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SYNTHESIS AND EVALUATION OF SYMMETRIC AND ASYMMETRIC BIS-MANNICH BASES OF PIPERAZINE

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ABSTRACT

A series six derivatives of symmetric and asymmetric bis-mannich bases of piperazine **(3a-3f)** were synthesized and evaluated for anticonvulsant activity using subcutaneous pentylenetetrazole (scPTZ) and maximal electroshock (MES) induced seizure methods at the dose of 50mg/kg, p.o. Of these compounds, **3d** and **3e** exhibited good anticonvulsant activity, which may be due to the presence of asymmetric phenyl mannich bases containing (2-nitrophenyl) and (3-nitrophenyl) substitutions respectively (P>0.0001). However, piperazine possessing 3-nitrophenyl substitutions on the 1,4-positions (**3b)** displayed good anti-MES protection. Further compounds **3d and 3e** which exhibited the significant anti-PTZ activity were selectively subjected to the dose-dependent activity in different doses (25, 50 and 100 mg/kg).

Keywords: bis-mannich bases of piperazine, subcutaneous pentylenetetrazole induced seizure methods, maximal electroshock induced seizure methods.

1. INTRODUCTION

Mannich bases containing bridge nitrogen atom exhibit diverse pharmacological activities like anti inflammatory, antihelminthic, antibacterial, antineoplastic, analgesic, anticonvulsant, antinociceptive etc (Orjales *et al.*, 1996; Sriram *et al.*, 2006; Obniska *et al.*, 2010). Therefore, these compounds have gained the importance in drug discovery. N-aryl piperazines were also reported as key pharmacophoric moieties for antidepressant, anticonvulsant, antinociceptive, anti inflammatory and TRPV1 antagonists (Minsoo Han et al., 2012). Based on the reported literature, it is designed to combine two pharmacophores and involved in the synthesis of symmetric and asymmetric bis-mannich bases of piperazine (3a-3f) and their evaluation for anticonvulsant activity.

2. EXPERIMENTAL METHODOLOGY

2.1 Materials and Methods:

Aldehydes and esters were procured from Sigma-Aldrich and Merck chemicals. Purity of the samples was monitored by TLC analysis using precoated aluminium plates (Merck), coated with silica gel (Kieselgel 60) with F_{254} indicator. Melting points were determined in open capillaries using Analab melting point apparatus and were uncorrected. IR spectra were recorded as KBr diluted pellets on a Jasco FTIR (FTIR-4100) Spectrophotometer. ^{1}H NMR spectra were carried out on Jeol-400 MHz NMR Spectrophotometer (JNM-400) using TMS as internal reference. Chemical shifts (δ) values are given in parts per million (ppm) using $CDCl_3$ as solvent and coupling constants (J) in Hz. Splitting patterns are designated as follows: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet. Mass spectral data was obtained on LCMS (schimadzu) APCI model LC-2010 EV.

Synthesis of symmetric bis mannich bases of piperazines (3a-3c).

An eqimolar mixture of piperazine and the aniline or substituted aniline in 1:2 molar ratio was dissolved in suitable quantity of ethanol by adding 1ml of formalin (37%). The reaction mixture was refluxed for 5-8 h. The completion of the reaction was monitored by TLC (n-hexane: methanol 8:2). After the completion of the reaction, poured the contents in ice water and filtered it off. The separated solid was recrystallized from water or aqueous alcohol to obtain the compounds **3a-3c (scheme 1).**

Synthesis of the N-[(4-((2-nitrophenylamino) methyl) piperazin-1-yl) methyl]-2-nitrobenzenamine (3a): 0.01 mol (0.86g) of piperazine and 0.02 mol (2.76 g) of 2-nitroaniline dissolved in suitable quantity of ethanol and 1ml of formalin (37%). The reaction mixture was refluxed for 6 h to obtain the compound **3a** as orange fine crystals.

Synthesis of asymmetric bis mannich bases of piperazines (3d-3f).

