A study on synthesis and biological evaluation of curcumin pyrazole derivatives for anticancer and antioxidant properties

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ABSTRACT: A series of curcumin pyrazole derivatives (2a & 2b) were synthesized and the structures were determined by ^{1}H and ^{13}C NMR spectroscopic techniques. The purity of the compounds was confirmed by LC-MS. The curcumin and its pyrazole series (2a & 2b) along with standard drugs were assessed for in vitro antioxidant and anticancer activities. From the present findings, the compound 2a possess promising in-vitro anticancer and antioxidant potency compared to that of natural curcumin. The synthetic analogue of curcumin, compound4,4'-(1E,1'E)-2,2'-(1-(4-chlorophenyl)-1H-pyrazole-3,5-diyl)bis(ethene-2,1-diyl)bis(2-methoxyphenol) (2a) could be a promising economical drug effective at non-toxic doses and necessitates in-vivo investigations.

Key words: Curcumin pyrazoles, antioxidant, trypan blue, anticancer

I INTRODUCTION

Plant derived drugs are gaining importance globally for its selective therapeutic properties which are based on the experimental evidences [1-3]. Curcumin is a hydrophobic polyphenol obtained from the rhizome (turmeric) of the herb *Curcuma longa*. Curcumin has been recognized as the major active component in turmeric and pharmacologically, curcumin has been found to be safe even at very high doses in human clinical trials [4]. It has been extensively used for a range of treatments due to its medicinal properties [5]. In spite of all these, curcumin is not a potent therapeutic molecule as the potential utility of curcumin is narrowed by its *in vivo* bioavailability [6, 7].

Many approaches are being sought to prevail over these limitations. Synthetic chemical modifications of curcumin have been studied & various curcumin analogues are synthesized to enhance the pharmacological profile of natural product. New analogues of curcumin exhibited growth suppressive activity than natural curcumin and an analogue of curcumin showed potent growth inhibitory activities on both prostate and breast cancer cell lines than curcumin [8, 9].

In our previous investigation, ethanone pyridine curcumin analogues and cyclopropoxy curcumin analogues were synthesized which exhibited *in-vivo* growth inhibitory and anti-angiogenic effects against a mouse tumor model [10-12]. Subsequently curcumin pyrazole derivatives were synthesized and investigated for the anti-proliferative, antioxidant, antidiabetic and antihyperlipidemic potential [13-16]. In this line of investigation, in the present study, a new series of curcumin pyrazole derivatives were synthesized and assessed for in-vitro antioxidant and anticancer properties in comparison with the natural curcumin.

II MATERIALS AND METHODS

2.1 MATERIALS

All chemicals and solvents were of analytical grade and obtained from Himedia chemicals, Mumbai, India. The chemicals and reagents used were 1, 1-Diphenyl-2-picryl hydrazyl (DPPH), sulphanilamide, sodium nitroprussude, o-phosphoric acid, N-(1-(Naphthyl ethylene diamine dihydrochloride), phenazine methosulfate, nicotinamide adenine dinucleotide reduced (NADH), riboflavin, sodium cyanide, ferrous bisulphate, thiobarbutyric acid (TBA), nitroblue tetrazolium (NBT), ethylene diamine tetra acetate (EDTA), RPM1 1640 media, fetal bovine serum, trichloroacetate (TCA), penicillin, streptomycin, paclitaxel, avastin and tamoxifen drugs.

The cell lines, HeLa (Human cervical cancer cell line), MCF-7 (Human breast cancer cell line), K-562 (human myelogenous leukemia cell line) were purchased from National centre for cell science, Pune, India.

2.2 METHODS:

2.2.1 SYNTHESIS OF CURCUMIN PYRAZOLE DERIVATIVES

Reactions were monitored by TLC using precoated sheets of silica gel G/UV-254 of 0.25 mm thickness (Merck 60F254) using UV light for visualization. ^{1}H and ^{13}C NMR spectra were recorded on an NMR spectrometer operating at 400 and 100 MHz respectively, using the residual solvent peaks as reference relative to $SiMe_4$. Mass spectra were recorded using high resolution mass spectrometer (HRMS). Infrared spectra were recorded on Shimadzu FT-IR model 8300 spectrophotometer.

2.2.1.1 General procedure for the synthesis of compounds (2a and 2b)

To a solution of curcumin (1 eq) in ethanol, $NaHCO_3$ (1.5 eq) and different phenyl hydrazine hydrochlorides (1.5 eq) were added. The resulting mixture was refluxed for 12-15 h and the reaction was monitored by TLC [17]. After completion of the reaction, mixture was evaporated under reduced pressure and the resulting residue was dissolved with ethyl acetate and washed with water followed by the brine solution. The organic layer was dried over anhydrous sodium sulfate and evaporated under vacuum to get the crude product which was purified by column chromatography using hexane: ethyl acetate as an eluent.

