourishment and water for about three weeks. Adult earthworms of approximately 4 cm in length and 0.2 - 0.3 cm in width were chosen for experiment. Different concentrations 50 and 100 mg/mL of samples was evaluated as per the standard method reported [26]. Four groups each with six earth worms were taken. Each *P. posthuma* was washed separately with normal saline before the initiation of experimental procedure and were placed into a 20 mL of normal saline. Group I earthworms were placed in 20 mL saline in a clean petri plate and Group II earthworms were placed in 20 mL saline containing standard drug piperazine citrate (50 mg/mL). Similarly, Group III and Group IV earthworms were placed in a 20 mL saline containing 50 and 100 mg/mL of test samples respectively. Observation was done keeping time taken for paralysis and the time taken for death as objective and was documented in minutes. Paralysis time was analyzed based on behavior of the worms with no revival body state in normal saline medium. Death was concluded based on total loss of motility with faded body color and the result are illustrated in **Table 4**.

Anti-Inflammatory Activity: Effect of 2-[5-(2-fluorophenyl)-3-(trifluoromethyl)-1H-pyrazol-1-yl] benzoic acid compound on carrageenan induced paw edema was studied on albino Wistar rats of either sex which were obtained from Sree Venkateshwara Enterprises Bangalore. The animals were acclimatized, maintained under standard laboratory condition for a week. Given free access to UV purified, filtered water and standard pelleted feed procured from M/s Pranava Agro Industries Sangli, Maharastra, ad libitum. Studies conducted were approved by the Institutional Animal Ethics Committee (IAEC) of Sree Siddaganga College of Pharmacy, Tumkur (Ref No: SSCPT/IAEC.Clear/141/2012-13). Standard used was Indomethacin which was procured from Research Lab Fine Chem Industries, (B.N:99550509, Mfg date: May 2009, Exp. Date: May 2014). Test compound (100 mg/kg body weight) and indomethacin (10 mg/kg body weight). Test compound was made into suspension by using 1% carboxy methyl cellulose (vehicle) and administered through oral route.Carrageenan induced paw edema [27] experiment was carried out on test system (albino wistar rats) weighing between 150 - 180 g was randomly divided into three groups of six animals each and was fasted overnight. Group I served as control and received vehicle, Group II standard indomethacin (10 mg/kg bw) through oral route. Group III was administrated with test compound 2-(5-(3,5-difluorophenyl)-3-isobutyl-1H-pyrazol -1-yl)benzoic acid at the dose of 100 mg/kg bw and after administering sample / Indomethacin / vehicle, test systems were kept under clinical sign observations for 30 min. The suspension of carrageenan (0.1 mL of 1% w/v) was injected into the sub-plantar region of right hind paw of each test system. The paw volume was measured by using digital Plethysmometer (IITc Life science, USA), immediately after injection, again at 30, 60, 120, and 180 min intervals, results are given in Table 5.

***In vitro* Antioxidant Assay: DPPH Scavenging Assay:** Antiradical activity of the compound was measured by a decrease in the absorbance at 516 nm of a methanolic solution of colored DPPH. [28]. A stock solution of DPPH (1.3 mg/mL in methanol) was prepared such that 75 µL of it in 3 mL methanol gave an initial absorbance of 0.9. Decrease in the absorbance in the presence of the compound **3** at different concentrations was noted after 15 min. EC_{50} (i.e. the concentration of the test solution required to give a 50% decrease in the absorbance compared to that of blank solution) was calculated from percent inhibition. A blank reading was obtained using methanol instead of the compound. Butylated Hydroxytoluene (BHT) was used as standard. The percentage inhibition of antiradical activity was calculated using the formula.

$$\% \text{ inhibition} = \frac{\text{Absorbance of blank} - \text{Absorbance of test sample}}{\text{Absorbance of blank}} \text{ X } 100$$