Step 1: An equimolar concentration of Boc-piperazine and the substituted aniline dissolved in suitable quantity of ethanol, 1ml of formalin (37%) was also added to the reaction mixture and refluxed for 5-hours. The completion of the reaction was monitored by TLC (n-hexane: methanol 8:2). After the completion of the reaction, poured the contents in ice water and filtered it off to obtain Boc-protected intermediate **(intermediate 1).**

Step 2: Deprotection was performed by dissolving the intermediate Boc-protected derivative in 6N HCl (60 ml) and washed with ether (3×50 ml). The aqueous phase was basified with solid KOH to pH 11. It was then extracted with ethyl acetate (3×100 ml). The combined organic phases were dried over Na_2SO_4 and concentrated to make the compounds (intermediate 2) for the repetition of step 1 with different amine **3d-3f** (Repine *et al*., 2007).

Synthesis of the N-[(4-((2-nitrophenylamino) methyl) piperazin-1-yl) methyl] benzenamine (3d): 0.01 mol (1.86 g) of Boc-piperazine and 0.01 mol of 2-nitroaniline were dissolved in suitable quantity of ethanol. To the stirred reaction mixture 1ml of formalin (37 %) was added and refluxed for 5 h and the completion of the reaction was monitored by TLC. After the completion of the reaction, poured the contents in ice water and filtered it off. The resulting piperazine was deprotected by dissolving in 6N HCl (60 ml) and washed with ether (3×50 ml). The aqueous phase was basified with solid KOH to pH 11. It was then extracted with ethyl acetate each quantity of 100ml for 3-4times. The combined organic phases were dried over Na_2SO_4 and concentrated to obtain the compounds for the repetition of mannich reaction. 0.01mol (2.36 g) of the compound and 0.01 mol (1 ml) of aniline dissolved in suitable quantity of ethanol and refluxed for 5-6h and obtained the compound as dark yellow powder.

2.1 PHYSICAL AND SPECTRAL DATA OF COMPOUNDS

N-((4-((2-nitrophenylamino) methyl) piperazin-1-yl) methyl)-2-nitro benzenamine (compound **3a, R= -2-NO_2, R^1= CH_2-NH-C_6H_4-2NO_2): R_f: (**n-hexane : methanol, 8:2**) 0.45; λ_{max} 407nm. **IR(KBr) ν_{max} , cm^{-1} :** 3449 (2°amine, N-H str), 3059, 3024 (Ar, C-H str), 2992 & 2951 (alkyl, C-H str), 1493 (N-O asym N-O Str), 1300 (N-O sym N-O str). **^{1}H NMR (400MHz, $CDCl_3$) δ (ppm):** 2.90-3.25 (br t, 4H, 2CH_2 of piperazine), 3.65-3.80 (br d, 4H, 2CH_2 of piperazine), 4.25 (s, 2H, CH_2), 6.05 (br s, 2H, NH), 6.65-8.19 (m, 8H, Ar-H, $C_6H_4NO_2$). **APCI-MS:** m/z = 385.9 $(M)^+$.

N-((4-((3-nitrophenylamino) methyl) piperazin-1-yl) methyl)-3-nitrobenzenamine (compound **3b, R= -3-NO_2, R^1= CH_2-NH-C_6H_4-3NO_2):** 0.01mol (0.86g) of piperazine and 0.02mol (2.76g) of 3-nitroaniline dissolved in suitable quantity of ethanol and proceeded as in **3a** and obtained the compound **3b** as yellow fine powder. **λmax** 402nm; **R_f: (**n-hexane : methanol, 8:2**)** 0.50. **IR (KBr) ν_{max} , cm^{-1} :** 3446 (2°amine,N-H str), 3087 & 3043 (Ar, C-H str), 2926 (alkyl, C-H str), 1536 (N-O asym N-O Str), 1348 (N-O sym N-O str). **^{1}H NMR (400MHz, $CDCl_3$) δ (ppm):** 3.25-3.45 (br d, 8H, 4CH_2 of piperazine), 4.25 (s, 4H, CH_2), 5.75 (br s, 2H, NH), 7.23-7.98 (m, 8H, Ar-H $C_6H_4N0_2$). **^{13}C NMR (100MHz, $CDCl_3$) δ (ppm):** 47.9 (**C**H_2-N), 50.7 (-HN-**C**H_2-N), 116.8, 120.9, 127.7, 135.3, 141.8 (CH_{ar}, C_{ar}), 150.6 (C-NO_2, C_{ar}). **APCI-MS:** m/z = 386.0 $(M)^+$. Anal. Calc. for $C_{18}H_{22}N_6O_4$: C, 55.95; H, 5.74; N, 21.75. Found: C, 55.85; H, 5.69; N, 21.68.

