DEVELOPMENT OF *N*-ALKYLATED BENZIMIDAZOLE BASED CUBOSOMES HYDROGEL FOR TOPICAL TREATMENT OF BURNS

Supplementary File

API 1 (*N*-ethyl benzimidazole)

Yield: 1.34 g (91%), B.P. (1 mm tube): 184-186 °C. FT-IR (liquid, ν_{max}, cm^{-1}): 3409 (C_{aliph}-$N_{benzimi}$); 3084 (C-H_{arom}); 2981, 2929 (C-H_{aliph}); 1037 (C_{arom}-$N_{benzimi}$). ^{1}H NMR (300 MHz, DMSO-d_6) δ ppm: 1.34 (t, J= 7.2 Hz, 3H, CH_3), 4.19 (q, 2H, CH_2), 7.18 (qnt, 2H, Ar-H) 7.47 (d, *J*=9.9 Hz, IH, Ar-H), 7.65 (d, *J*=9.9 Hz. IH, Ar-H), 8.24 (s, 1H, NCHN); ^{13}C {^{1}H} NMR (75.5 MHz, DMSO-d_6); δ ppm: 14.5 (CH_3), 38.7 (N- CH_2-R), 109.5, 119.1, 120.9, 121.8, 134.5, 142.7 (Ar-C), 143.6 (NCHN).**API 2 (***N*-propyl benzimidazole)

Yield: 1.45 g (87.3%), B.P. (1 mm tube): 236-238 °C. FT-IR (liquid, ν_{max}, cm^{-1}): 3402 (C_{aliph}-$N_{benzimi}$); 3084, 3055 (C-H_{arom}): 2966, 2929, 2878 (C-H_{aliph}); 1030, 1211 (C_{arom}-$N_{benzimi}$). ^{1}H NMR (300 MHz, Acetonitrile-d_3) δ ppm: 0.85 (t, *J*=7.3 Hz, 3H, CH_3), 1.82 (sext, 2H, alkyl chain-CH_2), 4.10 (t, 2H, *J*=7.0 Hz, N-CH_2-R), 7.25 (m, 2H, Ar- H), 7.43 (d, *J*=7.2 Hz, 1H, Ar-H), 7.68 (d, *J*=7.2 Hz, 1H, Ar-H) 7.94 (s, 1H, NCHN); ^{13}C {^{1}H} NMR (100.10 MHz, Acetonitrile-d_3) δ ppm: 11.0 (CH_3), 23.3 (CH_2), 46.7 (N-CH_2-R), 110.4, 120.2, 122.0, 122.9 and 134.5 (Ar-C) 143.8 (NCHN), 144.2 (Ar-C

API 3 (*N*-butyl benzimidazole)

Yield: 1.33 g (75.1%), B.P (1 mm tube): 256-258 °C. FT-IR (liquid, ν_{max}, cm^{-1}): 3416 (C_{aliph}-$N_{benzimi}$); 3077 (C-H_{arom}); 2959, 2929, 2863 (C-H_{aliph}); 1048, 1165 (C_{arom}-$N_{benzimi}$). ^{1}H NMR (400 MHz, Acetonitrile-d_3) δ ppm: 0.71 (t, *J*=3.8 Hz, 3H, CH_3), 1.39 (sext, 2H, CH_2), 1.82 (qnt, 2H, CH_2), 4.20 (t, *J*=7.2 Hz, 2H, N-CH_2-R), 7.09 (qnt, 2H, Ar-H), 7.38 (d, *J*=2.8 Hz, 1H, Ar-H), 7.56 (d, *J*=2.8 Hz, 1H, Ar-H), 7.94 (s, 1H, NCHN); ^{13}C {^{1}H} NMR (100.10 MHz, Acetonitrile-d_3) δ ppm: 13.2 (CH_3), 19.2 (CH_2), 31.3 (CH_2), 43.7 (N-CH_2-R) 109.9, 119.3, 121.1, 122.0, 133.6 and 141.5 (Ar-C), 143.5 (NCHN).**API 4 (***N*-pentyl benzimidazole)Yield: 1.47 g (78.2%), B.P. (1 mm tube): 262-263 °C. FT-IR (liquid, ν_{max}, cm^{-1}): 3417 (C_{aliph}-$N_{benzimi}$; 3078(C-H_{arom}); 2962, 2927, 2860 (C-H_{aliph}); 1053, 1163 (C_{arom}-$N_{benzimi}$). ^{1}H NMR (400 MHz, Acetonitrile-d_3) δ ppm: 0.71 (t, *J*=3.8 Hz, 3H, CH_3), 1.41 (br.m, 4H, CH_2), 1.82 (qnt, 2H, CH_2), 4.20 (t, *J*=7.2 Hz, 2H, N-CH_2-

R), 7.09 (qnt, 2H, Ar-H), 7.38 (d, *J*=2.8 Hz, 1H, Ar-H), 7,56 (d, *J*=2.8 Hz, 1H, Ar-H), 7.94 (s. 1H, NCHN); ^{13}C {^{1}H} NMR (100.10 MHz, Acetonitrile-d_3) δ ppm: 13.2 (CH_3), 192, 21.4, 31.3 (3 × CH_2), 43.7 (N-CH_2-R), 109.9, 119.3, 121.1, 122.0, 133.6 and 11.5(Ar-C), 143.5 (NCHN).