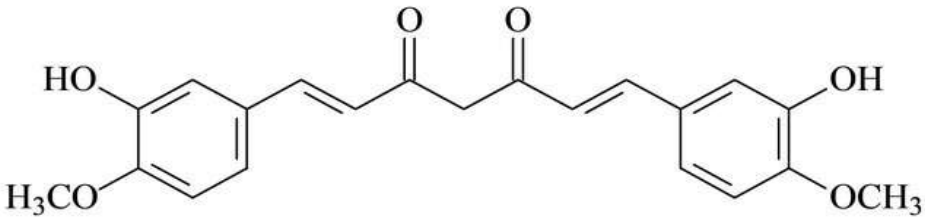
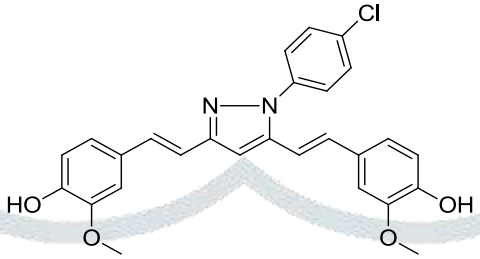


Fig 1. Structure of curcumin
(1,7-bis(4-hydroxy-3- methoxyphenyl)-1,6-heptadiene-3,5-dione).

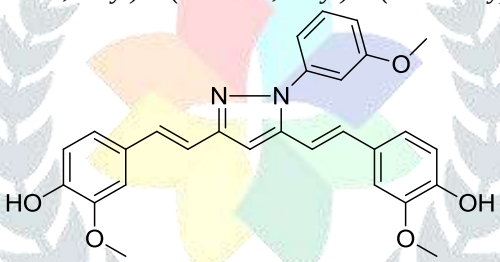
Characterization data

4,4'-(1E,1'E)-2,2'-(1-(4-chlorophenyl)-1H-pyrazole-3,5-diyl)bis(ethene-2,1-diyl)bis(2-methoxyphenol) (2a)



The title compound was prepared from curcumin (1 eq) and 4-chloro phenylhydrazine hydrochloride (1.5 eq) according to the general procedure. The product obtained as a pale brown solid which was purified from methanol. Yield: 90 %; **^{1}H NMR** (400 MHz, DMSO-d6): δ 3.80 (s, 3H, OCH_3), 3.83 (s, 3H, OCH_3), 6.80-6.86 (m, 4H), 6.98-7.19 (m, 7H), 7.50-7.66 (m, 4H, ArH), 9.19 (s, 1H, OH), 9.30 (s, 1H, OH). **^{13}C NMR** (100 MHz, DMSO-d6): δ 56.08 (OCH_3), 56.12 (OCH_3), 101.8 (ArC-H), 110.1 (ArC-H), 110.8 (Pyrazole C=C), 111.4 (ArC-H), 116.0 (ArC-H), 116.9 (C=C), 117.4 (ArC-H), 120.7 (ArC-H), 121.1 (ArC-H), 124.7 (C=C), 127.9 (Ar-C), 129.0 (Ar-c), 131.2 (C=C), 131.8 (ArC-H), 133.5 (C=C), 134.1 (ArC-Cl), 141.1 (Pyrazole C-N), 142.9 (ArC-N), 147.3 (ArC-OH), 148.0 (ArC-OH), 148.2 (ArC-OCH_3), 148.3 (ArC-OCH_3), 151.9 (Pyrazole C=N). **LCMS** (ESI) *m/z* $[M + 1]^+$475.97; Anal. Calcd for $C_{27}H_{23}ClN_2O_4$: C, 68.28; H, 4.88; Cl, 7.46; N, 5.90; O, 13.48 Found: C, 68.31; H, 4.89; Cl, 7.50; N, 5.91; O, 13.51.

*4,4'-(1E,1'E)-2,2'-(1-(**3**-methoxyphenyl)-1H-pyrazole-3,5-diyl)bis(ethene-2,1-diyl)bis(2-methoxyphenol) (2b)*



The title compound was prepared from curcumin (1 eq) and 3-methoxy phenylhydrazine hydrochloride (1.5 eq) according to the general procedure. The product obtained as a brown solid was purified from methanol. Yield: 91 %; **^{1}H NMR** (400 MHz, DMSO-d6): δ 3.89 (s, 3H, OCH_3), 3.91 (s, 3H, OCH_3), 3.92 (s, 3H, OCH_3), 6.80 (s, 1H), 6.99-7.04 (m, 3H), 7.09-7.21 (m, 6H), 7.30-7.39 (m, 5H). **^{13}C NMR** (100 MHz, DMSO-d6): δ 55.4 (OCH_3), 56.4 (OCH_3), 56.8 (OCH_3), 107.0 (ArC-H), 107.9 (ArC-H), 110.3 (ArC-H), 110.8 (Pyrazole C=C), 111.9 (ArC-H), 112.9 (ArC-H), 114.9 (ArC-H), 115.5 (ArC-H), 117.9 (C=C), 118.2 (ArC-H), 119.5 (ArC-H), 122.2 (ArC-H), 124.6 (C=C), 129.0 (Ar-C), 129.6 (Ar-C), 129.9 (C=C), 130.7 (C=C), 132.3 (Pyrazole C-N), 137.8 (ArC-N), 145.8 (ArC-OH), 146.3 (ArC-OH), 146.8 (ArC-OCH_3), 148.0 (ArC-OCH_3), 148.9 (ArC-OCH_3), 154.1 (Pyrazole C=N). **LCMS** (ESI) m/z $[M + 1]^+$ 471.54 Anal. Calcd for $C_{28}H_{26}N_2O_5$: C, 71.47; H, 5.57; N, 5.95; O, 17.00 Found : C, 71.50; H, 5.59; N, 5.97; O, 17.01.