N-((4-((4-nitrophenylamino)methyl) piperazin-1-yl) methyl)-4-nitro benzenamine (compound **3c, R= -4-NO_2, R^1= CH_2-NH-C_6H_4-4NO_2):** 0.01mol (0.86g) of piperazine and 0.02mol (2.76g) of 4-nitroaniline dissolved in suitable quantity of ethanol and proceeded as in **3a** and obtained the compound **3c** as yellowish orange fine crystals. **R_f: (**n-hexane :

methanol, 8:2**)** 0.49; **λmax** 391nm. **IR (KBr) ν_{max}, cm^{-1}:** 3451 (2°amine, N-H str), 3065 & 3041 (Ar, C-H str), 1492 (N-O asym N-O Str), 1354 (N-O sym N-O str). **^{1}H NMR (400MHz, $CDCl_3$) δ (ppm):** 2.33-2.55 (br t, 4H, $2CH_2$ of piperazine), 3.45-3.65 (br d, 4H, $2CH_2$ of piperazine), 4.1 (s, 4H, CH_2), 5.47 (br s, 2H, NH), 6.7-8.2 (m, 8H, Ar-H of P-NO_2 C_6H_4). **APCI-MS:** m/z = 386.0 $(M)^+$. Anal. Calc. for $C_{18}H_{22}N_6O_4$: C, 55.95; H, 5.74; N, 21.75. Found: C, 55.79; H, 5.67; N, 21.72.

N-[(4-[(2-nitrophenylamino) methyl] piperazin-1-yl) methyl] benzenamine (compound **3d, R= -2-NO_2, R^1= CH_2-NH-C_6H_5): R_f: (**n-hexane : methanol, 8:2**)** 0.64; **λmax** 407nm. **IR (KBr) ν_{max} , cm^{-1}:** 3482& 3452 (2°amine,N-H str), 3097 & 3015 (Ar, C-H str), 2875 & 2932 (Alkyl, C-H str), 1505 (N-O asym N-O Str), 1366 (N-O sym N-O str). **^{1}H NMR (400MHz, $CDCl_3$) δ (ppm):** 3.2-3.4 (br d, 8H, $4CH_2$ of piperazine), 4.32 (s, 4H, $2CH_2$), 5.07 (br s, 1H, NH), 6.85-7.55 (m, 9H, Ar-H, nitrophenyl and phenyl). **APCI-MS:** m/z = 341.0 $(M)^+$.

N-[(4-[(3-nitrophenylamino) methyl] piperazin-1-yl) methyl] benzenamine (compound **3e, R= -3-NO_2, R^1= CH_2-NH-C_6H_5):** 0.01 mol (1.86 g) of Boc-piperazine and 0.01 mol of 3-nitroaniline were dissolved in suitable quantity of ethanol and proceeded as in **3d** and obtained the compound **3e** as yellow powder. **R_f: (**n-hexane: methanol, 8:2**)** 0.55; **λmax** 391nm.

N-[(4-[(4-nitrophenylamino) methyl] piperazin-1-yl) methyl] benzenamine (compound **3f, R= -4-NO_2, R^1= CH_2-NH-C_6H_5):** 0.01 mol (1.86 g) of Boc-piperazine and 0.01 mol of 4-nitroaniline were dissolved in suitable quantity of ethanol and proceeded as in **3d** and obtained the compound **3f** as yellow powder. **R_f: (**n-hexane: methanol, 8:2**)** 0.46; **λmax** 393nm.

2.3. PHARMACOLOGICAL STUDIES

2.3.1. Experimental animals

Male Swiss albino mice (18-22g) and male Wistar rats (150-200g) were used as experimental animals. They were obtained from King Institute of Preventive Medicine, Chennai. The animals were acclimatized for a week under standard husbandary conditions, room temperature of 24±1°C, relative humidity 45-55% and 12: 12 h light/dark cycle. The animals had free access to rodent pellet diet (Pranav Agro Industry, Bangalore) and water under strict hygienic conditions. All animal experiment protocols were approved by the Institutional Animal Ethical Committee (IAEC) of Annamacharya college of Pharmacy, Rajampet, India.

