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Biological Activities of 4-(Substitued phenyl)-5-(2,4-dinitrophenyl)-3-methyl-1,3a,4,5-tetrahydropyrazolo-[3,4-c]pyrazole

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Ethyl acetoacetate (**I**) was stirred with hydrazine hydrate in ethanol to get 5-methyl-2,4-dihydro-3*H*-pyrazol-3-one (**II**). This was refluxed with different aryl aldehydes in glacial acetic acid in presence of fused sodium acetate to get corresponding (4E)-4-(substituted benzylidene)-5-methyl-2,4-dihydro-3*H*-pyrazol-3-one (**IIIa-f**). These were treated with 2,4-dinitro phenyl hydrazine to get a series of 4-(substituted phenyl)-5-(2,4-dinitrophenyl)-3-methyl-1,3a,4,5-tetrahydro-pyrazolo-[3,4-c]pyrazole (**IVa-f**). The structures of the compounds have been established based on their analytical and spectral data. All the compounds have been screened for their analgesic, antibacterial and antifungal activity. Compound **IVc**, **IVe** and **IVf** exhibited significant analgesic activity. Compound **IVa** was found to be more active against *Streptococcus mutans*. Compound **IVf** was found to be more active against both fungal strains.

Key Words: Analgesic, Antibacterial, Antifungal activity, Pyrazole.

INTRODUCTION

Pyrazolines, as a class of heterocyclic compounds, have been studied extensively for the past several years because of their broad spectrum biological activities. The pharmaceutical importance of pyrazoline compounds lies in the fact that they can be effectively utilized as antibacterial[1-5], antifungal[1,3-5], antiinflammatory[6], antitubercular[7], analgesic[8], insecticidal[9], antiparasitic[10] and antiviral[11] agents. Some of these compounds have also anticonvulsant[12], cardiovascular[13] and anticancer[14] properties. In addition, pyrazolines have played a crucial part in the development of theory in heterocyclic chemistry and also used extensively in organic synthesis. Recent literature[4] describes the investigations of a few derivatives of pyrazoles as

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possible antibacterial agents. These literature reports prompt us to synthesize 4-(substituted phenyl)-5-(2,4-dinitrophenyl)-3-methyl-1,3a,4,5-tetrahydro-pyrazolo-[3,4-c]pyrazole (**IVa-f**) and to evaluate their analgesic, antibacterial and antifungal activities.

EXPERIMENTAL

The melting points of newly synthesized compounds were determined by open capillary method and were uncorrected. Purity of the compounds was checked by micro TLC using silica gel G-coated glass plates using benzene and acetone (1:1; v/v) as irrigant and iodine vapour as detecting agent. The IR spectra of the compounds were recorded on Jasco FT/IR-5300 spectrophotometer using KBr pellet. ^{1}H NMR spectra were recorded in a Bruker DPX-400 MHz spectrometer; chemical shifts (δ) were reported in ppm, using TMS as internal standard. GCM spectra were recorded in Shimadzu QP 50000. Elemental analysis for C, H and N were performed on a Perkin-Elmer 240 C elemental analyzer and were found to be with in ± 0.4 % of the theoretical values.

Synthesis of (4E)-4-(substituted benzylidene)-5-methyl-2,4-dihydro-3*H*-pyrazol-3-one (IIIa-f): 5-Methyl-2,4-dihydro-3*H*-pyrazol-3-one (**II**) (0.98 g, 0.01 mol) and aryl aldehyde (0.01 mol) were refluxed with 40 mL glacial acetic acid in presence of anhydrous sodium acetate (0.82 g, 0.01 mol) for 4 h, cooled to room temperature and poured in an ice cold water. The solid separated was filtered, washed with water and recrystallized from appropriate solvent system.

(4E)-4-Benzylidene-5-methyl-2,4-dihydro-3*H*-pyrazol-3-one (IIIa): IR (KBr, ν_{max}, cm^{-1}): 3416 (N-H), 1680 (C=O), 1585 (C=N). ^{1}H NMR ($CDCl_3$) δ: 7.5-7.8 (m, 5H, Ar-H), 6.63 (s, 1H, N-H), 2.4 (s, 3H, -CH_3), 5.2 (s, 1H, Ar-CH=C); M/e: 186; Anal. ($C_{11}H_{10}N_2O$) Found %: C, 70.71; H, 5.2; N, 5.01; Calculated %: C, 70.95; H, 5.41; N, 15.04.

