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# NANO-CATALYZED GREEN SYNTHESIS OF PYRAZOLE DERIVATIVES & ITS BIOLOGICAL ACTIVITY AS EAC RECEPTOR ANTAGONISTS

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**ABSTRACT**

Different derivatives of Cinnamaldehydes were prepared by Claisen-Schmidt condensation (by using Strong basic reagent). The prepared Cinnamaldehydes were treated with Hydrazine hydrate in presence of ZnO Nano-catalyst under microwave assisted solvent-free conditions to afford different substituted Pyrazoles. Green chemistry was employed. Comparisons of both microwave & conventional methods were studied & found that the first was more potent than the later. The synthesized compounds were characterized by FT-IR, $^1$HNMR & elemental analysis. All synthesized compounds were screened for in vitro test against EAC cell lines. Most compounds exhibited good inhibitor potency with $IC_{50}$ values.



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## Introduction

Different substituted Pyrazoles were prepared by microwave method via Cinnamaldehydes with Hydrazine hydrate. [1-4] ZnO catalyst with solvent free conditions was used for both microwave & conventional methods. [5, 6] Comparison studies were done & found that microwave method was good in yield of compounds as well as in time completion of reactions than conventional method. Green synthesis was employed by using nano-catalyst under solvent free conditions**.** Synthesized compounds were characterized by FT-IR, $^1$HNMR, Elemental analysis and confirmed. As of our literature study, [7, 8] Pyrazoles possess anticancer activity by their function as antagonists for various cancer cell lines. [9, 10] We planned to screen these molecules on abnormal EAC cells. Ehrlich Ascites carcinoma enzymes are the substances which can regulate the cell cycle of the human body. Unbalancing secretion of EAC lead to cause cancer cells due to abnormality cell cycle. Herein we reported that synthesized pyrazoles can inhibit EAC cell lines. 3c, 3d and 3e compounds exhibited excellent inhibition against EAC cell lines by calculating $IC_{50}$ values.

## Instruments Used:

Required chemicals were purchased by SDFCL Company & nano-ZnO was facilitated by Dept. Of Physics, Govt. Science College, Chitradurga. Melting point was determined in open capillary tubes in Buchi B-540 melting point apparatus. The reaction was monitored by thin layer chromatography using silica gel glass plates. The reaction was visualized by short Ultraviolet lamp & isolated in iodine chamber. FT-IR spectrometer (Vertex series from Bruker), $^1$HNMR (400MHz) & Thermo Fischer elemental analyzer (BR422710716) were used. Biological activity (EAC cell lines) has been screened by **Cytxon Biosolutions Pvt. Ltd.**, Hubballi – 580031, Karnataka, India.

**Materials Used and Experimental procedure for the biological study:**

- **MTT assay Protocol**

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**Principle:**

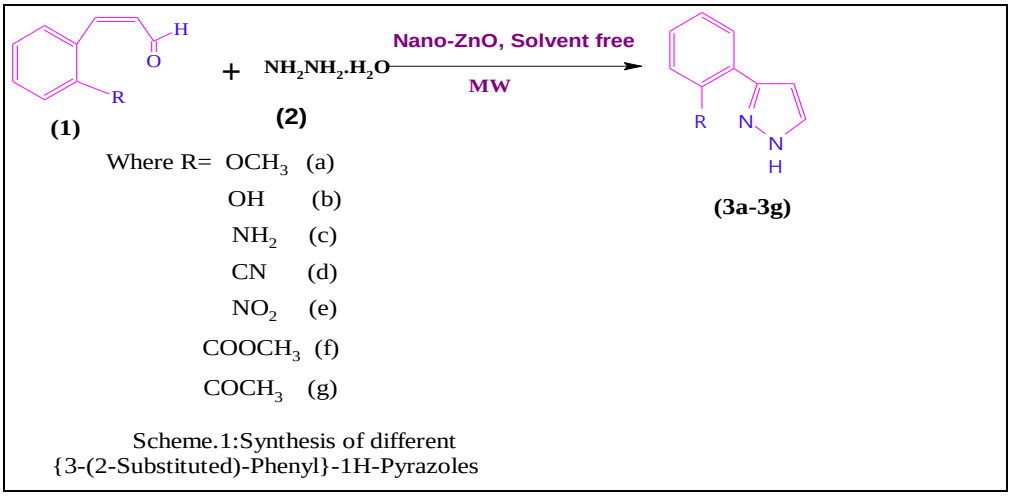
The reduction of tetrazolium salts is now widely accepted as a reliable way to examine cell proliferation. The yellow tetrazolium MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) is reduced by metabolically active cells, in part by the action of dehydrogenase enzymes, to generate reducing equivalents such as NADH and NADPH. The resulting intracellular purple formazan can be solubilized and quantified by spectrophotometric means. The assay measures the cell proliferation rate and conversely, when metabolic events lead to apoptosis or necrosis, and the reduction in cell viability.