Estimation of Superoxide Anion Scavenging Activity: The superoxide anion scavenging activity of the compound **3** was determined by the method described by [29]. About 1 ml NBT solution containing 156 µM NBT dissolved in 1.0 mL 100 mM phosphate buffer, pH 7.4, 1 mL NADH solution containing 468 µM NADH dissolved in 1 mL 100 mM phosphate buffer, pH 7.4, and 0.1 ml of various concentration of test sample and reference compound (100 and 120 µg) were mixed and the reaction was started by adding 100 µL phenazinemethosulfate solution containing 60 µM phenazinemethosulfate in 100 mM phosphate buffer, pH 7.4. The reaction mixture was incubated at 25ºC for 5 min and absorbance at 560 nm was measured against control samples. BHT was used as reference compound. Decreased absorbance of the reaction mixture indicated increased superoxide anion scavenging activity. All tests were performed in triplicate. The capability of scavenging the superoxide anion radicals was calculated using the following equation,

$$\% \text{ scavenging effect} = \left[\frac{(A_0 - A_1)}{A_0} \right] \text{X } 100$$

Where,
A_0 is the absorbance of the control (without test samples)
A_1 is the absorbance of test samples.

Lipid Peroxidation Assay: A modified Thiobarbituric acid reactive species (TBARS) assay was used to measure lipid peroxidation [30]. In a test tube 0.5mL (10% in distilled water) of egg homogenate, 0.1 mL of test sample was taken. The volume was made up to 1 mL by adding distilled water. To induce lipid peroxidation, 0.05 mL ferrous sulphate (0.07M) was added to the above mixture and incubated for 30 min. There after add 1.5 mL of 20% acetic acid (pH 3.5), 1.5 mL of 0.8% TBA and 0.05 mL 20% TCA was added, vortexed and heated in boiling water bath for 60 min. After cooling 5 mL of Butanol was added to all the test tubes and centrifuged at 3000 rpm for 10 min. Measure the absorbance of the organic upper layer at 532 nm in uv-vis Spectrophotometer (Shimadzu UV-2450). The experiment was performed in triplicate. The capability of scavenging the free radicals was calculated using the following equation,

$$\% \text{ Scavenging effect} = \frac{\text{Control} - \text{Test}}{\text{Control}} \text{ X } 100$$

Results of the *in vitro* antioxidant assay data are summarized in the result and discussion section.

Antiproliferative Activity: The HepG2 and EAT cell lines were grown in RPMI 1640 medium containing 10% fetal bovine serum and 2mM L-glutamine. Compound **3** was evaluated for *in vitro* cytotoxicity

against HepG2 and EAT cell lines by MTT assay [31]. For present screening experiment, cells were inoculated into 96 microliter plates in 100µL at plating densities depending on the doubling time of individual cell lines. After cell inoculation, the microliter plates were incubated at 37^0C, 5%, CO_2, 95% air and 100% relative humidity for 24 h prior to addition of **3** compound at a concentration range of 1 µg/mL after 48 h. 50 µL of 1 mg/mL solution of MTT in RPMI-1640 medium was added to each well. The culture plates were gently shaken and incubated for another 4 h. MTT was removed carefully and DMSO (100 µL) was added and shaken well. The absorbance was measured at 570 nm in an automated plate reader and the percentage of cells growth inhibition was calculated using the following formulae [31] and given in result and discussion section of the paper.

$$\% \text{ inhibition} = \frac{\text{Absorbance control} - \text{Absorbance test}}{\text{Absorbance test}} \text{ X } 100$$

Statistical Analysis: The data of antibacterial and anthelmintic was expressed as Mean±S.E. of triplicates and six Pheretima posthuma in each group respectively. The difference in values at **p≤0.01 was considered as statistically significant. The analysis of variance (ANOVA) was performed using ezANOVA (version 0.98) software to determine the mean and standard error of the inhibition zone in antibacterial activity and standard error of paralysis and death time of earthworms. Anti-inflammatory data was expressed as Mean±S.E. the difference in values at *p<0.05, **p<0.01 and ***p<0.001 when compared with carrageenan control using ANOVA followed by Dunnett's Multiple Comparison Test on Graph Pad Prism 5.

RESULTS AND DISCUSSION

Characterization of synthesized compound was done by LC-MS, IR, ^{1}H NMR spectra, elemental analysis and X-ray Crystallography. Data's interpreted in the table 2 are in agreement with the structure proposed.