API 5 (*N*-hexyl benzimidazole)

Yield 5.72 g (94.4%). FT-IR (liquid, ν_{max}, cm^{-1}): 3434 (C_{aliph}-$N_{benzimi}$); 3077, 3056 (C-H_{arom}): 2957, 2931, 2859 (C-H_{aliph}); 1615 (C_{arom}-C_{arom}); 1496, 1459, 1286, 1365 (C_{arom}-$N_{benzimi}$). ^{1}H NMR (500 MHz, DMSO-d_6) δ ppm: δ 0.77 (t, *J*=7.0 Hz,3H, CH_3), 1.19 (br.m, 6H,3 × CH_2) 1.72 (qnt, 2H, CH_2), 4.14 (t, *J*=7.0 Hz, 2H, CH_2-N), 7.17 (m, 2H, Ar 2 × CH), 7.47 (d, *J*=7.5 Hz, 2H, Ar 1 × CH),7.65 d, *J*=8.0 Hz, 1H, Ar 1 × CH), 8.12 (s, 1H, NCHN); ^{13}C {^{1}H} NMR (125.5 MHz, DMSO-d_6) δ ppm: 13.5 (CH_3), 21.8, 25.6, 29.2, 30.5 (4 × CH_2), 44.0 (R-CH_2-N) 109.8, 119.2, 121.0, 121.9, 133.6 (Ar-C) 143.5 (NCHN).**API 6** (*N*-benzyl benzimidazole)

Yield 83% (2.937 g). M.p 110-120 ℃ FTIR stretching vibrations (3080 – 3356 cm^{-1}), C-H stretching (2374 - 3032 cm^{-1}), -HC = N- (1450 -1500 cm^{-1}), bending vibrations of CH_2 and CH (1350 – 974 cm^{-1}). ^{1}H NMR (500 MHz, $CDCl_3$, δ ppm) 5.32 (2H, s, 1 × CH_2), 7.33 - 7.15 (6H, m, 6 × $CH_{aromatic}$), 7.15 (d, 2H, $CH_{aromatic}$, J=8 Hz) 7.81 (2H, d.d, J = 7.5 Hz, 2 × CH), 7.9 (1H, s, N-CH).^{13}C NMR (100.62 MHz, $CDCl_3$, δ ppm) 143.1 (C=N-), 129.0, 128.2, 127.0, 123.0, 122.2, 120.4, 110.0, 48.8 (CH_2 Benzyl).