(4E)-4-(2-Chlorobenzylidene)-5-methyl-2,4-dihydro-3*H*-pyrazol-3-one (IIIb): IR (KBr, ν_{max}, cm^{-1}): 3456 (N-H), 1687 (C=O), 1556 (C=N). ^{1}H NMR ($CDCl_3$) δ: 7.2-7.5 (m, 4H, Ar-H), 6.3 (s, 1H, N-H), 2.3 (s, 3H, -CH_3), 5.1 (s, 1H, Ar-CH=C); M/e: 220; Anal. ($C_{11}H_9ClN_2O$) Found %: C, 59.7; H, 4.1; N, 12.5; Calculated %: C, 59.87; H, 4.11; N, 12.70.

(4E)-4-(2-Hydroxybenzylidene)-5-methyl-2,4-dihydro-3*H*-pyrazol-3-one (IIIc): IR (KBr, ν_{max}, cm^{-1}): 3412 (OH and N-H), 1680 (C=O), 1530 (C=N). ^{1}H NMR ($CDCl_3$) δ: 7.3-7.5 (m, 4H, Ar-H), 6.2 (s, 1H, N-H), 2.2 (s, 3H, -CH_3), 5.3 (s, 1H, Ar-CH=C); M/e: 202; Anal. ($C_{11}H_{10}N_2O_2$) Found %: C, 65.2; H, 4.8; N, 13.80; Calculated %: C, 65.33; H, 4.98; N, 13.85.

(4E)-4-(2,4-Dichlorobenzylidene)-5-methyl-2,4-dihydro-3*H*-pyrazol-3-one (IIId): IR (KBr, ν_{max}, cm^{-1}): 3280 (N-H), 1700 (C=O), 1585 (C=N). ^{1}H NMR ($CDCl_3$) δ: 7.2-7.4 (m, 4H, Ar-H), 6.4 (s, 1H, N-H), 2.2 (s, 3H,

-CH_3), 5.3 (s, 1H, Ar-CH=C); M/e: 255; Anal. ($C_{11}H_8Cl_2N_2O$) Found %: C, 51.72; H, 3.09; N, 10.97; Calculated %: C, 51.79; H, 3.16; N, 10.98.

(4E)-4-(4-Dimethylaminobenzylidene)-5-methyl-2,4-dihydro-3*H*-pyrazol-3-one (IIIe): IR (KBr, ν_{max}, cm^{-1}): 3285 (N-H), 1715 (C=O), 1560 (C=N). ^1H NMR ($CDCl_3$) δ: 7.3-7.5 (m, 4H, Ar-H), 6.3 (s, 1H, N-H) 3.1 (s, 6H, -$N(CH_3)_2$), 2.3 (s, 3H, -CH_3), 5.3 (s, 1H, Ar-CH=C); M/e: 229; Anal. ($C_{13}H_{15}N_3O$) Found %: C, 68.09; H, 6.57; N, 18.31; Calculated %: C, 68.10; H, 6.59; N, 18.32.

(4E)-4-(3,4,5-Trimethoxybenzylidene)-5-methyl-2,4-dihydro-3*H*-pyrazol-3-one (IIIf): IR (KBr, ν_{max}, cm^{-1}): 3262 (N-H), 1712 (C=O), 1582 (C=N). ^1H NMR ($CDCl_3$) δ: 7.3-7.4 (m, 2H, Ar-H), 6.5 (s, 1H, N-H) 3.2 (s, 9H, -$(OCH_3)_3$), 2.2 (s, 3H, -CH_3), 5.2 (s, 1H, Ar-CH=C); M/e: 276; Anal. ($C_{14}H_{16}N_2O_4$) Found %: C, 60.85; H, 5.82; N, 10.11; Calculated %: C, 60.86; H, 5.84; N, 10.14.