2.2.2 ANTIOXIDANT ACTIVITIES

2.2.2.2 Antioxidant activity by DPPH method

The antioxidant activity of the curcumin and its analogues (2a & 2b) was determined using 1, 1-Diphenyl-2-picryl hydrazyl radical (DPPH). DPPH scavenging activity was measured by the spectrophotometric method with minor modifications [18]. 0.05 mL of the curcumin and the compounds (2a & 2b) dissolved in DMSO were added to a methanolic solution of DPPH (200 µM), at different concentrations (25-100 µM). An equal amount of DMSO was added to the control. The decrease in the absorbance of test compounds was read at 517 nm after 20 min using spectrophotometer (Shimadzu UV-1800) and the percentage inhibition was calculated by using the formula:

$$\%\ scavenging\ activity = \frac{(control\ absorbance - sample\ absorbance)}{(control\ absorbance)} \times 100$$

Where control absorbance is the measurement of DPPH solution without compound and sample absorbance is the measurement of DPPH solution with compound.

2.2.2.3 Superoxide anion radical scavenging assay

The scavenging activity of the superoxide anion radical (O_2^-) was measured in terms of inhibition of production of O_2^- according to the method described with minor modifications [19]. To 1 mL of nitro blue tetrazolium (NBT) solution (156 mM NBT in 100 mM phosphate buffer, pH 7.4), 1 mL NADH solution (468 mM NADH in 100 mM phosphate buffer, pH 7.4), and 1 mL of curcumin and its derivatives (2a & 2b) in DMSO was mixed at different concentrations (25-100 µM). The reaction was started by adding 100 mL of phenazine methosulfate (PMS)

solution (60 µM PMS in 100 mM phosphate buffer, pH 7.4) to the mixture. The reaction mixture was incubated at 25 °C for 5 min and the absorbance was measured at 560 nm against blank and compared with the standard. The decreased absorbance of the reaction mixture indicated increased superoxide anion scavenging activity. The percentage inhibition of superoxide anion generated was calculated.

$$\%\ scavenging\ activity = \frac{(control\ absorbance - sample\ absorbance)}{(control\ absorbance)} \times 100$$

2.2.2.4 Nitric oxide scavenging activity

Nitric oxide scavenging activity was measured spectrophotometrically with minor modifications [20]. Sodium nitroprusside (5 mM) in phosphate buffered saline, pH 7.4 was mixed with different concentrations of the curcumin and its pyrazoles, 2a and 2b at 25-100 µM concentrations prepared in DMSO and incubated at 25 °C for 150 min. A control without test compound, but with an equivalent amount of DMSO; was taken. After 150 min, 1.5 mL of the incubated solution was removed and diluted with 1.5 mL of Griess reagent [1 % sulphanilamide, 2 % phosphoric acid and 0.1 % N-(1-(Naphthylethylenediamine dihydrochloride). Absorbance of the chromophore formed during diazotization of the nitrite with sulphanilamide and subsequent coupling with N-(1-(Naphthylethylenediamine dihydrochloride) was measured at 546 nm. The percentage scavenging activity was measured and compared with the effects of ascorbic acid.

$$\%\ scavenging\ activity = \frac{(control\ absorbance - sample\ absorbance)}{(control\ absorbance)} \times 100$$

2.2.3 ANTICANCER ACTIVITY

Cell lines and culture

Three different cancer cell lines, MCF-7, HeLa and K-562 cells were selected for the anticancer screening of curcumin and its pyrazole derivatives (2a & 2b). Cells were grown in RPM1 1640 supplemented with 10% heat inactivated fetal bovine serum (FBS), 100 U/mL of penicillin and 100 µg of streptomycin/mL and incubated at 37 °C in a humidified atmosphere with 5% CO_2. MTT assay was employed to assess the growth inhibitory potential of the curcumin and its analogues [21] with few modifications.