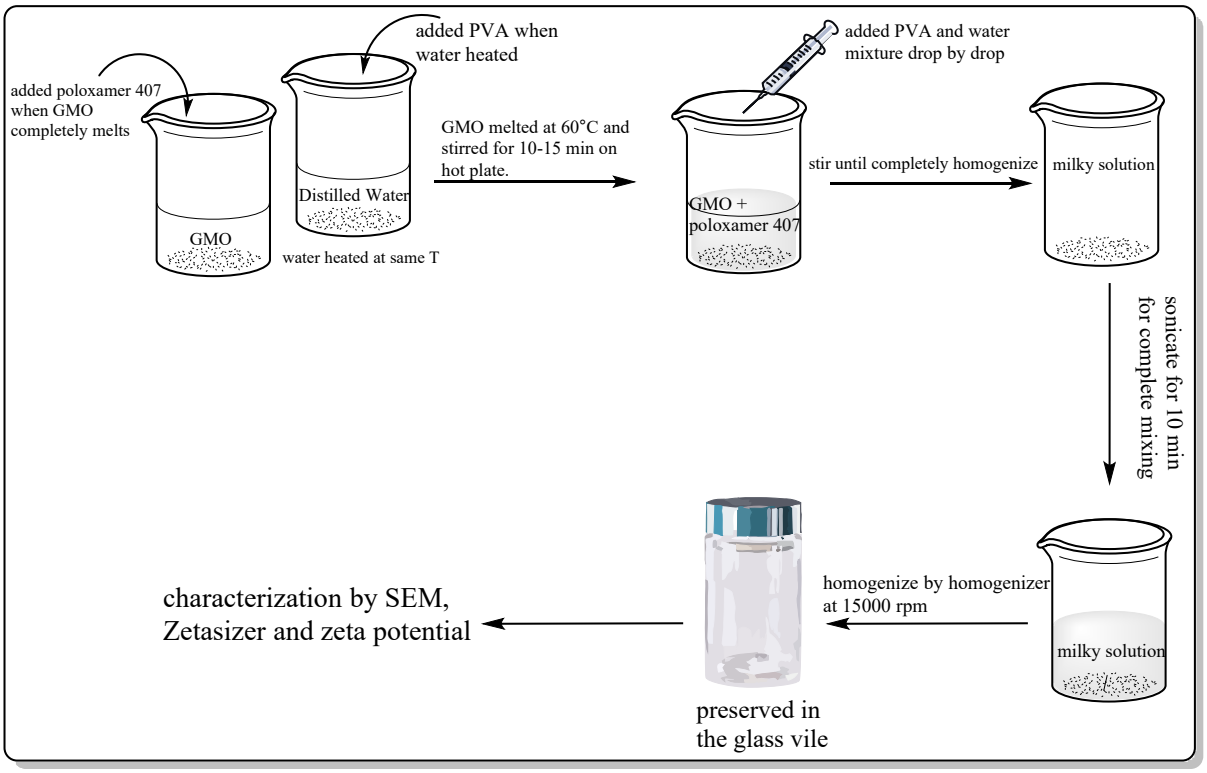


Fig. S1. Schematic diagram of the formation of Cubosomes

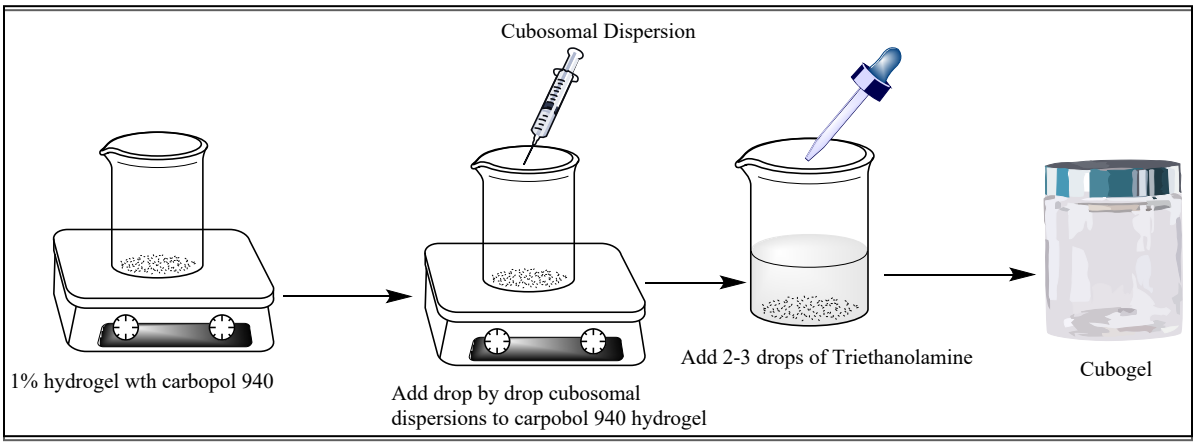
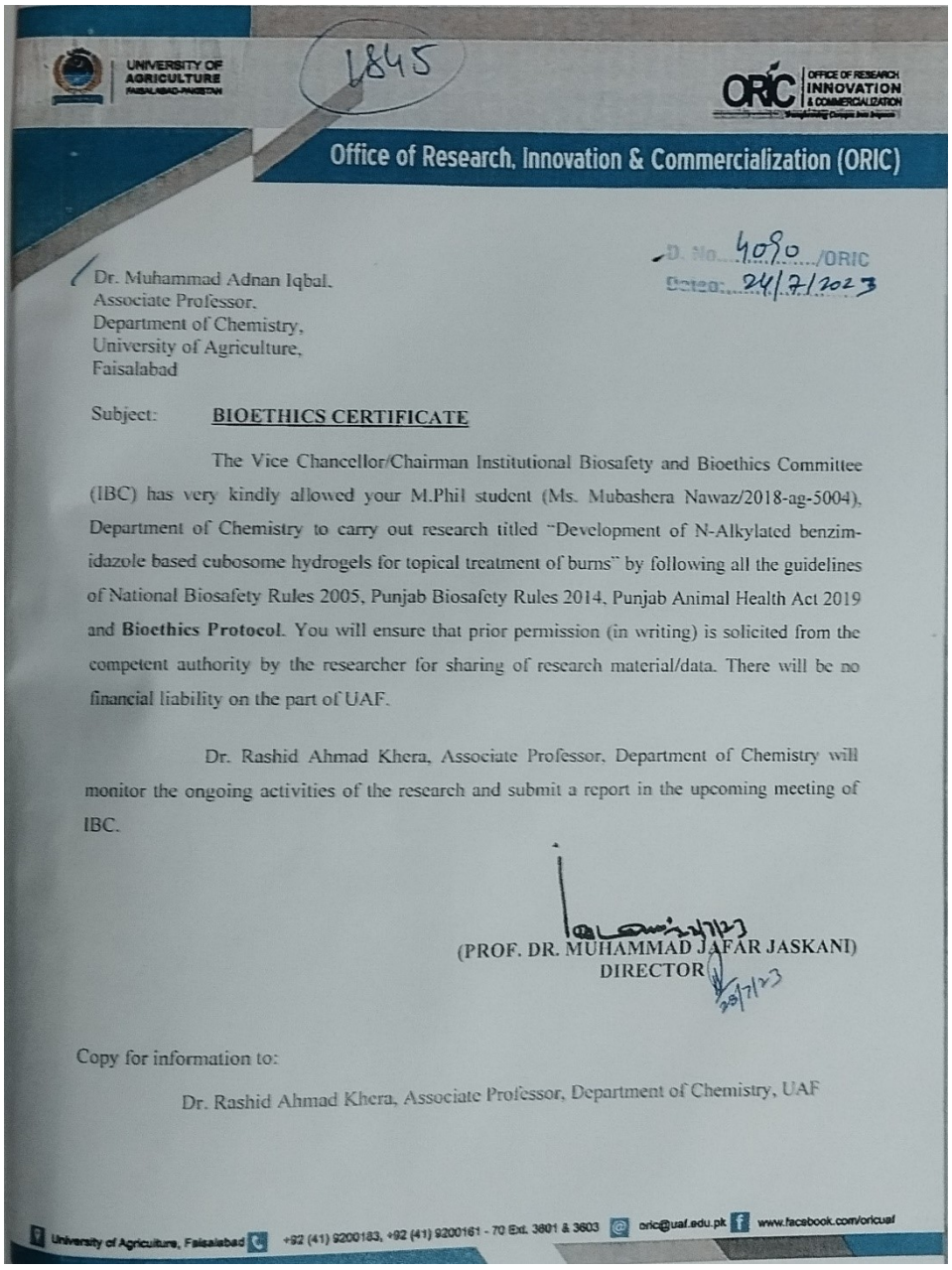


