Synthesis and biological evaluation of formazan derivatives

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Abstract
The formazan derivatives (FM1–FM5) were synthesized by the reaction of benzaldehyde phenylhydrazone with substituted aromatic and hetero aromatic amines. The structures of the synthesized compounds were then elucidated using UV, IR, 1H NMR and mass spectral data. The synthesized derivatives were screened for anticonvulsant, antibacterial and antiviral activities. All the compounds showed remarkable antibacterial activity at 250 µg/ml, but FM4 and FM3 did not show any inhibition on *Staphylococcus aureus* and *Vibriocholera*, respectively. All the compounds showed significant anticonvulsant effect at 100 mg/kg p.o. and the experimental data were statistically significant at $P<0.001$ level. But none of the compounds was effective against Japanese encephalitis virus.

Key words: Anticonvulsant, antibacterial, antiviral, formazan, Ranikhet disease virus

INTRODUCTION
Formazans have been found to possess important medical applications due to their various activities such as antiviral,[1] antimicrobial,[2,3] anti-inflammatory, analgesic,[4] antifungal,[5] anticancer, anti-HIV,[6] etc. Several formazans showed promising anti-fertility,[7] anti-parkinsonian[8] and anticonvulsant activities.[9] The antiviral activity has been shown by formazan derivatives in both plants and animals. Various authors have reported that this class of compounds is active against the Ranikhet disease virus, Tobacco mosaic virus (TMV) and Gomphrena mosaic virus (GMV).[10] The antiviral properties of some formazan derivatives have been reported in which the antiviral effect is attributed to the presence of an intact C=NNH grouping and C–N=N grouping [Figure 1].[11] Formazan derivatives have been widely used to evaluate cell viability and to screen anti-HIV agents and the cytotoxicity of these agents. Synthetic formazan effectively inhibited the replication of laboratory-adapted and primary HIV-1 isolates and cell-to-cell fusion, with low cytotoxicity. It blocks the six-helix bundle formation between peptides derived from the N- and C-terminal heptad repeat regions of the gp41 ectodomain of HIV-1.[12,13] It has been reported that members of a series of formazan are used for detecting and quantifying the presence of a target molecule, such as an antigen, an antibody or a polynucleotide. The crown formazan derivative, used as a replicase inhibitor specific to the bovine viral diarrhea virus, has been recently identified.[14] Moreover, they have been studied extensively because of their ready accessibility, diverse chemical reactivity and broad spectrum of biological activities.[15,16]

Taking into consideration the referred important biological activities of formazan derivatives, our study was designed to synthesize some potent molecule for biological screening.

MATERIALS AND METHODS

Instruments and Equipments
The melting points were determined using open capillary tubes and are uncorrected. The lambda max of the
compounds was measured by UV–visible spectrophotometer (UV-Pharma Spec 1700, Shimadzu, Kyoto, Japan). The infrared (IR) spectra were recorded on FT-IR8400S, Fourier Transform (Shimadzu) Infrared spectrophotometer using KBr disk method. The proton magnetic resonance spectra were recorded on Perkin Elmer spectrophotometer [300 MHz in dimethyl sulfoxide (DMSO)-d6] using Tetramethylsilane as an internal standard and chemical shifts were expressed in δ ppm. The mass spectra were recorded on a JEOL SX-102 (FAB) spectrometer.

**Chemicals and Drugs**

All the chemicals and reagents were of synthetic grade and commercially procured from s.d. Fine Chem. Ltd. (Mumbai, India). Diazepam (Calmposie inj. Ranbaxy, Gurgaon India) was purchased from local medical stores (Majhitar, East Sikkim), Streptomycin was obtained from Alkem Pvt. Ltd. (East Sikkim, India) as a gift sample.

**Animals**

Male Swiss Albino mice weighing 22–25 g were used for the pharmacological and toxicological studies. Animals were housed in groups of 6–8 per cage at a temperature of 25±1°C and a relative humidity of 45–55%. A 12:12 dark:light cycle was followed during the experiments. Animals had free access to food and water ad libitum. During the study period, guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Institutional Animals Ethics Committee (IAEC), were followed for the maintenance of animals.