Table 2: Physical and Spectral data of 2-[5-(2-fluorophenyl)-3-(trifluoromethyl)-1H-pyrazol-1-yl] benzoic acid **(3)**

Compound Code	**3**
Molecular Formula	$C_{17}H_{10}F_4N_2O_2$
Color and Nature	Colorless crystalline solid
Yield in %	68 % (1.02 g)
LCMS: *m/z*	351.2 (M+ 1)
IR: νmax/cm^{-1}	3453-3319 (very broad strong H-bond), 1650 (C=O broad stretching), 1571 (C-O stretching), 1340-1138 (CF3 stretching)
^{1}H NMR	12.39 (s, 1H, OH), 8.10 (t, *J* = 7.59 Hz, 2H, Ar-H), 7.5-5-7.08 (m, 6H, Ar-H), 6.84 (s, 1H, Pyrazole-H)
Elemental Analysis: Calculated % (Found %)	C = 58.17 % (58.06 %); H = 2.86 % (2.45 %); N = 8.00 % (8.12 %); O = 9.14 % (9.26 %).
MP ^{o}C	221–223 ^{o}C

Crystallographic Study: The title compound crystallizes in the triclinic crystal system and *P*-1 space group with the unit cell parameters given in **Table 1**. The number of molecules in the asymmetric unit is two. In the first molecule, the dihedral angle between the pyrazole and the fluorobenzene ring is 52.85°, while that between the fluorobenzene and the benzoic acid ring is 71.82°, and that between pyrazole and the benzoic acid ring is 60.24°. Similarly, in the second molecule, the respective dihedral angles are 53.80°, 70.30° and 55.96°. In the crystal structure, the molecules are linked into one another through C5-H5…π interactions of pyrazole ring (**Fig 2.**)

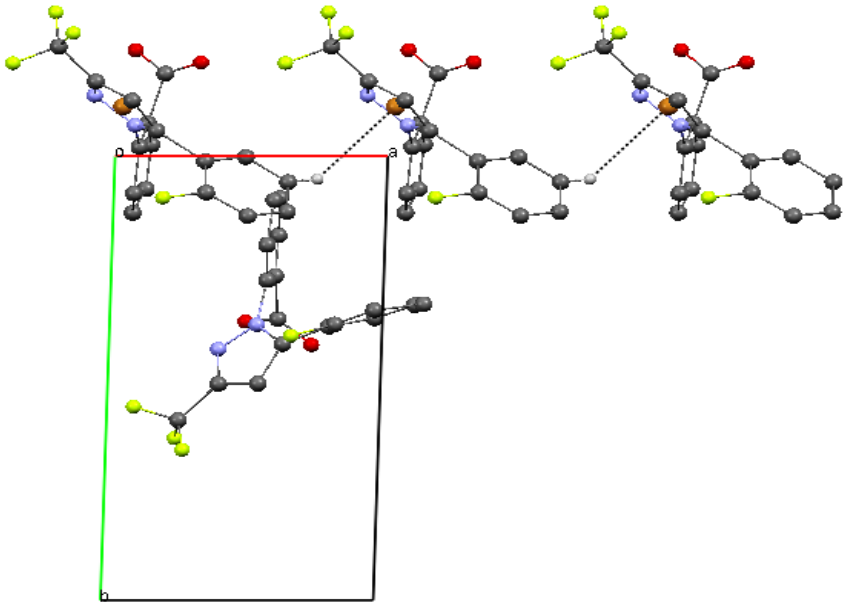


Fig. 2: The packing of molecules along a *axis* via C-H… Cg interactions. Here Cg is the centroid of the pyrazole ring. Hydrogen atom not involved in C-H……Cg interactions are omitted for clarity purpose.

Table 3: Geometry of C5-H5…π interactions (Å, deg)

D-H…A	**d(D-H)**	**d(H-A)**	**d(D-A)**	**D-H-A**
C5-H5… π*	0.93	2.99(3)	3.6766(3)	132(3)

* -1+X, Y, Z

Table 4. Antibacterial activity of compound 3

Samples	**Concentration (µg/mL)**	**Bacterial Strains**	
		E. Coli	***S. Aureus***
Standard Ciprofloxacin	10	15.12 ± 0.43	12.10 ± 0.39
Compound 3	200	4.82 ± 0.31**	1.01 ± 0.34*
	400	6.33 ± 0.52**	3.21 ± 0.31**
Solvent DMSO	-	NS	NS