Fig. S2. Schematic diagram of the Development of cubogel



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Office of Research, Innovation & Commercialization (ORIC)

D. No. 4080 /ORIC
Dated: 24/7/2023

Dr. Muhammad Adnan Iqbal,
Associate Professor,
Department of Chemistry,
University of Agriculture,
Faisalabad

Subject: **BIOETHICS CERTIFICATE**

The Vice Chancellor/Chairman Institutional Biosafety and Bioethics Committee (IBC) has very kindly allowed your M.Phil student (Ms. Mubashera Nawaz/2018-ag-5004), Department of Chemistry to carry out research titled "Development of N-Alkylated benzimidazole based cubosome hydrogels for topical treatment of burns" by following all the guidelines of National Biosafety Rules 2005, Punjab Biosafety Rules 2014, Punjab Animal Health Act 2019 and **Bioethics Protocol**. You will ensure that prior permission (in writing) is solicited from the competent authority by the researcher for sharing of research material/data. There will be no financial liability on the part of UAF.

Dr. Rashid Ahmad Khera, Associate Professor, Department of Chemistry will monitor the ongoing activities of the research and submit a report in the upcoming meeting of IBC.

(PROF. DR. MUHAMMAD JAFAR JASKANI)
DIRECTOR
24/7/23

Copy for information to:

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Fig. S3. Bioethics certificate for animal study