**Preparation of benzaldehyde phenylhydrazone derivatives**

0.01 mol of phenyl hydrazine was added dropwise to a well-stirred mixture of 0.01 mol benzaldehyde in dilute acetic acid (2 ml in 10 ml water) in a 100 ml conical flask at room temperature. The reaction mixture was further stirred for 1 hour and kept at room temperature for 30 minutes. The precipitated yellow crystalline mass was filtered and dried in an oven at 60°C. The crude product was recrystallized from rectified spirit with charcoal treatment. Benzaldehyde phenylhydrazone was obtained as fine colorless needles, with melting point 156°C, and % yield was 80%.

### Synthesis of 1-phenyl-3-phenyl-5-[aryl/heteroaryl] formazan derivatives

0.01 mol of substituted aryl amine was dissolved in a mixture of 5 ml concentrated hydrochloric acid and 5 ml water taken in a 100 ml conical flask, with constant stirring. The reaction mixture was cooled in ice bath until the temperature fell below 5°C. Separately, 1.6 g of sodium nitrite was dissolved in 7.5 ml of water and chilled in an ice bath below 5°C. The sodium nitrite solution was filtered to obtain a clear solution and then added dropwise to the aniline mixture with vigorous shaking and the temperature was not allowed to rise above 10°C. This diazonium salt solution of aryl and heteroaryl amine was filtered to obtain a clear solution and then added dropwise with continuous stirring to a solution of benzaldehyde phenyl hydrazone (0.01 mol) in pyridine (20 ml), maintaining the temperature below 10°C. The reaction mixture was allowed to stand for about 4 hours and was then poured into 250 ml of ice-cold water with continuous stirring. The dark colored solid which separated out was filtered, washed successively with cold water followed by hot water, finally with methanol and dried in air as well. The formazans thus synthesized were recrystallized from the mixture of chloroform and petroleum ether. The scheme of synthesis is given in Figure 2 and the physicochemical data of the title compounds are presented in Table 1.

### Table 1: Physicochemical data of the synthesized compounds

| Compound code | –R     | Physical state | Molecular weight | Molecular formula | Melting point (°C) | % Yield |
|---------------|--------|----------------|------------------|------------------|-------------------|--------|
| FM1           | 4-NO2  | Dark red powder | 346              | C19H15N5O2       | 236–238           | 94.28  |
| FM2           | 4-Cl   | Red powder     | 335              | C19H15NCl        | 168–170           | 98.93  |
| FM3           | 2-Cl, 3-Cl | Dark red powder | 370              | C19H14N4Cl2      | 208–210           | 80.35  |
| FM4           | 3-Cl   | Dark red powder | 335              | C19H15NCl        | 180–182           | 89.70  |
| FM5           |        | Colorless powder | 287              | C18H15N4         | 216–218           | 91.00  |
Figure 2: Scheme of synthesis

1H, NH), 6.84–6.96 (m, 4H, Ar–H), 7.08–7.3 (m, 5H, Ar–H), 7.33–7.37 (m, 5H, Ar–H); MS: m/z 287 [M]+, 286 [M−1]+, 285 [M−2]+.

FM4: 1-phenyl-3-phenyl-5-[3-chlorophenyl] formazan

λ max (nm) ethanol: 476; IR (KBr, cm−1): 3450 (N–H str.), 1654 (C=N str.), 1581 (N=N str.); 1H NMR (CDCl3): δ 15.31 (s, 1H, NH), 7.90–8.31 (m, 5H, Ar–H), 7.01–7.62 (m, 5H, Ar–H), 6.6–6.9 (m, 3H, Ar–H); MS: m/z 370 [M]+, 369 [M−1]+.