**Experimental section:**

**General Procedure for the Synthesis of 2-Substituted-3-Phenyl-1H-Pyrazoles:**

To a solution of Cinnamaldehydes (0.01M), 0.5 g of Hydrazine hydrate (0.01M) was added & 2 equiv. of Nano-ZnO was mixed to the solution. The reaction mixture was kept under microwave oven without solvents used **(Scheme 1).** The reaction was monitored by TLC & iodine chamber. The crude compound along with Nano-ZnO powder was washed with very hot ethanol/water. The compound obtained was filtered, recrystallized by ethanol, and finally dried.

**Reaction Scheme-1:**




Scheme.1:Synthesis of different {3-(2-Substituted)-Phenyl}-1H-Pyrazoles

**3-(2-methoxyphenyl)-1*H*-pyrazole(3a)**: Orange solid, %yield= 88.00, m p: 146$^0$c, IR (KBr): 3412 cm$^{-1}$ (N-H stretch), 3060 cm$^{-1}$(=C-H, stretch), 2867 cm$^{-1}$ (-CH, stretch), 1175 cm$^{-1}$(C-O-C), 1212cm$^{-1}$ (C=N, stretch); 2.509- 2.527 (CH=N, α to nitrogen), 3.333 ($OCH_3$), 7.486 -7.644 (Ar-H),.$^1$H NMR (400MHz, DMSO-$d_6$): δ 2.509- 2.527 (1H, d, CH=N, α to nitrogen), 3.33 (3H, s), 7.45 (1H, d, *J* = 2.5 Hz), 7.57 (1H, ddd, *J* = 8.2, 1.2, 0.5 Hz), 7.58-7.59 (2H, 7.14 (td, *J* = 8.2, 1.5 Hz), 7.6(ddd, *J* = 8.2, 8.0, 1.2 Hz)), 7.61-7.62 (2H, 7.52 (d, *J* = 2.5 Hz), 7.64 (ddd, *J* = 8.0, 1.5, 0.5 Hz), 8.169 - 8.19 ( 1H, s, N-NH-CH).

Anal. Calcd. For **$C_{10}H_{10}N_2O$** (174.00%): C, 68.97; N, 16.1; O, 9.19; H, 5.74; Found: C, 69.14; N, 15.89; O, 9.14; H, 5.83.

**2-(1*H*-pyrazol-3-yl)phenol(3b)**: Brown solid, %yield= 94.00, m p: 171$^0$c IR (KBr): 3432 cm$^{-1}$ (N-H, stretch), 2919 cm$^{-1}$ (-C-H, stretch), 1601 cm$^{-1}$ (C=C, aromatic), 1180 cm$^{-1}$ (C-OH, stretch); HNMR (σ ppm): 1.926-3.335 (-CH=N, α to nitrogen), 4.717 (Ar-OH),$^1$H NMR (400MHz, DMSO-$d_6$): δ 6.77 (1H, d, *J* = 2.5 Hz), 6.96-6.94 (1H, ddd, *J* = 8.2, 1.5, 1.4 Hz), 7.28 (1H, ddd, *J* = 7.6, 1.5, 1.5 Hz), 7.4 (1H, td, *J* = 1.4, 0.5 Hz), 7.77 (1H, ddd, *J* = 8.2, 7.6, 0.5 Hz), 8.1 (1H, d, *J* = 2.5 Hz)., 9.687(=N-NH).

Anal. Calcd. For **$C_9H_8N_2O$** (160.00%): C, 67.50; N, 17.5; O, 10.00; H, 5.00; Found: C, 68.14; N, 17.59; O, 9.44; H, 4.83.