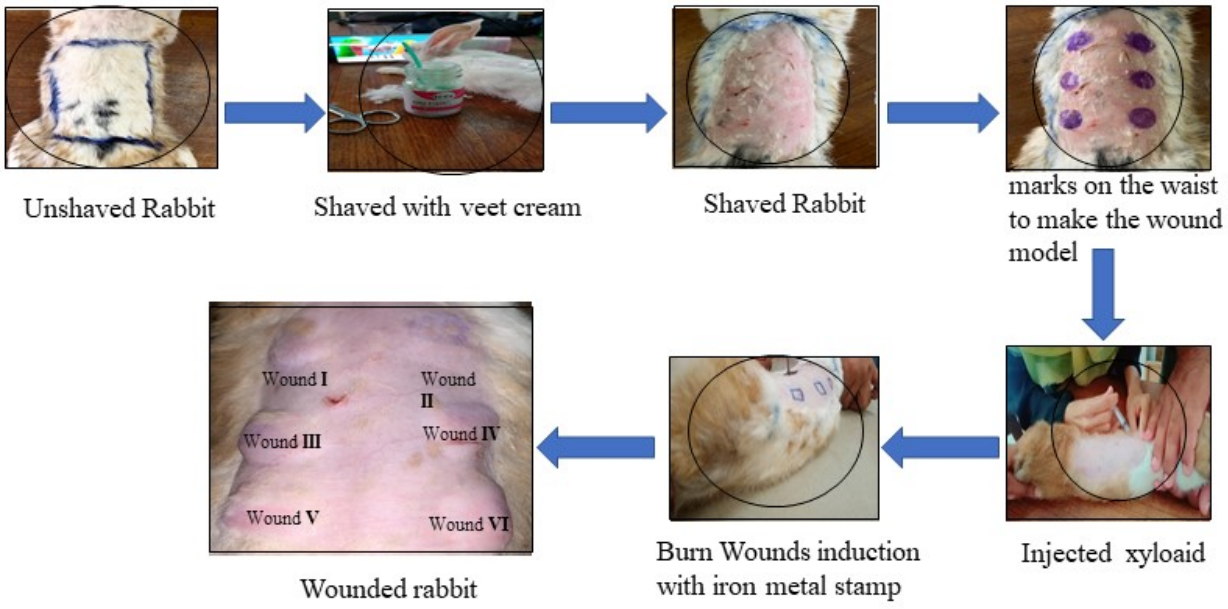

Fig. S4. The schematic diagram of burn induction method

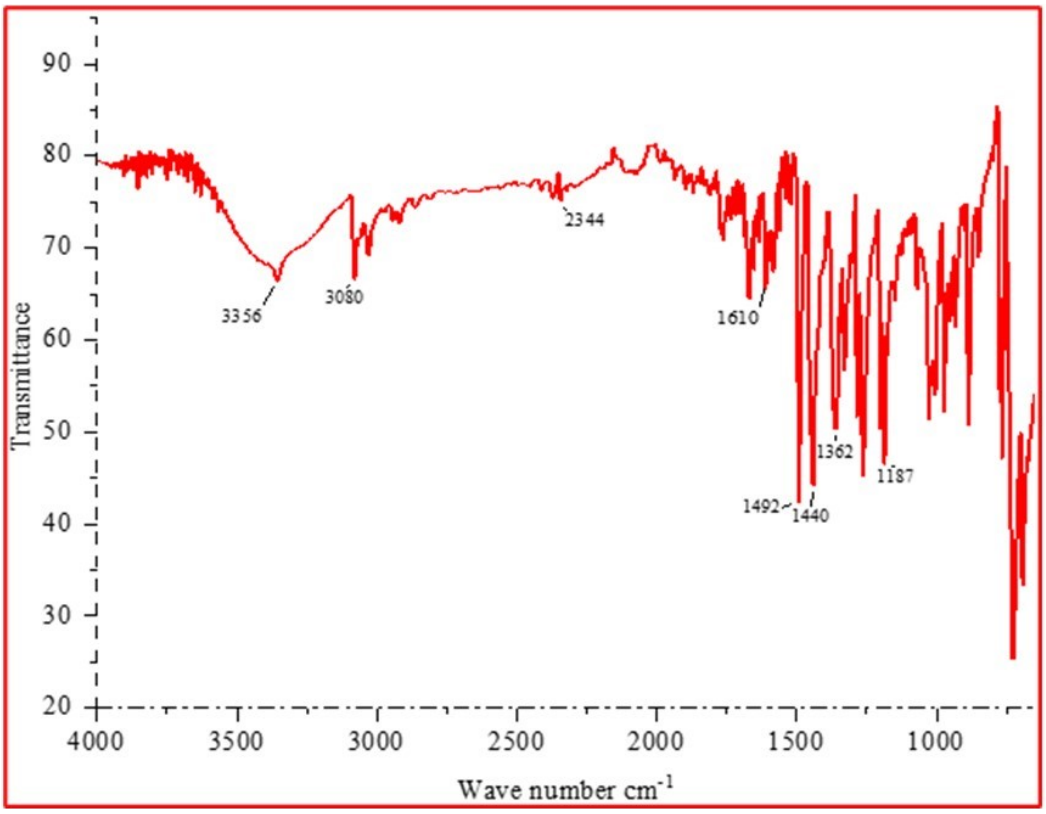

Fig. S5. FT-IR spectrum of *N*-alkylated benzimidazole **API 6** (1-benzyl-1-benzoimidazole).

Antibacterial Activity

The well-diffusion method was used to assess the antibacterial activity of loaded cubosome formulations [1]. The findings demonstrated that, in comparison to the formulations API 1, API 2, API 3, API 5, and API 6 with 2, 5, 3, 4, and 1% loading respectively, the formulation API4 (6 % loading of N-Alkylated benzimidazole) showed greater potential against the growth of *Escherichia coli* and *Staphylococcus aureus* bacteria. The inhibition zones were measured in order to assess the effectiveness of loaded formulations that had been created. Table 1, displays the zone of inhibitions, which represent the highest level of antibacterial activity against *Escherichia coli* and *Staphylococcus aureus*.

Table S1: The zone of inhibition of API (1-cyclopentyl benzimidazole) loaded formulations against *S. aureus* and *E. coli* bacterial growth.

Formulations	**Zone of inhibition against *S. aureus*, size (mm)**	**Formulations**	**Zone of inhibition against *E. coli*, size (mm)**
API 1	11±1.2	**API 1**	14±0.9
API 2	14±1.6	**API 2**	13 ±1.1
API 3	12±1.1	**API 3**	15±1.2
API4	15±1.4	**API4**	17±2.0
API5	13±2.0	**API5**	11±1.9
API6	09±1.3	**API6**	10±1.5