2.2.3.1 MTT assay

The cytotoxic effects of the curcumin and synthesized compounds against the cervical carcinoma, breast carcinoma and leukemic cells (5 x 10^5 cells) were assessed using 3-(4, 5 dimethyl-2-yl)-2, 5 diphenyl tetrazolium bromide (MTT) assay. The test compounds are dissolved in DMSO and treated with different concentrations of curcumin and synthesized compounds 2a and 2b (10, 20 and 40 µM respectively). Cells in the control wells received the same volume of the media containing DMSO. After 48 and 72 h treatment, cells were harvested and incubated with MTT (0.5 µg/mL) for 4 h at 37 °C in 96 well plate. The blue MTT formazan precipitate formed in the viable cells was solubilized by the addition of 70 µL DMSO. The suspension is placed in microvibrator for 5 min and absorbance was measured at 540 nm using multimode reader (Varioskan Flash Multimode, Thermo scientific, USA). The experiment was performed in triplicates and repeated at least for three times.

2.2.3.2 Trypan blue dye exclusion assay

To study the growth suppressive effects of the curcumin derivatives (2a & 2b) along with curcumin, 5 X 10^5 cells were plated in a 24 well plate (Corning, USA.) in 1 ml of complete medium [22]. All the three cancer cell lines were treated with various concentrations of curcumin and its derivatives, 2a and 2b (10, 20 and 40 µM respectively). DMSO treated cells were used as control. Cells were harvested after 48h and stained with 0.4% trypan blue and calculated using a haemocytometer for viable cells. Experiments were done in triplicates and the percentage of growth inhibition by different samples at different concentrations was plotted against time (48h). Tamoxifen, avastin and paclitaxel were used as standard anticancer drugs for MCF-7, HeLa and K562 cell lines respectively.

STATISTICAL ANALYSIS

Analyses were performed by using graph pad software prism 5.1. All the values are expressed as mean ± SEM for samples and are statistically analyzed. One way ANOVA followed by Dunnett test, in each case experimental sample were compared with control and significance was determined. p-value ≤0.05 was considered as statistically significant.

III RESULTS AND DISCUSSION

3.1 Antioxidant studies

3.1.1 DPPH free radical scavenging assay

The free radical scavenging ability of curcumin and its pyrazoles (2a & 2b) along with ascorbic acid were analyzed by the DPPH method. The free radical scavenging activity of each compound at a concentration range of 25-100 µM was evaluated by measuring the change of absorbance formed by the reduction of DPPH. The scavenging activity of ascorbic acid against DPPH was found to be 39.24, 72.58 & 86.23% at 25, 50 & 100 µM concentrations respectively. The scavenging activity of curcumin was found to be 31.94, 49.21 & 78.23% at 25, 50 & 100 µM concentrations respectively. Compound 2a showed an effective quenching with 37.51, 68.53 & 88.58% inhibition at 25, 50 & 100 µM concentrations respectively. Compound 2b was also found to be efficient in quenching free radical with 29.46, 51.41 & 78.12% inhibition at 25, 50 & 100 µM concentrations respectively. Compound 2a was found to be potent as ascorbic acid in quenching DPPH free radicals followed by curcumin and 2b respectively.

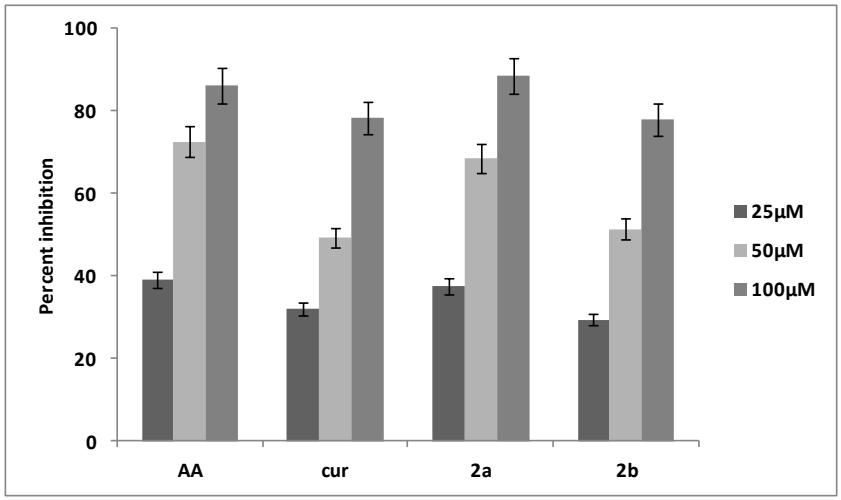


Fig 2. DPPH free radical scavenging activity of curcumin and its derivatives 2a and 2b. (AA: Ascorbic acid)
Values are mean ± SEM (n=3) analyzed by one-way ANOVA followed by Dunnet's test. Data are pooled from three independent experiments. ($p \leq 0.05$ is considered as statistically significant).