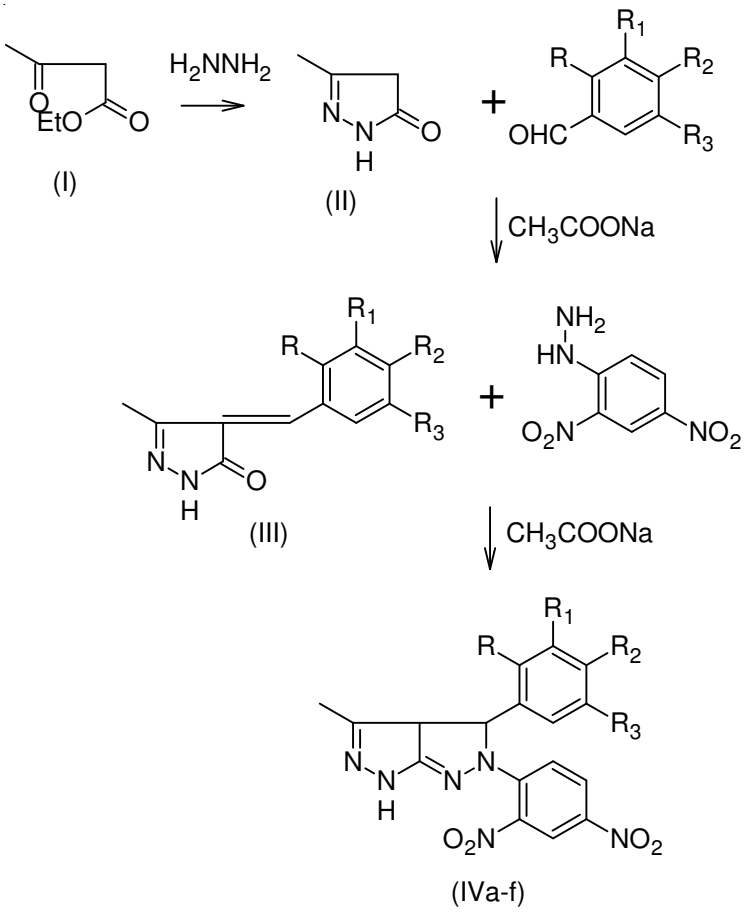
Synthesis of 4-(substituted phenyl)-5-(2,4-dinitrophenyl)-3-methyl-1,3a,4,5-tetrahydropyrazolo-[3,4-c]pyrazole (IVa-f): A mixture of (4E)-4-arylidene-5-methyl-2,4-dihydro-3*H*-pyrazol-3-one (**IIIa-f**) (0.01 mol), 2,4-dinitrophenylhydrazine (1.98 g, 0.01 mol) and anhydrous sodium acetate (0.82 g, 0.01 mol), was added to glacial acetic acid (40 mL), refluxed for 4 h, cooled to room temperature and poured into cold water. The dark orange-yellow solid thus separated were filtered, washed with water and crystallized from appropriate recrystallizing solvent as orange yellow crystals (**Scheme-I**). The physical properties are given in Table-1.

TABLE-1
PHYSICAL DATA OF COMPOUNDS (**IVa-f**)

Compd.	R	R_1	R_2	R_3	Yield (%)	m.p. (ºC)	m.f.	m.w.	R_f value*
IVa	H	H	H	H	55	228	$C_{17}H_{14}N_6O_4$	366	0.67
IVb	Cl	H	H	H	59	215	$C_{17}H_{13}ClN_6O_4$	400	0.43
IVc	OH	H	H	H	53	207	$C_{17}H_{14}N_6O_5$	382	0.62
IVd	Cl	H	Cl	H	68	227	$C_{17}H_{12}Cl_2N_6O_4$	435	0.60
IVe	H	H	$N(CH_3)_2$	H	61	245	$C_{19}H_{19}N_7O_4$	409	0.44
IVf	H	OCH_3	OCH_3	OCH_3	67	236	$C_{20}H_{20}N_6O_7$	456	0.53

*R_f value was determined in benzene:acetone (1:1).

4-Phenyl-5-(2,4-dinitrophenyl)-3-methyl-1,3a,4,5-tetrahydropyrazolo-[3,4-c]pyrazole (IVa): IR (KBr, ν_{max}, cm^{-1}): 3287 (N-H), 1585 (C=N). ^1H NMR ($CDCl_3$) δ: 8.5 (d, 1H, C3a -H), 7.8 (d, 1H, C4-H), 7.2-7.5 (m, 8H, Ar-H), 6.6(s, 1H, N-H), 2.4 (s, 3H, -CH_3). M/e: 366; Anal. ($C_{17}H_{14}N_6O_4$) Found %: C, 55.71; H, 3.84; N, 22.93; Calculated %: C, 55.73; H, 3.85; N, 22.94.