2.3.2. Acute toxicity studies

The study was conducted as per OECD-425 guide lines for testing of chemicals acute oral toxicity. The test was used to fix the safe dose for the compounds **3a-3f**. Swiss albino mice were divided into six groups each containing 10 animals and repeated for all the drugs. Drugs were administered by oral route in different concentrations (2000, 1000, 500, 250, 100 and 50mg/kg body weight). The animals were observed for their death over a period of 7days. The LD_{50} values were calculated by up and down method and dose was fixed as 50mg/kg body weight.

2.3.3. Methods

Evaluation of Anticonvulsant Activity

Subcutaneous Pentylenetetrazole Seizure test (Sc PTZ)

This method utilizes a dose of Pentylenetetrazole (PTZ) 80mg/kg subcutaneously, in rats that produces clonic seizures. The rats were divided into several groups of six rats each. Group 1 animals were kept as control and were received vehicle; Group 2 received Diazepam (5 mg/kg, intraperitoneally), other Groups received the test compounds **3a-3f** respectively (50mg/kg, oral), which were prepared by suspending in 0.5% sodiumcarboxymethylcellulose. 1 h after administration of vehicle, diazepam and test compounds **3a-3f**, PTZ (80mg/kg) was injected subcutaneously. The time of onset of clonic convulsions and the protection against mortality were observed (Subudhi *et al.*, 2009). The percentage protection against mortality was calculated.

Maximal Electric Shock test (MES)

Anticonvulsant property of the test compounds in this model was assessed by its ability to protect against Maximal Electric Shock induced convulsions. Male Wistar albino rats were divided into several groups of six rats each. Group 1 was the control group which received vehicle (0.5% sodium carboxy methyl cellulose, oral); Group 2 received Phenytoin (30 mg/kg, oral), other Groups received each of test compounds **3a-3f** respectively (50 mg/kg, oral), which were prepared by suspending in 0.5% sodium carboxymethyl cellulose. 1 h after the administration of vehicle, Phenytoin, test compounds, Maximal Electric Shock of 150 mA current for 0.2 sec was applied through corneal electrodes to induce convulsions using an Electroconvulsiometer in the control, standard and test compounds treated animals. Duration of hind limb tonic extension was noted. Abolition or reduction in the duration of tonic extension was considered as the index for antiepileptic activity (Obniska. J. *et al.*, 2010). The percentage protection against shock induced seizures was calculated using the formula: $[C_{\text{hind limb extension}} - T_{\text{hind limb extension}} / C_{\text{hind limb extension}}] 100$.

3. RESULTS AND DISCUSSION:

In the present study, the synthesis of the N-[substituted piperazine-1-yl) methyl] benzenamine derivatives **(3a-3f)** was carried out via the symmetric bis-mannich reaction (Abuo-Rahama *et al.*, 2009). The condensation of commercially available piperazine or Boc-piperazines and various substituted anilines with formaldehyde in equimolar or 1:2 molar ratios resulted in the compounds **(3a-3f)** in good yields (52-80%, **scheme 1)**. In the synthesis of **3d-3f,** Boc-protected piperazine was used in the above method and the reaction was further continued for the deprotection of piperazinyl moiety by acidification followed by basification to obtain the compounds (Repine *et al.*, 2007).

The IR spectra of the title compounds showed a broad band at 3500-3400cm^{-1} assignable to the secondary amine group. A band at 1450-1350 cm^{-1} indicated symmetric and asymmetric stretching of nitro functional group and at 1050-1020 cm^{-1} showed C-N stretching. The ^{1}H NMR spectrum of the compounds supported the structures of **3a-3f**. These compounds showed a broad singlet at 2.3-2.8 ppm which indicated the NH proton of piperazinyl moiety and a triplet or doublet in the region of 2.4-3.8 ppm representing alkyl protons of piperazine ring. A singlet was observed in the region of 4.2-5.2 ppm assignable to the methylene protons flanked by two nitrogens and multiplets in the region of 6.4-8.2 ppm due to the presence of aryl protons. The mass spectra of the compounds **(3a-3f)** showed the molecular ion peaks at their respective molecular weights as M^{+} and $(M+H)^{+}$.

Pharmacological evaluation of N-[substituted piperazine-1-yl)methyl] benzenamine derivatives (3a-3f).

