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Potential anti-amoebic effects of synthetic 1,4-benzothiazine derivatives against *Acanthamoeba castellanii*

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A B S T R A C T

A rare but lethal central nervous system disease known as granulomatous amoebic encephalitis (GAE) and potentially blinding *Acanthamoeba* keratitis are diseases caused by free-living *Acanthamoeba*. Currently, no therapeutic agent can completely eradicate or prevent GAE. Synthetic compounds are a likely source of bioactive compounds for developing new drugs. This study synthesized seventeen 1,4-benzothiazine derivatives (I –XVII) by a base-catalyzed one-pot reaction of 2-amino thiophenol with substituted bromo acetophenones. Different spectroscopic techniques, such as EI-MS, ^{1}H-, and ^{13}C NMR (only for the new compounds), were used for the structural characterization and conformation of compounds. These compounds were assessed for the first time against *Acanthamoeba castellanii.* All compounds showed anti-amoebic potential *in vitro* against *A. castellanii*, reducing its ability to encyst and excyst at 100 μM. Compounds **IX**, **X**, and **XVI** showed the most potent activities among all derivatives and significantly reduced the viability to 5.3×10^4 ($p < 0.0003$), 2×10^5 ($p < 0.006$), and 2.4×10^5 ($p < 0.002$) cells/mL, respectively. The cytotoxicity profile revealed that these molecules showed lower to moderate cytotoxicity, i.e., 36 %, 2 %, and 21 %, respectively, against human keratinocytes *in vitro*. These results indicate that 1,4-benzothiazines showed potent *in vitro* activity against trophozoites and cysts of *A. castellanii*. Hence, these 1,4-benzothiazine derivatives should be considered to develop new potential therapeutic agents against *Acanthamoeba* infections.

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1. Introduction

Acanthamoeba species are free-living, opportunistic protists isolated from a wide range of natural and artificial environments [1]. *Acanthamoeba castellanii*'s life cycle comprises two alternative stages: a growing, pathogenic trophozoite stage and a dormant, resistant cyst stage. *Acanthamoeba* species can cause rare but severe human infections such as *Acanthamoeba* keratitis (AK), a corneal infection that occurs mostly in contact lens wearers, and granulomatous amoebic encephalitis (GAE), a central nervous system (CNS) infection with a mortality rate of more than 90 % [2]. Based on ribosomal RNA gene sequence, the *Acanthamoeba* species are distributed among 23 different types of genotypes, i.e., T1 to T23, among which T4 is the most prevalent genotype [3,4]. Most AK cases are associated with T4 [5], while T2, T4, T5, T10, and T12 are mostly associated with GAE infection [5,6].

Typically, antimicrobial agents treat infections caused by *Acanthamoeba*, but most drugs are ineffective due to the double-walled cyst stage. Therefore, a combination of chlorhexidine, pentamidine, amphotericin B, etc., is recommended to treat infections [7,8]. The most effective drugs against both phenotypes of *Acanthamoeba* are biguanides, but they are very toxic for corneal cells. On the other hand, chlorhexidine can be used as a potent therapeutic agent against both forms of *Acanthamoeba* [9,10]. However, chlorhexidine is mostly combined with diamidines and shows promising results only with the early diagnosis of infection [11]. The lack of an effective therapeutic agent against *Acanthamoeba* infection worsens the disease burden [6].

Therefore, an urgent need is to develop new effective therapeutic compounds that have potential against both phenotypic forms of *Acanthamoeba*, show lower toxicity against human cells, and can cross the blood-brain barrier (BBB). For this purpose, various synthetic classes of compounds have been screened against *Acanthamoeba in vitro* [3]. Those compounds, especially synthetic heterocyclic compounds, have shown effective activity against *Acanthamoeba castellanii*.