Scheme-I

4-(2-Chlorophenyl)-5-(2,4-dinitrophenyl)-3-methyl-1,3a,4,5-tetrahydropyrazolo-[3,4-c]pyrazole (IVb): IR (KBr, ν_{max}, cm^{-1}): 3262 (N-H), 1520 (C=N). ^1H NMR ($CDCl_3$) δ: 8.7 (d, 1H, C3a-H), 6.9 (d, 1H, C4-H), 7.41-7.54 (m, 7H, Ar-H), 6.2 (s, 1H, N-H), 2.43 (s, 3H, CH_3). M/e: 400; Anal. ($C_{17}H_{13}ClN_6O_4$) Found %: C, 50.55; H, 3.12; N, 21.65; Calculated %: C, 50.95; H, 3.27; N, 20.97.

4-(2-Hydroxyphenyl)-5-(2,4-dinitrophenyl)-3-methyl-1,3a,4,5-tetrahydropyrazolo-[3,4-c]pyrazole (IVc): IR (KBr, ν_{max}, cm^{-1}): 3428 (OH and N-H), 1509 (C=N). ^1H NMR ($CDCl_3$) δ: 9.1 (s, 1H, OH), 8.6 (d, 1H, C3a-H), 7.3-7.5 (m, 7H, Ar-H), 7.0 (d, 1H, C4-H), 6.3 (s, 1H, N-H), 2.5 (s, 3H, CH_3). M/e: 382; Anal. ($C_{17}H_{14}N_6O_5$) Found %: C, 49.32; H, 3.68; N, 20.46; Calculated %: C, 49.04; H, 3.87; N, 20.18.

4-(2,4-Dichlorophenyl)-5-(2,4-dinitrophenyl)-3-methyl-1,3a,4,5 tetrahydropyrazolo-[3,4-c]pyrazole (IVd): IR (KBr, ν_{max}, cm^{-1}): 3281 (N-H),

1581 (C=N). ^{1}H NMR ($CDCl_3$) δ: 8.0 (d, 1H, C3a-H), 7.2-7.5 (m, 6H, Ar-H), 6.8 (d, 1H, C4-H), 6.2 (s, 1H, N-H), 2.5 (s, 3H, CH_3). M/e: 435; Anal. ($C_{17}H_{12}Cl_2N_6O_4$) Found %: C, 46.80; H, 2.54; N, 19.10; Calculated %: C, 46.91; H, 2.78; N, 19.31.

4-(4-Diaminomethylphenyl)-5-(2,4-dinitrophenyl)-3-methyl-1,3a, 4,5-tetrahydropyrazolo-[3,4-c]pyrazole (IVe): IR (KBr, ν_{max}, cm^{-1}): 3281 (N-H), 1507 (C=N). ^{1}H NMR ($CDCl_3$) δ: 8.07 (d, 1H, C3a-H), 7.2 (d, 1H, C4-H), 7.37-7.45 (m, 7H, Ar-H), 6.5(s, 1H, N-H), 3.1 (s, 6H,-N $(CH_3)_2$), 2.5 (s, 3H, CH_3). M/e: 409; Anal. ($C_{19}H_{19}N_7O_4$) Found %: C, 55.60; H, 4.32; N, 23.80; Calculated %: C, 55.74; H, 4.68; N, 23.95.

4-(3,4,5-Trimethoxyphenyl)-5-(2,4-dinitrophenyl)-3-methyl-1,3a, 4,5-tetrahydropyrazolo-[3,4-c]pyrazole (IVf): IR (KBr, ν_{max}, cm^{-1}): 3268 (N-H), 1586 (C=N). ^{1}H NMR ($CDCl_3$) δ: 8.07 (d, 1H, C3a-H), 6.9 (d, 1H, C4-H), 7.39-7.56 (m, 5H, Ar-H), 6.2 (s, 1H, N-H), 3.1 (s, 9H, -$(OCH_3)_3$), 2.5 (s, 3H, CH_3). M/e: 456; Anal. ($C_{20}H_{20}N_6O_7$) Found %: C, 52.46; H, 4.10; N, 18.24; Calculated %: C, 52.63; H, 4.41; N, 18.41.

Analgesic activity: The synthesized compounds were assessed for the analgesic activity using Wistar Albino mice of either sex. Analgesic activity was measured by acetic acid induced writhings method[15]. Control group received vehicle (1 mL, 0.25 % CMC solution). Standard drug used was aspirin (100 mg/kg). Six group of animals were pretreated with the synthesized compounds and two groups were pretreated with standard and vehicle, respectively. Under similar conditions, after 0.5 h they were injected with 1 % (v/v) acetic acid (1 mL/100 g body weight, i.p.) and number of abdominal contractions, trunk twist response and extension of hind limbs as well as number of animals showing such response during 5 min were recorded. Mean writhings scores in all groups were calculated.