Antibacterial activity result showed that compound **3** is active at higher concentrations 200 and 400 µg /mL. Values given are the mean ± SEM of clear zone and symbols represent statistical significance, **P* < 0.05, ***P* < 0.0. NS - No significance observed

Table 5: Anthelmintic activity of compound 3 against *Pheretima posthuma*

Test Samples	Concentration (mg/mL)	Time taken for paralysis (min)	Time taken for death (min)
Control (Saline)	-	142.33± 0.49	167.17±0.87
Standard Piperazine citrate	50	39.17± 0.48**	57.00± 0.58**
Compound 3	100	37.00± 1.59	66.50 ± 0.76
	50	69.00 ± 1.46**	109.00 ± 0.86**

Anthelmintic activity result showed that compound **3** is moderately active compared to standard drug piperazine citrate. Values given are the mean±S.E.M of three earthworms and symbols represent statistical significance *p<0.05, **p<0.01.

Table 6: Effect of 3 on Carrageenan induced paw edema

Concentration (Dose mg/kg. p.o)	Time in Minutes			
	30	**60**	**120**	**180**
Standard Indomethacin (10 mg/kg)	0.27±0.03	0.20±0.06***	0.23±0.06***	0.29±0.06**
Carrageenan (1%, 0.1 mL)	0.22±0.100	0.44±0.04	0.54±0.03	0.48±0.09
Compound 3 (100 mg/kg)	0.28±0.073	0.13±0.03 ***	0.02± 0.00 ***	0.09 ± 0.04***

Compound **3** showed significant anti-inflammatory activity at time intervals 60,120 and 180 min compared to control Carrageenan. Proving that compound **3** is a potent anti-inflammatory agent. Values are given the mean ± S.E.M for six animals each group ANOVA followed by Dunnett's Multiple Comparison Test. Values are statistically **p<0.01 and ***p<0.001 when compared with Carrageenan control.

DPPH Radical Scavenging Activity: The free radical scavenging activity was investigated in DPPH assay. **3** at concentration 100 µg/mL showed 23.16 % scavenging the free radicals compared with that of BHT [32] which showed 75 % inhibition at 75 µg/mL. **3** showed DPPH scavenging activity in concentration dependent manner.

Superoxide anion scavenging activity: The superoxide anion scavenging activity was reported in **3** at concentration of 100µg/mL showed 18.21 % inhibition when compared with the vitamin C. The superoxide anion derived from dissolved oxygen by Phenazinemetho sulphate/NADH coupling reaction

reduces nitroblue tetrazolium. The decrease in the absorbance at 560 nm with the active constituents of the compounds indicates the consumption of superoxide anion in the reaction mixture.

Lipid peroxidation inhibition activity: Compound **3** was screened against non-enzymatic in vitro lipid peroxidation in egg homogenate was evaluated by method of determination of thiobarbituric acid reactive species (TBARS). Compound **3** showed low absorbance values, which indicated a high level of antioxidant activity. Scavenging activity of the compound **3** at concentration 100 µg/mL was 16.55 % shows its ability to scavenge free radicals, and its inhibition value was comparable to standard Isoascorbic acid.

Anti-proliferative studies: The experiments were carried out in triplicates and to the average values were plotted graphically. The percentage of inhibition was calculated and they were compared with the positive control drug Aderomycin (ADR). [33] The results showed that the compound **3** was active in the assay system used. The compound **3** showed 57.42±6.13% inhibition on HEPG2 and 73.33±8.17% on EAT cells when compared to the standard drug ADR.

APPLICATIONS

Results obtained by the pharmacological studies indicate that this 2-[5-(2-fluorophenyl)-3-(trifluoromethyl)-1H-pyrazol-1-yl] benzoic acid possess promising pharmacophore property.

CONCLUSIONS

Tested compound 2-[5-(2-fluorophenyl)-3-(trifluoromethyl)-1H-pyrazol-1-yl] benzoic acid is an active antibacterial, anthelmintic, anti-inflammatory, antioxidant and anti-proliferative agent. It can be concluded that compounds of this class certainly hold great promise for discovering new antibacterial, anthelmintic, anti-inflammatory, antioxidant and anti-proliferative agents.

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