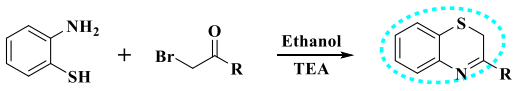
Research on heterocyclic compounds has proven to be a fruitful avenue in drug discovery against a variety of diseases [12,13]. It is desirable to discover new antimicrobial compounds that inhibit specific proteins in pathogens [14,15]. Synthetic heterocyclic compounds have been investigated against several pathogens, including free-living amoebae [3]. A few examples of these are oxadiazole, benzimidazole, chromene, triazol, etc. [15,16]. Literature shows that thiophene and its derivatives possess diverse biological effects. A series of 4-amino-thiophene and thienopyrimidine derivatives was investigated for possible anti-microbial and anticancer activities and has demonstrated potent activity against microbial and cancer cells [15]. Heterocyclic compounds also act as good ligands for different metals, and the metal heterocyclic complexes exhibit significant biological utilization. For example, Au(I) carbene complexes recently showed potent activity against cancer cells [17]. 1,4-benzothiazine is a dynamic heterocyclic class of compounds involved in versatile biological activities. These compounds have attracted the persistent interest of medicinal chemists and have become an important motif for developing new drug candidates [18]. Previous studies showed that compounds having a 1,4-benzothiazine skeleton exhibit a significant therapeutic role in central nervous system (CNS) diseases and also possess anti-malarial [19], anti-inflammatory [20,21], antimicrobial [22], atherosclerosis [23], and anticancer activities [24]. The structure-activity relationship showed that benzothiazine has sulfur and nitrogen atoms in a six-member ring, which are believed to have a critical role in bioactivities [25]. Since benzothiazines and their derivatives are still unexplored for their activities against free-living amoebae. We hypothesized that benzothiazine may exhibit potent anti-amoebic activity against both stages of *Acanthamoeba* and can inhibit the re-occurrence of *Acanthamoeba* infections (GAE and AK). This work synthesized a library of seventeen 1,4-benzothiazine derivatives and evaluated their anti-acanthamoeba potential against trophozoite and cyst stages (Scheme 1). The possible toxicity of the tested compounds was also evaluated against the human keratinocyte (HaCaT) cell line.

Considering the challenges in finding novel and efficient therapies to treat neglected diseases, these benzothiazine derivatives might serve as effective therapeutics against *Acanthamoeba castellanii* infections.

2. Materials and methods

2.1. Experimental Design

To find novel alternative therapeutic agents against *Acanthamoeba* infection, we synthesized a library of seventeen benzothiazine derivatives (**I** –**XVII**) by a base-catalyzed one-pot reaction of 2-amino thiophenol with substituted bromoacetophenones. Different spectroscopic techniques were used for the structural characterization and confirmation of compounds. The compounds were then assessed against *Acanthamoeba castellanii* to determine their anti-amoebic potential. Encystation and excystation assays were performed to examine their ability to inhibit the phenotypic alterations of *Acanthamoeba*. Finally, the cytotoxicity assay was performed to investigate the possible toxicity of compounds to host cells.



I-XVII

Scheme-1. Synthesis of 1,4-benzothiazines (**I-XVII**) and their substitution patterns.

2.2. Synthesis and structural characterization

The ^{13}C and ^{1}H- NMR data were collected using Bruker Avance Neo spectrometers (300–500 MHz), and deuterated solvents were used to solubilize the compounds. The chemical shift values (δ) were expressed in ppm, and the coupling constant (J) was measured in Hz. EI-MS spectra were recorded on MAT 312, and HREI-MS spectra were recorded on MAT 113D mass spectrometers. The pre-coated silica gel GF-254 (Merck, Germany) plates were used for thin-layer chromatography. Iodine spray and ultraviolet light (254 and 366 nm) were used for TLC monitoring. The chemicals used throughout the research were bought from Sigma Aldrich (USA), and their purity was confirmed by thin-layer chromatography. Double-distilled solvents were used throughout the studies. For measuring the melting points of all the synthetic compounds, a Stuart® SMP10 apparatus and open capillaries were used.

The general method for the synthesis of compounds I- XVII (Scheme 1).

A suspension of 2-amino thiophenol (1.0 eq.) in diethyl ether (10 mL) and a few drops of trimethylamine were added and stirred for 5 min. Next, a suspension of bromo-1-phenylethanones (1.0 eq.) in the same solvent was added gradually to the above reaction mixture and then refluxed at 75–80 °C. TLC analysis was performed to check the progress of the reaction until the product was formed and 2-bromo-1-phenylethanone disappeared. The eluent used for the TLC *n*-hexane and ethyl acetate in a ratio of (9:1). When the product formed and the finishing point of the reaction was indicated on TLC, the reaction mixture was allowed to cool down for half an hour and then decanted into cold water. The precipitate produced in ice water was strained and washed with plenty of water and then *n*-hexane. Finally, ethanol was used to crystallize the products.