Antibacterial activity: The newly synthesized compounds were evaluated for the antibacterial activity against bacterial strains by using zone of inhibition method[16]. The test drug solutions were prepared by dissolving the test drugs in DMSO. The solutions of the test drugs were prepared at the concentration of 1000 µg/mL in DMSO. The solutions of standard drugs amoxicillin/clavulanic acid and cefixime were prepared at the concentration of 1000 µg/mL in DMSO. Previously sterilized, liquified Muller Hinton Agar media was inoculated with the requisite quantity of the suspension of the microorganism at a temperature between 40-50 ºC and the inoculated medium was poured immediately aseptically into Petri dish, previously sterilized to occupy a depth of 3-4 mm. The Whatmann filter paper no. 2 was cut down into a small disc (6 mm in diameter) sterilized in the hot air oven and then impregnated with the test solutions and standard solution. The dried discs were placed on the surface of the medium aseptically. All the Petri dishes were left standing for 1 to 4 h at room temperature, as a

period of pre-incubation diffusion to minimize the effects of variation in time between the application of different solutions. All the Petri dishes were incubated for 24 h at 37 ºC. After incubation the diameters of the circular inhibition zones were measured.

Antifungal activity: The newly synthesized compounds were evaluated for the antifungal activity against fungal strains by using zone of inhibition method[16]. The solutions of the test drugs as well as the standard drug, ketoconozole were prepared at the concentration of 1000 µg/mL in DMSO seperately. Previously sterilized, liquefied Muller Hinton Agar media was inoculated aseptically with the requisite quantity of the suspension of the microorganism, at a temperature between 40-50 ºC and the inoculated medium was poured immediately into dry, sterile Petri dishes to occupy a depth of 3-4 mm. The Whatmann filter paper no. 2 was cut down into a small disc (6 mm in diameter) and sterilized in the hot air oven and then impregnated with the test solutions and standard solution. The dried discs were placed aseptically on the surface of the medium. After all the drugs were added, Petri dishes were left standing for 1-4 h at room temperature, as a period of pre-incubation diffusion to minimize the effects of variation in time between the application of different solutions. All the Petri dishes were incubated for 48 h at 25 ºC. After incubation the diameters of the circular inhibition zones were measured.

RESULTS AND DISCUSSION

5-Methyl-2,4-dihydro-3*H*-pyrazol-3-one (**II**) was prepared, by the cyclization of ethyl acetoacetate (**1**) with hydrazine hydrate by strring in ethanol. 5-Methyl-2,4-dihydro-3*H*-pyrazol-3-one (**II**) was refluxed with different aryl aldehydes in glacial acetic acid in presence of fused sodium acetate to get different (4E)-4-(substituted benzylidene)-5-methyl-2,4-dihydro-3*H*-pyrazol-3-one (**IIIa-f**). These were confirmed by ^{1}H NMR, mass and IR spectral studies. (4E)-4-(Substituted benzylidene)-5-methyl-2,4-dihydro-3*H*-pyrazol-3-one (**IIIa-f**) were treated with 2,4-dinitrophenyl-hydrazine to get a series of 4-(substituted phenyl)-5-(2,4-dinitrophenyl)-3-methyl-1,3a,4,5-tetrahydropyrazolo-[3,4-c]pyrazole (**IVa-f**).

Analgesic activity: The newly synthesized compounds were evaluated for their analgesic activity in Wistar Albino mice (weighing 25-30 g) by acetic acid induced writhings method. Asprin was employed as standard drug. The results of the activity are given in Table-2.

Analgesic studies revealed that the compounds **IVc** with 2-hydroxyphenyl, **IVe** with 4-dimethylaminophenyl and **IVf** with 3,4,5-trimethoxyphenyl moieties were found to possess significant analgesic activity when compared with the standard drug.

TABLE-2
ANALGESIC ACTIVITY OF COMPOUNDS (**IVa-f**)

Design of treatment (groups)	Dose (mg/kg, po)	Number of writhings in 5 min
Control	–	180.00 ± 0.607
Aspirin	100	79.43 ± 0.600
IVa	150	41.83 ± 0.477**
IVb	125	65.50 ± 0.763**
IVc	140	76.00 ± 0.577*
IVd	130	58.67 ± 0.881**
IVe	135	69.71 ± 0.666*
IVf	130	71.17 ± 0.600*

Values are expressed as mean ± SEM, n = 6 When compared with control.
*$p < 0.01$, **$p < 0.001$
(One way Anova followed by Dunnett's multiple comparison test).