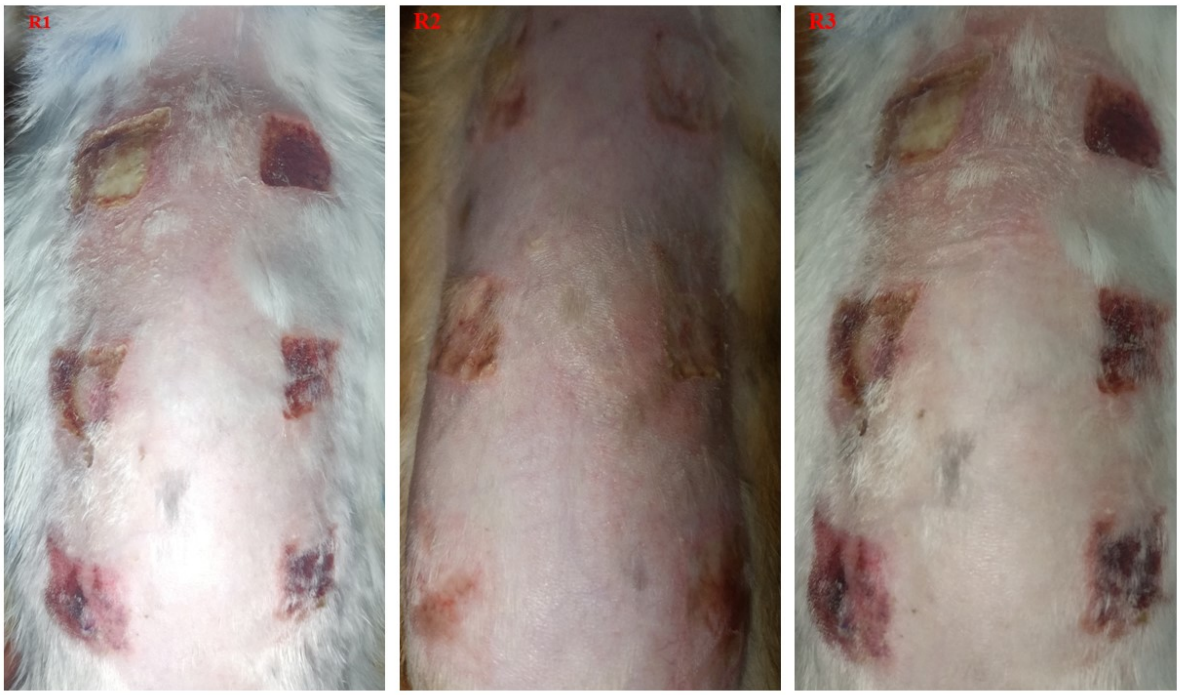

Figure S6: Three rabbits are under treatment.

Table S2: Wound contraction

Days	Wound I	Wound II	Wound III	Clotrimazole	Polyfax	Negative control
	cm^2	cm^2	cm^2	cm^2	cm^2	cm^2
1	20±1.3	14±1.6	18±1.5	17±1.2	18±1.9	7±1.7
3	31±1.5	22±0.8	26±1.7	23±1.7	25±2.1	16±1.3
5	39±1.9	36±2.3	39±1.2	36±0.9	34±1.7	22±1.9
7	46±1.2	50±1.3	52±1.2	44±1.6	43±0.9	28±0.9
9	59±2.0	61±1.7	63±0.9	58±0.9	55±1.3	32±1.5
11	65±0.6	69±0.9	72±2.3	63±1.2	65±1.5	39±1.5
14	72±1.8	78±2.1	81±1.9	70±1.5	72±2.5	45±0.9
16	80±1.2	84±2.5	89±2.5	81±2.3	83±1.9	51±1.2
21	86±1.5	94±1.2	96±2.1	88±1.7	91±1.5	59±1.7