FM5: 1-phenyl-3-phenyl-5-[2-pyridyl] formazan

λ max (nm) ethanol: 473; IR (KBr, cm−1): 3502 (N–H str.), 1653 (C=N str.), 1589 (N=N str.); 1H NMR (CDCl3): δ 16.23 (s, 1H, NH), 7.71–8.27 (m, 5H), 7.24–7.61 Ar–H (m, 5H, Ar–H), 6.73–7.50 (m, 4H, Ar–H); MS: m/z 335 [M]+, 333[M−2]+.

Acute Toxicity Studies

Groups of six albino mice, weighing 20–25 g, were fasted overnight and treated perorally with the test compounds. The dosage was varied from 100 to 1000 mg/kg body weight. The animals were observed for 24 hours for any signs of acute toxicity such as increased or decreased motor activity, tremors, convulsion, sedation, lacrimation, etc. All the animal experiments were performed on obtaining the approval of Institutional Animal Ethics Committee, Himalayan Pharmacy Institute, East Sikkim, India (IAECNo: HPI/10/59/IAEC/0081).

Antibacterial Activity

The antibacterial activity was determined using agar cup-plate method.[21] Exactly 20 ml of sterile nutrient agar medium was poured into sterile petri dishes and allowed to solidify. The petri dishes were incubated at 37°C for 24 hours to check for sterility. The medium was seeded with the micro-organisms by pour plate method using sterile top agar (4 ml) containing 1 ml culture. The bores were made on the medium using sterile borer. Test compounds were dissolved in DMSO and 0.1 ml (250 μg/ml) of the different test compounds was added to the respective bores. 0.1 ml of streptomycin at a concentration of 250 μg/ml was taken as standard reference and 0.1 ml of DMSO was used as control. The plates were incubated overnight at 37°C with appropriate positive and negative controls. The petri dishes were kept in refrigerator at 4°C for half an hour for diffusion. Then, the petri dishes were incubated at 37°C for 24 hours and zone of inhibition were observed and measured in millimeters. The results of determination of antibacterial activity are summarized in Table 2 with that of the standard drug.
Anticonvulsant Activity (Maximum Electroshock Method)
Several animal models of convulsions have been developed to evaluate anti-seizure activity. Many drugs that increase the brain contents of gamma aminobutyric acid (GABA) have exhibited anticonvulsant activity against seizures induced by maximum electroshock (MES), pentylentetrazol (PTZ) and lithium-pilocarpine (Li-Pilo). The MES is probably the best validated method for assessment of anti-epileptic drugs in generalized tonic-clonic seizures.[22,23] Male Swiss albino mice (22–25 g) of either sex, maintained in standard conditions for temperature, relative humidity, light/day cycles and fed with normal diet and water ad libitum, were used. All the synthesized compounds were screened for their anticonvulsant activity using electroconvulsometer. Maximal seizures were induced by application of an electrical current across the brain via corneal electrodes. Diazepam 4 mg/kg i.p. was used as standard drug. It was administered at a dose of 100 mg/kg p.o. in 0.5% Carboxymethyl cellulose to groups of mice (n=6), 30 minutes before the application of electric shock (42 mA, 0.2 seconds) using corneal electrodes. After 30 minutes and 4 hours of drug administration, electroshock was applied using corneal electrodes. The disappearance of the hind leg extensor component of convulsion was used as a positive criterion. The incidence and onset of tonic seizures were noted. The results are summarized in Table 3.

Antiviral Activity
All the compounds were tested for their antiviral activity against Japanese encephalitis virus [P20778].[24,25] The observed results are presented in Table 4.

RESULTS AND DISCUSSION

Almost all the compounds are active against Gram-positive organism S. aureus, except FM4. The compound FM5 exhibited significant zone of inhibition (13.2 mm) followed by FM3 (13 mm). Compounds FM1 and FM2 had moderate to fewer zones of inhibition but FM4 did not show inhibition on S. aureus. In the case of Gram-negative bacteria, all the compounds had shown remarkable antibacterial activities except FM3 on V. cholerae. Comparatively, all the compounds had shown lesser antibacterial activities than that of streptomycin at 250 µg/ml.