**2-(1*H*-pyrazol-3-yl)-aniline (3c)**: Yellow solid, % yield= 92.00, m p: 149$^0$c, IR (KBr): 3432 cm$^{-1}$ (N-H, stretch), 3251 cm$^{-1}$ ($NH_2$,stretch), 3098 cm$^{-1}$ (=C-H, stretch), 1422 cm$^{-1}$ (C=C, aromatic); $^1$H NMR (400MHz, DMSO-$d_6$): δ 3.715-3.883 (2H, $NH_2$), 2.009-2.517 (-CH=N, stretch), 6.82 (1H, d, *J* = 2.5 Hz), 7.04 (1H, td, *J* = 8.1, 1.1 Hz), 7.11 (1H, ddd, *J* = 8.3, 8.1, 1.4 Hz), 7.48(1H, ddd, *J* = 8.3, 1.1, 0.5 Hz), 7.50-7.81 (2H, 7.48 (d, *J* = 2.5 Hz), 7.84 (ddd, *J* = 8.1, 1.4, 0.5 Hz), 8.64 (=CH-NH).

Anal. Calcd. For **$C_9H_9N_3$**(159.00%): C, 67.92; N, 26.42; H, 5.66; Found: C, 68.12; N, 26.79; H, 5.09.

**2-(1*H*-pyrazol-3-yl)-Benzonitrile (3d):** Yellow solid, %yield= 82.00, m p: 168$^0$c, IR (KBr): 3390 cm$^{-1}$(N-H, stretch), 2191 cm$^{-1}$ (CN, stretch), 1597 cm$^{-1}$ (C=C, aromatic).$^1$H NMR (400MHz, DMSO-$d_6$): δ 2.506 (-CH=N), 7.50 (1H, d, *J* = 2.4 Hz), 7.51-7.54 (2H, 7.58 (ddd, *J* = 8.4, 7.6, 1.6 Hz), 7.8 (d, *J* = 2.4 Hz)), 7.89 (1H, ddd, *J* = 7.6, 1.6, 0.5 Hz), 7.9-7.91 (2H, 7.76 (ddd, *J* = 7.6, 7.6, 1.2 Hz), 7.78 (ddd, *J* = 8.4, 1.2, 0.5 Hz), 8.72 (s, =CH-NH).

Anal. Calcd. For **$C_{10}H_9N_3$** (171.00%): C, 70.17; N, 24.56; H, 5.26; Found: C, 71.12; N, 24.69; H, 4.19.

**3-(2-nitrophenyl)-1*H*-pyrazole (3e):** Brown solid, %yield= 89.00, m p: 164$^0$c, IR (KBr): 3439 cm$^{-1}$ (N-H, stretch), 3081 cm-1 (=C-H), 1630 cm$^{-1}$ (), 1596 cm$^{-1}$ ($NO_2$).$^1$H NMR (400MHz, DMSO-$d_6$): δ. 2.503-3.317 (=CH-N),7.18 (1H, d, *J* = 2.1 Hz), 7.33 (1H, ddd, *J* = 8.5, 8.1, 1.6 Hz), 7.49 (1H, d, *J* = 2.1 Hz), 7.62-7.75 (2H, 7.77 (ddd, *J* = 8.1, 7.5, 1.6 Hz), 7.8 (ddd, *J* = 7.5, 1.6, 0.5 Hz)), 8.4 (1H, ddd, *J* = 8.5, 1.6, 0.5 Hz), 9.7 (1H, =CH-NH).

Anal. Calcd. For **$C_9H_7N_3O_2$**(189.00%): C,57.14; N, 22.23; O, 16.93, H, 3.70; Found: C, 58.02; N, 21.89;O, 16.97 H, 3.2.

**Methyl 2-(1*H*-pyrazol-3-yl)-Benzoate (3f):** Yellow solid, %yield= 79.00. m p: 192$^0$c, IR (KBr): 3333 cm$^{-1}$ (N-H, stretch), 2921 cm$^{-1}$ (-C-H, stretch), 1732 cm$^{-1}$(C=O, ester).$^1$H NMR (400MHz, DMSO-$d_6$): δ 3.88 (3H, $CH_3$), 6.82 (1H, d, *J* = 2.4 Hz), 7.04 (1H, ddd, *J* = 8.2, 7.7, 1.4 Hz), 7.16 (1H, d, *J* = 2.4 Hz), 7.48-7.50 (2H, 7.77 (ddd, *J* = 7.7, 7.6, 1.3 Hz), 7.81 (ddd, *J* = 7.6, 1.4, 0.4 Hz)), 7.86 (1H, ddd, *J* = 8.2, 1.3, 0.4 Hz), 8.64 (s, =CH-NH).

Anal. Calcd. For **$C_{11}H_{10}N_2O_2$** (202.00%): C, 65.34; N, 13.86; O, 15.84, H, 4.96; Found: C, 66.02; N, 14.29; O, 15.27 H, 4.33.