3.1.2 Nitric oxide scavenging activity

Nitric oxide scavenging activities of curcumin and its pyrazoles (2a & 2b) along with ascorbic acid were tested. The nitric oxide scavenging activity of ascorbic acid was found to be 38.14, 69.11 & 93.59% at 25, 50 & 100 µM concentrations respectively. The curcumin was also effective in scavenging nitric oxide free radicals and was found to be 34.12, 49.76 & 74.13% at 25, 50 & 100 µM concentrations respectively. Compound 2a showed an effective quenching and was comparable to ascorbic acid activity with 38.94, 61.45 & 86.11 % inhibition at 25, 50 & 100 µM concentrations respectively. Compound 2b showed quenching with 31.45, 48.76 & 76.23% inhibition at 25, 50 & 100 µM concentrations respectively. Compound 2a exhibited more potency in scavenging nitric oxide radical than curcumin and compound 2b.

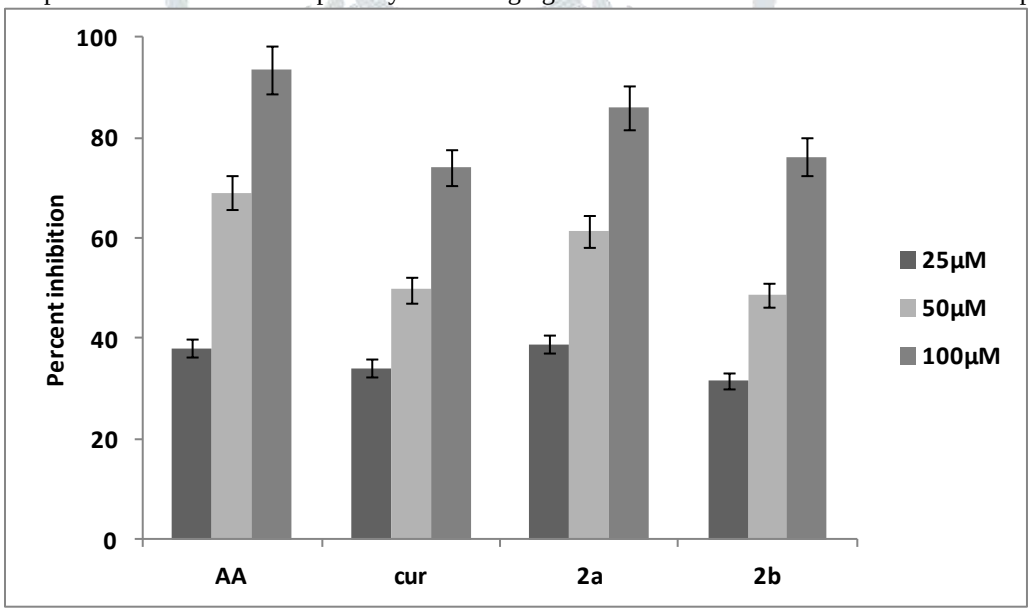


Fig 3. Nitric oxide scavenging activity of curcumin and its derivatives 2a and 2b. (AA: Ascorbic acid)
Values are mean ± SEM (n=3) analyzed by one-way ANOVA followed by Dunnet's test. Data are pooled from three independent experiments. ($p \leq 0.05$ is considered as statistically significant).

3.1.3 Superoxide anion scavenging activity

Superoxide anion scavenging activities of curcumin and its pyrazoles (2a & 2b) along with ascorbic acid were assessed. The nitric oxide scavenging activity of ascorbic acid was found to be 28.52, 66.89 & 81.74% at 25, 50 & 100 µM concentrations respectively. The scavenging activity of curcumin was found to be 27.59, 49.25 & 76.51% at 25, 50 & 100 µM concentrations respectively. Compound 2a showed an effective quenching with 25.46, 53.11 & 75.89% inhibition at 25, 50 & 100 µM concentrations respectively. Compound 2b showed quenching with 20.41, 43.56 & 70.79% inhibition at 25, 50 & 100 µM concentrations respectively. Compound 2a and 2b exhibited prominent activity and compound 2a was found to be potent as curcumin in scavenging superoxide anion free radicals.