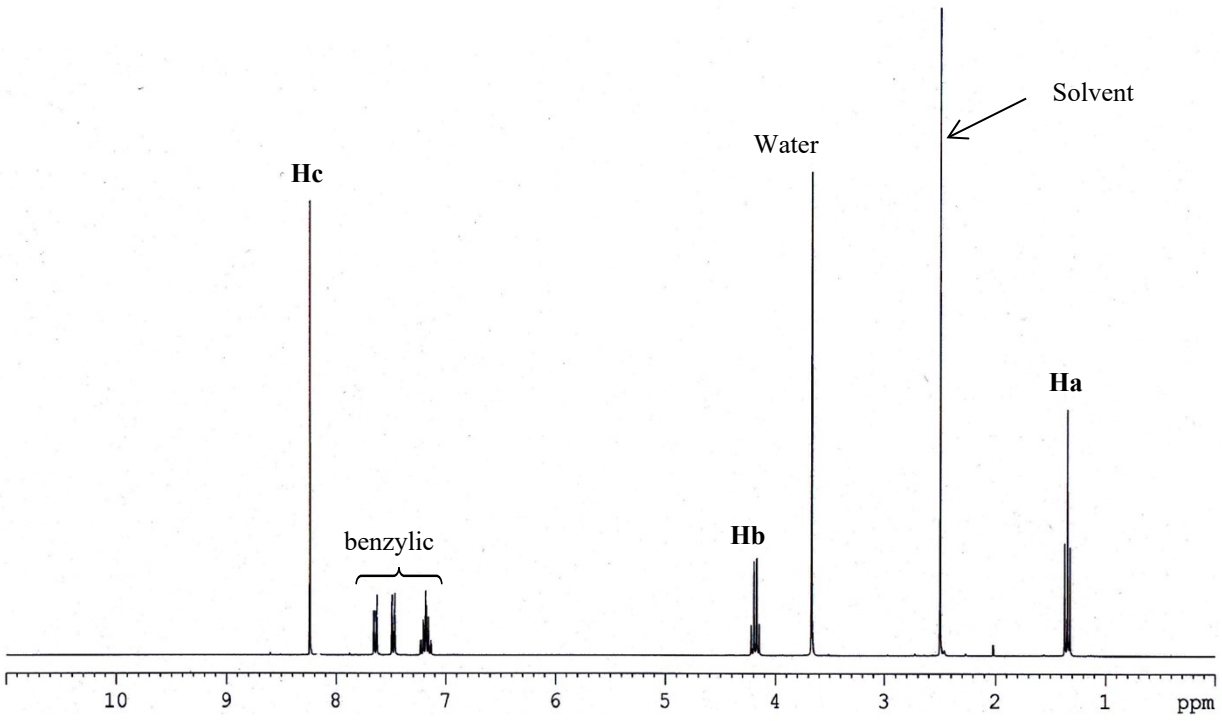

Fig. S7. API 1 (*N*-ethyl benzimidazole) [2].

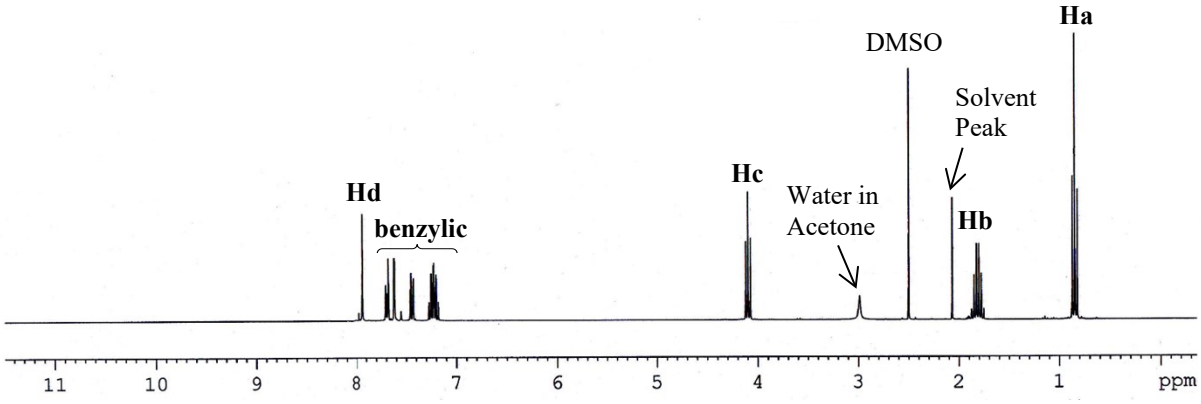

Fig. S8 API 2 (*N*-propyl benzimidazole) [2].