**1-[2-(1*H*-pyrazol-3-yl) phenyl]-Ethanone (3g):** Yellow solid, %yield= 81.00, m p:138$^0$c**,** IR (KBr): 3372 cm$^{-1}$ (-NH, stretch), 3147 cm$^{-1}$ (=CH, stretch),2942 cm-1(-CH, stretch), 1718 cm$^{-1}$ (C=O, stretch), 1605 cm$^{-1}$ =C, r, weak), 1438 cm$^{-1}$ ($CH_3$, bend); $^1$H NMR (400MHz, DMSO-$d_6$): δ 3.71 (3H, s), 2.315 (=CH-N), 6.82 (1H, d, *J* = 2.4Hz), 7.04 (1H, ddd, *J* = 7.9, 7.7, 1.5 Hz), 7.59-7.65 (2H, 7.64 (d, *J* = 2.4 Hz), 7.62 (ddd, *J* = 7.9, 1.3, 0.4), 7.48-7.51 (2H, 7.76 (td, *J* = 7.7, 1.3 Hz), 7.84 (ddd, *J* = 7.7, 1.5, 0.4 Hz, 8.64 (=CH-NH).

Anal. Calcd. For **$C_{11}H_{10}N_2O$** (186.00%): C, 70.97; N, 15.05; O, 8.60, H, 5.37; Found: C, 71.46; N, 15.55; O, 7.78 H, 5.21.

## Results and Discussion

### Cytotoxic studies

The cells were trypsinized and aspirated into a 5ml centrifuge tube. Cell pellet was obtained by centrifugation at 300 x g. The cell count was adjusted, using DMEM HG medium, such that 200µl of suspension contained approximately 10,000 cells. To each well of the 96 well micro-titre plate, 200µl of the cell suspension was added and the plate was incubated at 37$^o$C and 5% $CO_2$ atmosphere for 24 h. After 24 h, the spent medium was aspirated. 200µl of different test concentrations **(100, 200 and 300µg/ml from stock)** of test drugs were added to the respective wells. The plate was then incubated at 37$^o$C and 5% $CO_2$ atmosphere for **24 h**. The plate was removed from the incubator and the drug containing media was aspirated. 200µl of medium containing 10% MTT reagent was then added to each well to get a final concentration of 0.5mg/ml and the plate was incubated at 37$^o$C and 5% $CO_2$ atmosphere for 3 h.

The culture medium was removed completely without disturbing the crystals formed. Then 100µl of solubilization solution (DMSO) was added and the plate was gently shaken in a gyratory shaker to solubilize the formed formazan. The absorbance was measured using a micro plate reader at a wavelength of 570 nm and also at 630 nm. [11, 12] The percentage growth inhibition was calculated, after subtracting the background and the blank, and concentration of test drug needed to inhibit cell growth by 50% ($IC_{50}$) was generated from the dose-response curve for the cell line.

Compounds were confirmed by FT-IR, 3043 cm$^{-1}$ for N-H stretch, 1284, 1344, 1384 & 1441 cm$^{-1}$ (C-N stretch), 1583 cm$^{-1}$ (aromatic C=C) & $^1$HNMR confirmed by 8.0-11.5 for C-N=NH, 6.3-7.8 (Ar-H) (see data as separate file). Comparisons of microwave method and conventional method were made, and found that first one was more efficient in quick reactions and afforded more yield than the later one (See table 1). [13, 14] Compounds 3c, 3d and 3e revealed excellent inhibitory potency against EAC cell lines. [15] In the present investigation, *in vitro* screening of the compound against EAC cancer cell line by MTT assay based cytotoxicity study revealed that all the compounds among the test compound series **3a to 3g**, compound 3e showed significant cytotoxic property with $IC_{50}$ less than 60 µg/ml. Compounds **3c** and **3d** were also proved to be potential among the tested series with $IC_{50}$ less than 100 µg/ml. All compounds in the series expect 3f and 3h showed potential cytotoxic effect with $IC_{50}$ less than 100 µg/ml. Totally, compound **3e** exhibited the best potency among all compounds and Doxorubicin. The $IC_{50}$ value (Inhibition Concentration 50) corresponds to the concentration of the compound causing death in 50 % of cell population at the end of the incubation period of 24 h (see table 2).