Anticonvulsant activity:

The anticonvulsant activity profile of compounds **3a-3f** was evaluated by subcutaneous pentylenetetrazole (scPTZ) and maximal electroshock (MES) induced seizure methods after oral administration of the drug candidate to male Wistar albino rats at the dose of 50 mg/kg body mass. The dose was fixed by up and down method as per OECD-425 guidelines. Seizure inducing pentylenetetrazole (80mg/kg) or maximal electroshock (150 mAmp, 0.2 sec) was applied 1 hour after the administration of drug candidate. Compounds **3c-3f** showed significant anti-PTZ activity ($P<0.05$ Vs control). Of these compounds, **3d** and **3e** exhibited good anticonvulsant activity and delayed the onset of convulsions, which may be due to the presence of asymmetric phenyl mannich bases containing (2-nitrophenyl) and (3-nitrophenyl) substitutions respectively ($P>0.0001$). Other derivatives displayed moderate activity and are not comparable to diazepam as reference standard (**Table 1**). Compounds **3a** and **3c** were devoid of activity. The protection against PTZ induced mortality was also studied. Compounds **3d-3f** demonstrated moderate protection against mortality (66.6%) and other compounds did not exhibit significant protection.

In **MES model**, compounds **3a-3f** showed significant decrease in the duration of hind limb extension and percentage protection against electro convulsions ($P<0.05$ Vs control) (**Table 1**). Among these derivatives, piperazine possessing 3-nitrophenyl substitutions on the

1,4-positions (**3b)** displayed good anti-MES protection and other derivatives showed moderate protection.

However, the activities of the compounds **3a-3f** are less active than phenytoin. Further compounds **3d** and **3e** which exhibited the significant anti-PTZ activity were selectively subjected to the dose-dependent activity in different doses (25, 50 and 100 mg/kg) (**Table 2)**. It was observed that significant improvement in latency periods and protection against mortality dose dependently.

4. CONCLUSION

The present study revealed that compounds existing as asymmetric phenyl mannich bases containing (2-nitrophenyl) and (3-nitrophenyl) substitutions as in **3d** and **3e** displayed good anticonvulsant activity dose dependently.

Scheme 1: Synthetic protocol of the Symmetric and Asymmetric bis-mannich bases. (3a-3f)