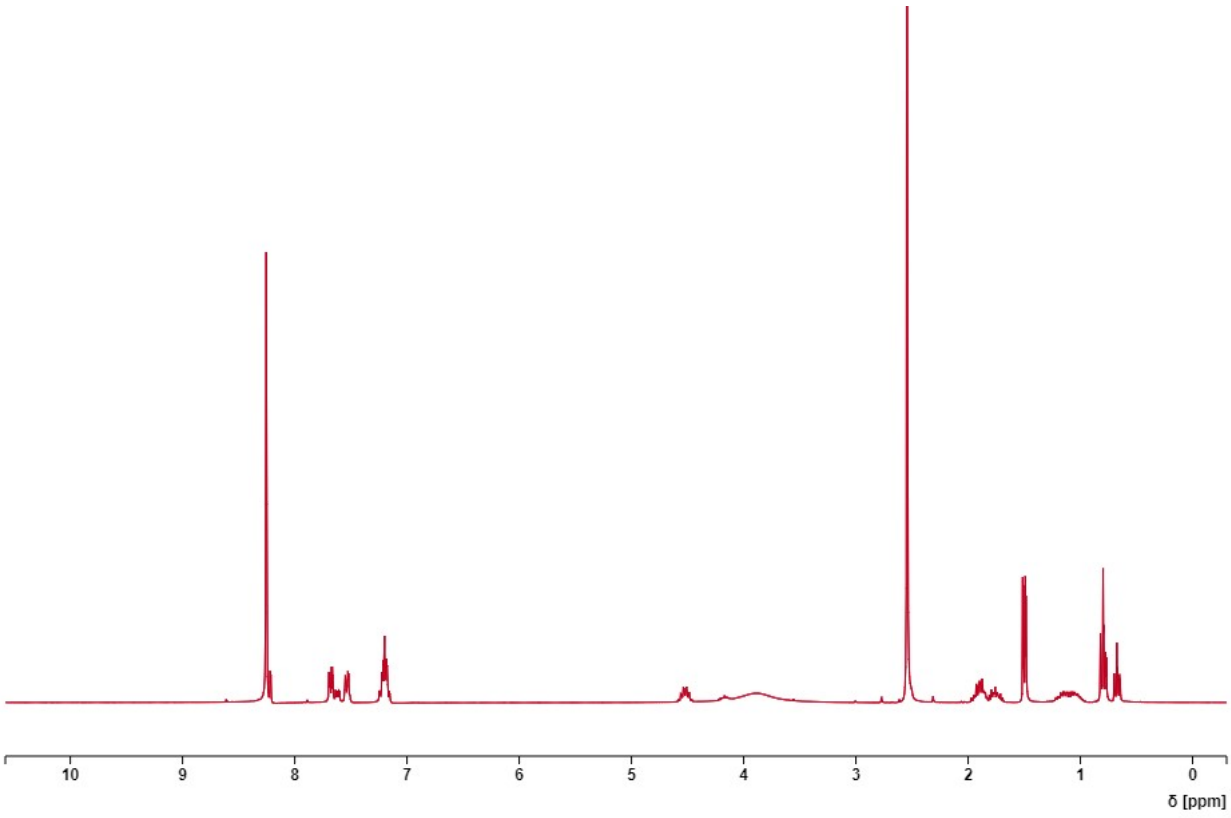

Fig. S9 API 4 (*N*-pentyl benzimidazole).

N-hexylbenzimidazole, 500 MHz, DMSO-d6

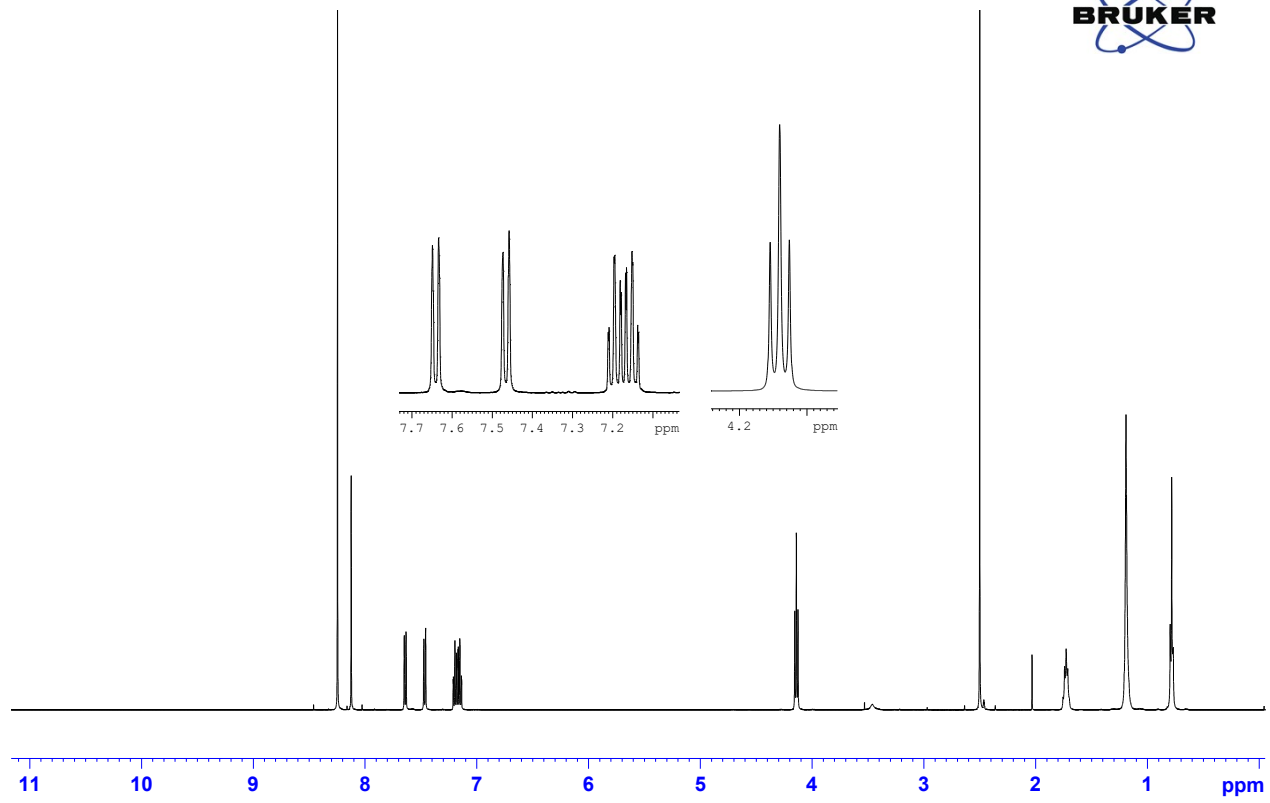

Fig. S10 API 5 (*N*-hexyl benzimidazole).

Supporting References

1. Alshawwa, S. Z.; El-Masry, T. A.; Nasr, M.; Kira, A. Y.; Alotaibi, H. F.; Sallam, A.-S.; Elekhnawy, E., Celecoxib-Loaded Cubosomal Nanoparticles as a Therapeutic Approach for Staphylococcus aureus In Vivo Infection. *Microorganisms* **2023,** *11*, 2247.
2. Haque, R. A.; Iqbal, M. A.; Khadeer Ahamed, M. B.; Majid, A. M. S. A.; Abdul Hameed, Z. A., Design, synthesis and structural studies of meta-xylyl linked bis-benzimidazolium salts: potential anticancer agents against 'human colon cancer'. *Chemistry Central Journal* **2012,** *6*, 68.