**Table 1:** Details of Comparison for MW & a Convention method

| Compounds | Microwave method | Yield (%) | Convention method | Yield (%) |
|---|---|---|---|---|
| 3a | 30 sec | 88.00 | 4 min | 74.00 |
| 3b | 60 sec | 94.00 | 6 min | 78.00 |
| 3c | 45 sec | 92.00 | 7 min | 79.00 |
| 3d | 60 sec | 85.00 | 5 min | 82.00 |
| 3e | 75 sec | 89.00 | 8 min | 68.00 |
| 3f | 40 sec | 79.00 | 6 min | 68.00 |
| 3g | 60 sec | 81.00 | 7 min | 72.00 |

**Table 2:** $IC_{50}$ values of 3a-3g compounds at 100, 200 and 300µg/ml concentrations

| Tested compounds | $IC_{50}$ (EAC) '100' µg/ml concentration | $IC_{50}$ (EAC) '200' µg/ml concentration | $IC_{50}$ (EAC) '300' µg/ml concentration |
|---|---|---|---|
| 3a | 98 | 89 | 75 |
| 3b | 102 | 97 | 79 |
| 3c | 79 | 76 | 65 |
| 3d | 86 | 62 | 53 |

| | | | |
|---|---|---|---|
| 3e | 57 | 38 | 32 |
| 3f | 143 | 148 | 134 |
| 3g | 99 | 94 | 93 |
| Doxorubicin | 40 | 40 | 40 |

**Results and Interpretation:**

The $IC_{50}$ values of the test compounds for EAC cell-line for 24-hour treatment were found: [16, 17]

**Table 3:** Expected $IC_{50}$ (µg/ml) values of compounds 3a-3g with reference drug Doxorubicin

| **Sample name** | Ehrlich Ascites Carcinoma **cell line** **$IC_{50}$ (in µg/ml) 24hr** |
|---|---|
| **Doxorubicin** | 40 µg/ml |
| ****Sample 3a** | <100µg/ml |
| **Sample 3b** | >100µg/ml |
| ****Sample 3c** | <100.00µg/ml<br>Calculated Value: 88.5µg/ml |
| ****Sample 3d** | <100.00µg/ml<br>Calculated Value: 69.0µg/ml |
| **Sample 3e** | <100.00 µg/ml |
| **Sample 3f** | >100.00 µg/ml |
| **Sample 3g** | >100.00 µg/ml |

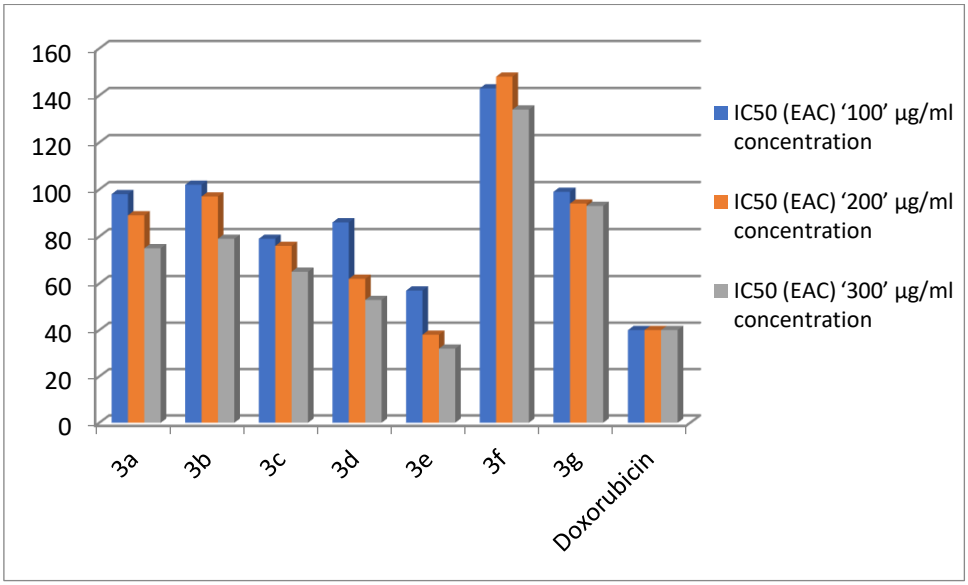



**Fig. 1:** Graphical representation for IC50 values at 100, 200 and 300µg/ml with Doxorubicin

**Conclusions**

Since Pyrazole derivatives possess various pharmacological efficacies, they were tested for anticancer activity. They showed excellent inhibition against the EAC cancer cell lines. Compound **3e** resulted as the best antagonist than Doxorubicin and among all other synthesized compounds. Nitro substituent of Pyrazole was found to be an effective molecule for excellent inhibition of EAC cancer cells. Microwave method without solvent was a simple reaction scheme and 2-MeIm-SCN catalyst was proved as an environmental benign catalyst.

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