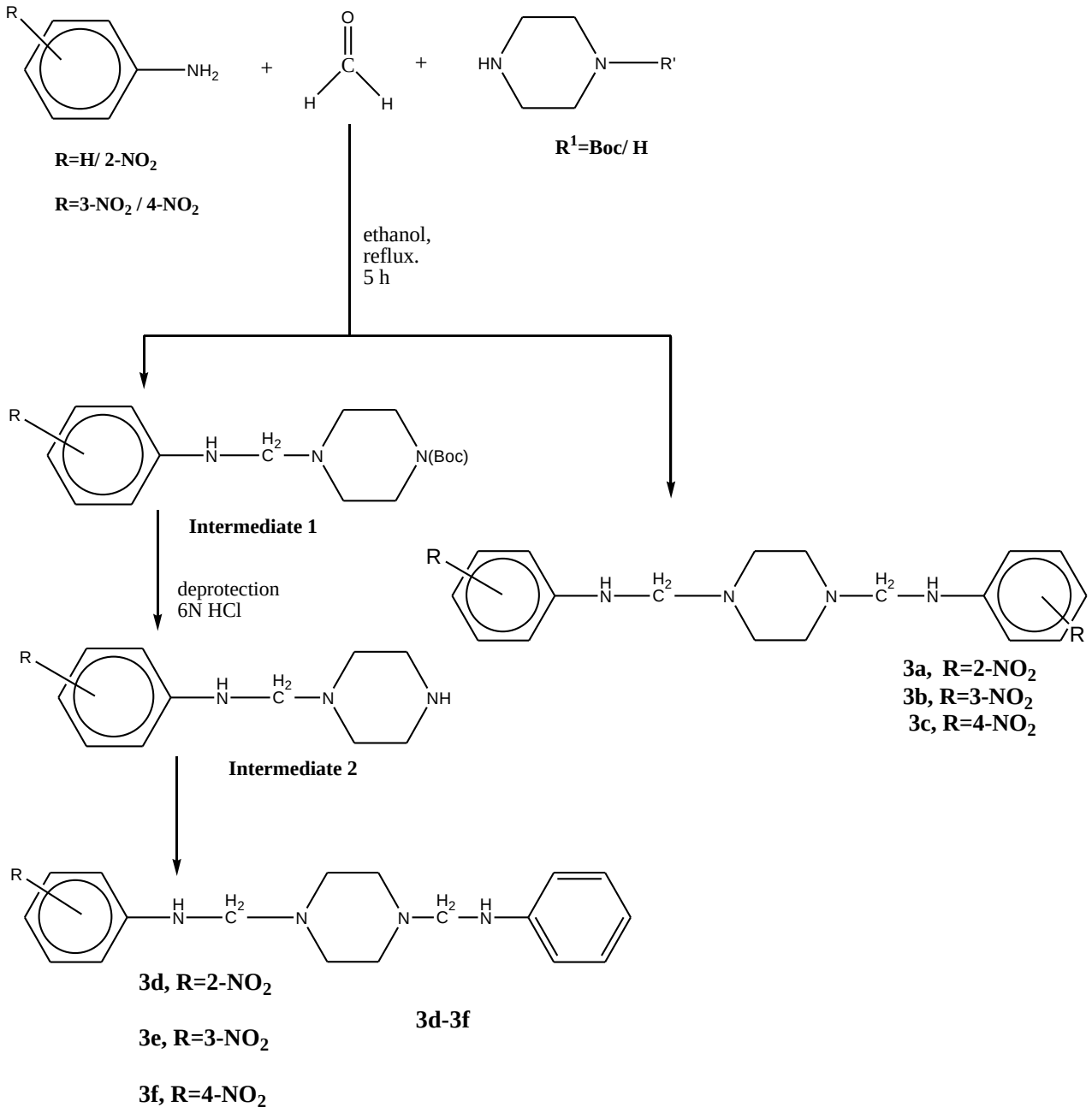

Table 1: Anticonvulsant activity of N-((substitutedpiperazin-1-yl) methyl) benzenamine derivatives **(3a-3f)** in pentylenetetrazole induced and maximal electro shock induced seizure models.

Compound	Latency Period mean ± SEM	Percentage mortality (%)	Duration of Limb Extension mean ± SEM	Percentage protection (%)
Control	66.67±2.789	000	21±1.317	----------
Diazepam	300±0.0***	100	------	--------
Phenytoin	----	-----	6.33±0.55***	69.8
3a	123.3±3.801^{ns}	00	14.33±0.4216***	31.7
3b	124.3±4.022 ^{ns}	00	8.33±0.557***	60.3
3c	126.7±4.595*	33.3	10.67±0.4216***	49.1
3d	246.7±8.102***	66.6	15±1.095***	28.57
3e	236.7±7.379***	66.6	12.33±0.9189***	41.2
3f	223.3±4.595***	66.6	14±0.6325***	33.3

The test compounds were administered orally (50mg/kg body weight) 1h before the injection of pentylenetetrzole (80mg/kg, i.p) or application of maximal electroshock (150mAmp, 0.2 sec). Values were expressed as mean±SEM, n=6. One-way analysis of variance (ANOVA) followed by Dunnet's multiple comparision test. *** P<0.0001 vs control, **P<0.05 vs control, ^{ns}P>0.05 vs control.

Table 2: Dose dependent study on anticonvulsant activity of N-[(substituted piperazin-1-yl) methyl]benzenamine derivatives 3d and 3e in PTZ and MES induced seizure models

Compound	Dose (mg/kg)	Latency period (s) mean±sem	% protection against mortality	Duration of limb extension	% protection
3d	25	127.5±3.3***	33.3	18±1.0^{ns}	15.4
	50	246.7±8.1***	66.6	15±1.0***	29.5
	100	289.2±2.7***	100	9.1±0.4***	57.2
3e	25	113.3±2.4***	33.3	13.3±0.5***	37.5
	50	236.7±7.3***	66.6	12.3±0.9***	42.2
	100	277.2±1.0***	83.3	9.3±0.42***	56.3
Diazepam	5	300.0±0.0***	83.3	------	-----
phenytoin	30	---------	-------	6.3±0.5***	70.4
control	---	66.6	--------	21.3±1.3	-----

The test compounds were administered orally (50mg/kg body weight) 1h before the injection of pentylenetetrzole (80mg/kg, i.p) or application of maximal electroshock (150mAmp, 0.2 sec). Values were expressed as mean±SEM, n=6. One-way analysis of variance (ANOVA) followed by Dunnet's multiple comparision test. *** P<0.0001 vs control, **P<0.05 vs control, ^{ns}P>0.05 vs control.

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