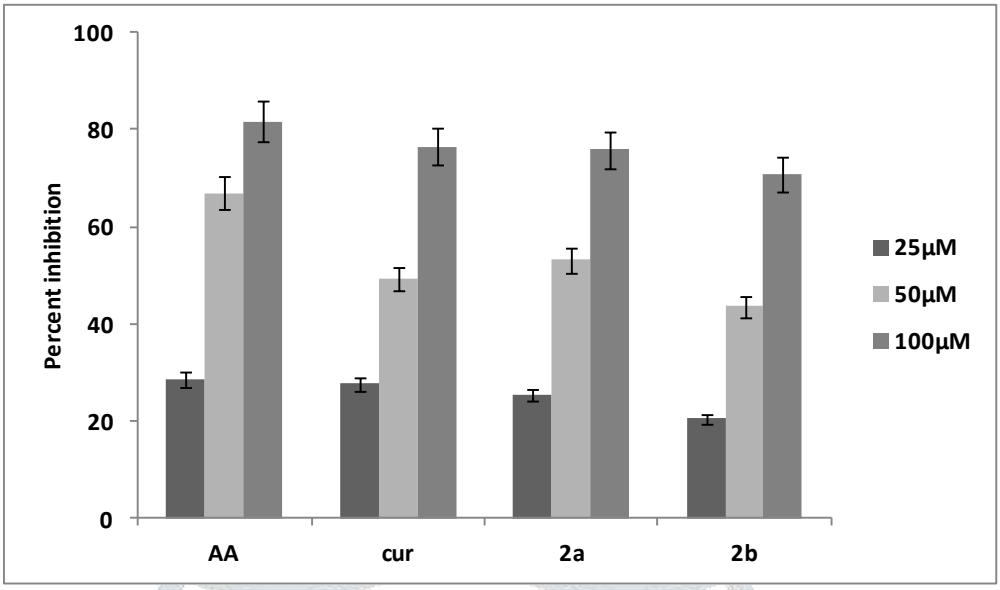


Fig 4. Superoxide anion scavenging activity of curcumin and its derivatives 2a and 2b. (AA: Ascorbic acid)
Values are mean ± SEM (n=3) analyzed by one-way ANOVA followed by Dunnet's test. Data are pooled from three independent experiments. ($p \leq 0.05$ is considered as statistically significant).

3.2 ANTICANCER ACTIVITY

All compounds were evaluated for *in-vitro* cytotoxicity against three different cancer cell lines, MCF-7, HeLa and K562 cells by MTT assay and trypan blue dye exclusion assay. Paclitaxel, avastin and tamoxifen drugs were used as positive control and curcumin was taken as a standard reference. The results of cytotoxicity studies of curcumin and its analogues (2a & 2b) were recorded at different concentrations (10, 20 and 40 µM respectively) and at different time intervals (48 & 72 h respectively).

3.2.1 MTT ASSAY

The data obtained by MTT assay showed that curcumin, compound 2a and 2b have considerable inhibitory effects on the growth of cancer cell lines after 48 and 72 h of treatment (Fig 5, 6 & 7). Compound 2a was found to be more effective and showed significant growth inhibitory effects against the cancer cell lines. Compound 2a was potent as curcumin and showed only 47.9% of viable cells at 40 µM concentrations after 72 h of treatment against HeLa cells, where as curcumin showed 47.7% of viable cells for the same and the compound 2b showed 51.36% viable cells at the same conditions. K-562 cells were more sensitive among the cell lines tested for the treatment and compound 2a, 2b and curcumin treated cells showed 22.85, 39.42 and 30.75% viable cells respectively at 40 µM concentration after 72 h of treatment. Curcumin was found to be quite better than compound 2a against MCF-7 cells and showed 37.49% of cell survival at 40 µM concentration after 72 h of treatment where as compound 2a showed 43.18% of viable cells. Compound 2b showed 57% of cell survival at 40 µM concentration after 72 h of treatment. Compound 2a was found to be more efficient in inhibiting cell growth in HeLa and K-562 cell lines than 2b and curcumin.

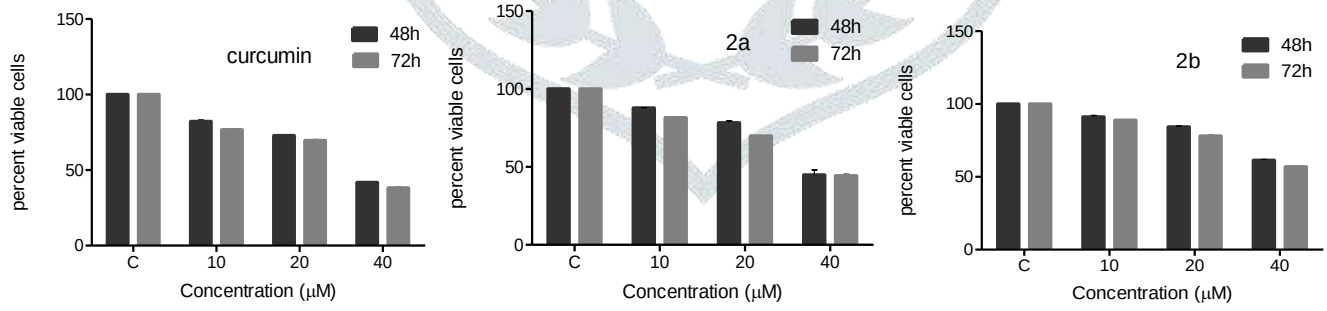


Fig 5.

Evaluation of cytotoxic effects of curcumin and its derivatives (2a & 2b) on MCF-7 cells by MTT assay.