Antibacterial activity: The newly synthesized compounds were screened for antibacterial activity which was carried out against *Streptococcus mutans* (gram positive), *Staphylococcus aureus* (gram positive), *Shigella dysenteriae* (gram negative) and *Escherichia coli* (gram negative) bacterial strains by determining the zone of inhibition using disc diffusion method. Amoxicilin-clavulanic acid (for gram positive bacteria) and cefixime (for gram negative bacteria) were used as standard drugs. Solvent and growth controls were kept for comparison and the zones of inhibition were noted (Table-3).

TABLE-3
ANTIBACTERIAL ACTIVITY OF COMPOUNDS (**IVa-f**)

Microorganism	% Diameter of zone (mm) (1 mg/mL)						
	IVa	**IVb**	**IVc**	**IVd**	**IVe**	**IVf**	Std.
Streptococcus mutans	94	87	NA	38	75	NA	100*
Staphylococcus aureus	57	NA	NA	28	28	85	100*
Shigella dysenteriae	67	50	NA	NA	50	67	100**
Escherichia coli	70	60	20	50	20	20	100**

NA = No Activity at this amount of test compound or standard.
*Zone of inhibition of amoxicillin-clavulanic acid = 16 mm (*S. mutans*) and 14 mm (*S. aureus*) (gram positive bacteria).
**Zone of inhibition of cefixime = 24 mm (*S. dysenteriae*) and 20 mm (*E. coli*) (gram negative bacteria).

As can be seen from Table-3, the compound **IVa** with 4-phenyl moiety was found to be more active against *Streptococcus mutans*. Compound **IVf**

with 3,4,5-trimethoxy phenyl moiety found to be possess significant activity against *Shigella dysenteriae*. Compound **IVa** with 4-phenyl, compound **IVb** with 2-chlorophenyl and compound **IVe** with dimethyl amino phenyl moiety showed moderate activity against *Staphylococcus aureus*, *Shigella dysenteriae* and *Escherichia coli*. Compound **IVd** showed moderate activity against *Escherichia coli*. Compound **IVc** with 2-hydroxy phenyl moiety showed no antibacterial activity.

Antifungal activity: The newly synthesized compounds were screened for their antifungal activity and the study was carried out against *Candida albicans* and *Rhizopus oryzae* fungal strains by determining the zone of inhibition using disc diffusion method (Table-4). Ketoconazole was used as standard drug. Solvent and growth controls were kept for comparison and the zone of inhibition were noted.

TABLE-4
ANTIFUNGAL ACTIVITY OF COMPOUNDS (**IVa-f**)

Microorganism	% Diameter of zone (mm) (1mg/mL)						
	IVa	**IVb**	**IVc**	**IVd**	**IVe**	**IVf**	Std.
Candida albicans	30	46	30	23	84	92	100*
Rhizopus oryzae	28	45	54	NA	72	81	100*

*Zone of inhibition of ketoconazole = 26 mm (*C. albicans*) and 22 mm (*R. oryzae*).

The antifungal studies revealed that the compounds **IVf** with 3,4,5-trimethoxyphenyl moiety was found to be more active against both the fungal strains. Compound **IVe** with 4-dimethylamino phenyl moiety was found to possess significant activity against both the fungal strains. All the other compounds exhibited moderate activity against fungi except compound **IVd** which shows no activity against *Rhizopus oryzae*.

Conclusion

A series of 4,5-disubtituted-3-methyl-1,3a,4,5-tetrahydropyrazolo[3,4-c]-pyrazoles (**IVa-f**) were prepared. All compounds were screened for analgesic, antibacterial and antifungal activity. Compounds **IVc** with 2-hydroxyphenyl, **IVe** with 4-dimethylaminophenyl and **IVf** with 3,4,5-trimethoxyphenyl moieties were found to possess significant analgesic activity. Compound **IVa** with 4-phenyl moiety was found to possess significant activity against *Streptococcus mutans*. Compound **IVf** with 3,4,5-trimethoxyphenyl moiety was found to possess significant activity against both fungal strains. Therefore, compounds **IVa**, **IVb**, **IVd** and **IVf** are recommended for further studies.

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