All the compounds were screened for their anticonvulsant activity by maximum electroshock method. Compound FM5 was found to be most active among all the synthesized compounds, whereas rest of the compounds were found to be moderate or less active. But none of the compounds showed neurotoxicity. An imbalance between the excitatory and inhibitory neurotransmitters is responsible for seizures.[26,27] At neuronal level, seizure activity often occurs when glutamatergic excitatory neurotransmitters override GABA mediated inhibition.[28] Several drugs are thought to inhibit seizures by regulating GABA mediated synaptic inhibition through an action at distinct sites of the synapse.[29] Hence, it can be expected that formazan may produce anticonvulsant effect by regulating GABA mediated synaptic inhibition. From the SAR point of view, it is interesting to observe that the formazan derived from hetero aniline FM5 displayed profound antibacterial and

### Table 2: Zone of inhibition (in mm) of 250 µg/ml of the compound against bacteria

| Compound | Zone of inhibition (in mm) of 250 µg/ml of compound |
|----------|-----------------------------------------------------|
|          | S. p       | S. a       | E. c       | S. s       | VI       |
| FM1      | 12.1       | 11         | 10.3       | 13.9       | 9.4      |
| FM2      | 9.1        | 14.2       | 7.9        | 13.3       | 13.8     |
| FM3      | 13         | 11         | 12.8       | 14.9       | —        |
| FM4      | —          | 15.7       | 9.6        | 9.7        | 15.3     |
| FM5      | 13.2       | 12.3       | 9.2        | 13.1       | 14.5     |
| Streptomycin | 15.3      | 21.3       | 17.4       | 16.1       | 22.1     |
| DMF      | —          | —          | —          | —          | —        |

*S. a = Staphylococcus aureus; NTCC-6571; E. c = Escherichia coli (TG1); S. s = Shigella sonnei; SG 4; S. p = Shigella paratyphi A2; VI = Vibrio cholerae DMF-dimethyl formamide

### Table 3: Anticonvulsant activity of synthesized compounds

| Entry | Treatment (100 mg/kg p.o.) | Onset of tonic seizures (in seconds) Mean±SEM | Incidence of the seizures % |
|-------|----------------------------|----------------------------------------------|-----------------------------|
| 01    | Control                    | 128.83±2.83*                                | 100                         |
| 02    | FM1                        | 350.33±13.35*                               | 83.33                       |
| 03    | FM2                        | 428.33±14.59*                               | 66.66                       |
| 04    | FM3                        | 483±4.28*                                   | 33.33                       |
| 05    | FM4                        | 518.5±4.28*                                 | 33.33                       |
| 06    | FM5                        | 643±16.87*                                  | 16.66                       |
| 07    | Diazepam (4 mg/kg i.p.)    | A                                            | 0                           |

A = Absent, *The results are statistically treated and significant at P<0.001 level

### Table 4: Antiviral activities of synthesized compounds

| Entry | Name of the virus          | Activity (in vitro) | Remarks |
|-------|----------------------------|---------------------|---------|
| FM1   | Japanese encephalitis virus| 500-4               | Inactive|
| FM2   | Japanese encephalitis virus| 500-4               | Inactive|
| FM3   | Japanese encephalitis virus| 500-4               | Inactive|
| FM4   | Japanese encephalitis virus| 500-4               | Inactive|
| FM5   | Japanese encephalitis virus| 500-4               | Inactive|

override GABA mediated inhibition.[28] Several drugs are thought to inhibit seizures by regulating GABA mediated synaptic inhibition through an action at distinct sites of the synapse.[29] Hence, it can be expected that formazan may produce anticonvulsant effect by regulating GABA mediated synaptic inhibition. From the SAR point of view, it is interesting to observe that the formazan derived from hetero aniline FM5 displayed profound antibacterial and
anticonvulsant activities. From the antiviral activity data, it was found that none of the compounds was effective.

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