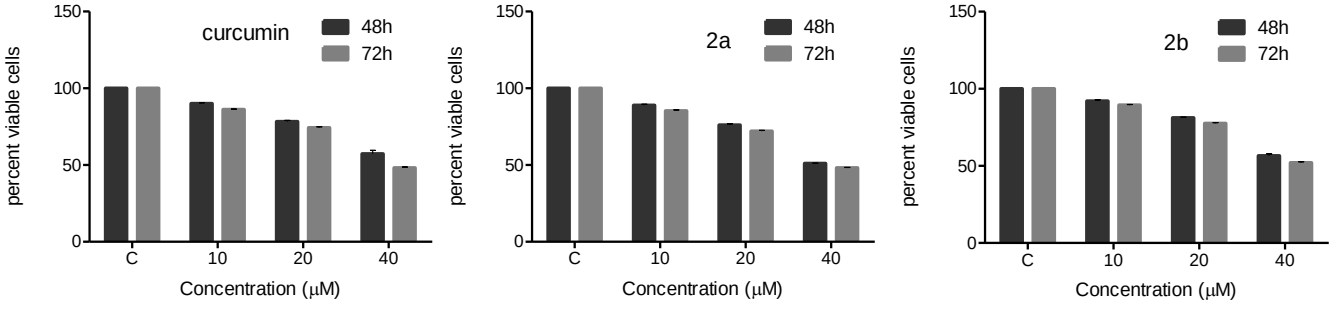


Fig 6.

Evaluation of cytotoxic effects of curcumin and its derivatives (2a & 2b) on HeLa cells by MTT assay.

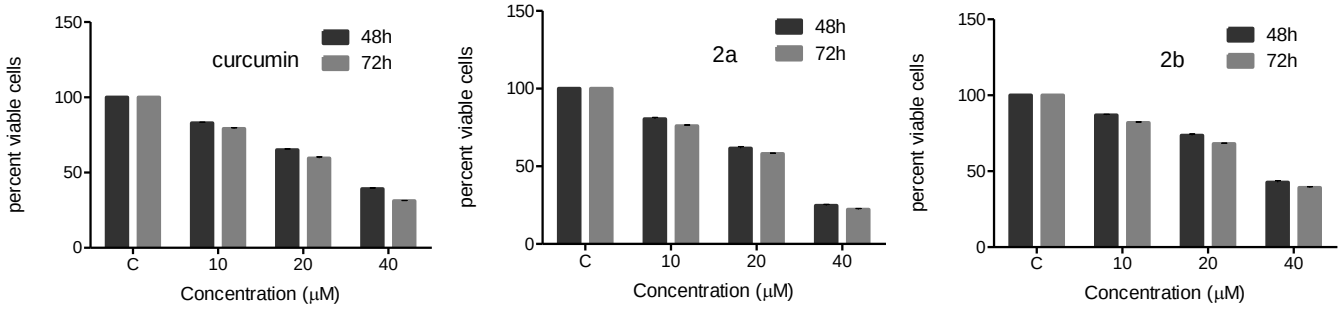


Fig 7.

Evaluation of cytotoxic effects of curcumin and its derivatives (2a & 2b) on K562 cells by MTT assay.
Values are mean ± SEM (n=3) analyzed by one-way ANOVA followed by Dunnet's test. Data are pooled from three independent experiments. ($p \leq 0.05$ is considered as statistically significant).

3.2.2 TRYPAN BLUE DYE EXCLUSION ASSAY

In trypan blue assay, tamoxifen, avastin and paclitaxel were used as reference anticancer drugs for MCF-7, HeLa and K-562 cells respectively and they exhibited 89.06, 78.86 & 92.75% inhibition of proliferation at 10 µM concentration respectively after 48 h. Further, in MCF-7 cells after 48 h exposure at 40 µM concentration (Fig 8), compound 2a showed highest inhibition of proliferation (69.27%) followed by curcumin and compound 2b (68.7 & 65.8% respectively). In HeLa cells, the growth suppressive effect was found to be 53.16, 48.58 and 46.89% respectively for compound 2a, curcumin and compound 2b after 48 h exposure at 40 µM concentrations (Fig 9). Cell proliferation in K-562 cells was inhibited prominently by compound 2a after 48 h exposure at 40 µM concentration and the inhibition was found to be 81.54%. Compound 2b and curcumin, it was found to be 71.72 and 77.93% respectively (Fig 10).

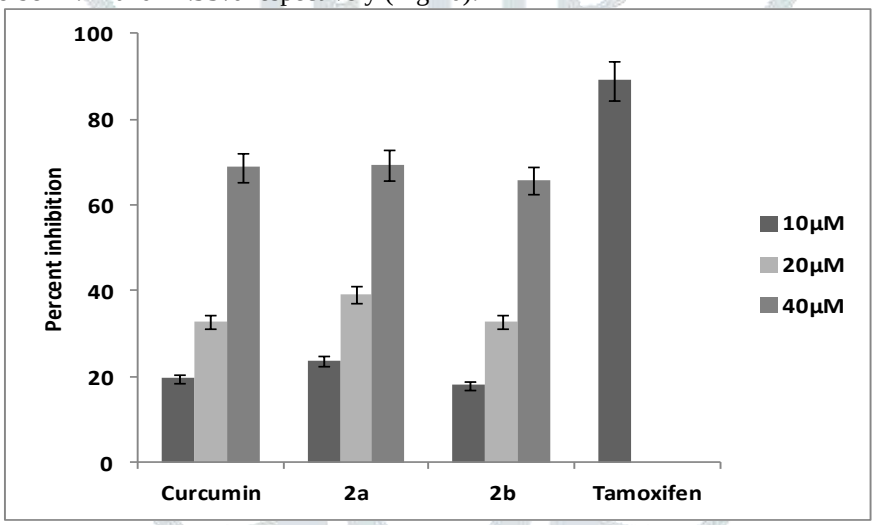


Fig 8. Evaluation of cytotoxic effects of curcumin and its derivatives (2a & 2b) on MCF-7 cells by trypan blue dye exclusion assay (48 h exposure).

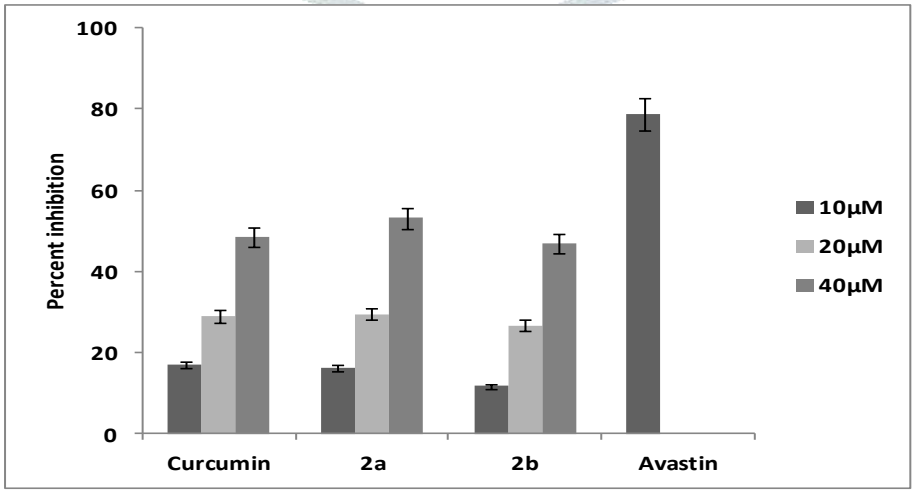


Fig 9. Evaluation of cytotoxic effects of curcumin and its derivatives (2a & 2b) on HeLa cells by trypan blue dye exclusion assay (48 h exposure).

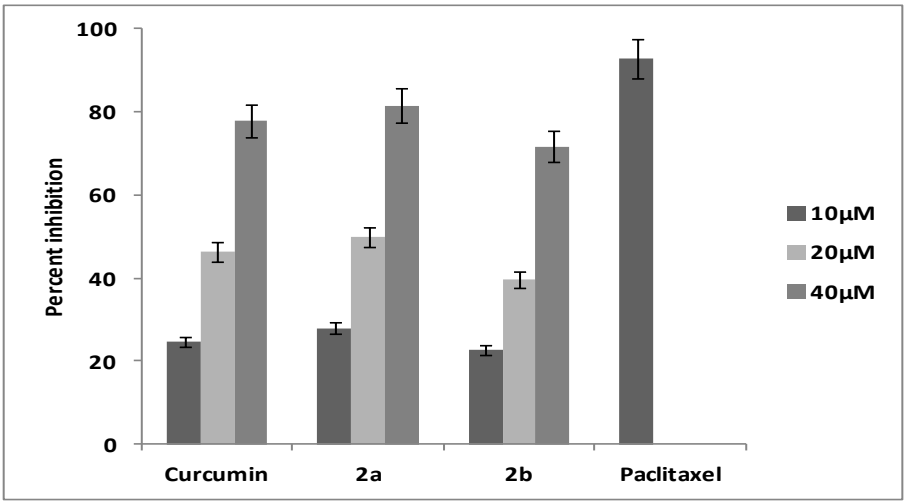


Fig 10. Evaluation of cytotoxic effects of curcumin and its derivatives (2a & 2b) on K562 cells by trypan blue dye exclusion assay (48 h exposure).

Values are mean ± SEM (n=3) analyzed by one-way ANOVA followed by Dunnet's test. Data are pooled from three independent experiments. ($p \leq 0.05$ is considered as statistically significant).

IV CONCLUSION:

Curcumin pyrazole derivatives (2a & 2b) were synthesized and are evaluated for their antioxidant and anticancer potential. They showed considerable antioxidant properties in-vitro and growth inhibitory activities on all the three different cancer cell lines (HeLa, MCF-7 and K-562), however compound 2a exhibited significant activity than that of the curcumin. Thus from the present findings we can conclude that, the synthetic analogue of curcumin, compound *4,4'-(1E,1'E)-2,2'-(1-(4-chlorophenyl)-1H-pyrazole-3,5-diyl)bis(ethene-2,1-diyl)bis(2-methoxyphenol)* (2a) could be a potent molecule and demand further assessment to study the *in vivo* molecular mechanism of the drug.

V ACKNOWLEDGEMENT

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