2.3. Structural characterization of 1,4-benzothiazine derivatives

2.3.1. 3-Phenyl-2H-benzo[b] [1,4]thiazine (I)

Off-white amorphous solid; Yield: 70 %; M.P.: 148–149 °C; EI-MS *m/z* (% rel. abund.): 224 [M^+, 100], 225 (51), 223 (28), 226 (11), 121 (7), 193 (7).^{1}H NMR (DMSO-d_6, 400 MHz): δ_H 7.60 (m, 3H, H-8, H-2′, H-6′), 7.44 (t, $J_{4'(3',5')} = 7.2$ Hz, 1H, H-4′), 7.38 (m, 1H, H-7), 7.29 (t, $J_{3'(2',4')} = 7.7$ Hz, $J_{5'(4',6')} = 7.7$ Hz, 2H, H-3′, H-5′), 7.15 (m, 1H, H-6), 6.87 (d, $J_{5,6} = 7.0$ Hz, 1H, H-5), 4.35 (s, 2H, H-2).

2.3.2. 3-(p-Tolyl)-2H-benzo[b] [1,4]thiazine (II)

Off-white amorphous solid; Yield: 69 %; M.P.: 153–155 °C; EI-MS *m/z* (% rel. abund.): 240 [M^+, 16], 239 (47), 238 (22), 236 (14), 223 (75), 121 (11).^{1}H NMR ($CDCl_3$, 400 MHz): δ_H 7.41 (ovp, 2H, H-8, H-7), 7.26 (ovp, 3H, H-5, H-2′, H-6′), 7.19 (m, 1H, H-6), 7.02 (d, $J_{3',2'/5',6'} = 8.0$ Hz, 2H, H-3′, H-5′), 4.02 (s, 1H, H-2).

2.3.3. 3-(3-Nitrophenyl)-2H-benzo[b] [1,4]thiazine (III)

Pale yellow amorphous solid; Yield: 68 %; M.P.:176–178 °C; EI-MS *m/z* (% rel. abund.): 270 [M^+, 58], 269 (73), 223 (100), 210 (8), 191 (18), 178 (6).^{1}H NMR ($CDCl_3$, 400 MHz): δ_H 8.22 (m, 1H, H-4′), 8.17 (m, 1H, H-2′), 7.81 (d, $J_{6,5} = 8.0$ Hz, H-6′), 7.47 (ovp, 2H, H-8, H-5′), 7.39 (ovp, 2H, H-6, H-7), 7.30 (m, 1H, H-5), 4.10 (s, 2H, H-2).

2.3.4. 3-(4-Bromophenyl)-2H-benzo[b] [1,4]thiazine (IV)

Pale yellow amorphous solid; Yield: 75 %; M.P.: 159–161 °C; EI-MS *m/z* (% rel. abund.): 303 [M^+, 100], 305 [M +2, 99], 223 (28), 121 (15), 273 (13); ^{1}H NMR ($CDCl_3$, 400 MHz): δ_H 7.61 (dd, $J_{8,7} = 7.92$ Hz, $J_{8,6} = 0.92$ Hz, 1H, H-8), 7.45 (d, $J_{2',3'/6',5'} = 8.6$ Hz, 2H, H-2′, H-6′), 7.33 (ovp, 3H, H-7, H-3′, H-5′), 7.14 (m, 1H, H-6), 6.92 (dd, $J_{5,6} = 7.7$ Hz, $J_{5,7} = 1$ Hz, 1H, H-5), 4.02 (s, 2H, H-2).

2.3.5. 3-(2-Methoxyphenyl)-2H-benzo[b] [1,4]thiazine (V)

Off-white amorphous solid; Yield: 71 %; M.P.: 140–141 °C; EI-MS *m/z* (% rel. abund.): 254 [M^+, 100], 255 (34), 239 (4), 223 (5), 136 (15).^{1}H NMR ($CDCl_3$, 400 MHz): δ_H 7.99 (dd, $J_{8,7} = 7.5$ Hz, $J_{8,6} = 1.6$ Hz, 1H, H-8), 7.45 (m, 1H, H-4′), 7.32 (m, 1H, H-7), 7.08 (m, 1H, H-6), 6.95 (m, 1H, H-3′), 6.88 (t, $J_{5'(4',6')} = 7.60$ Hz, 1H, H-5′), 6.66 (dd, $J_{5,6} = 8.5$ Hz, $J_{5,7} = 2.0$ Hz, 1H, H-5), 6.55 (d, $J_{6',5'} = 6.8$ Hz, 1H, H-6′), 4.48 (s, 1H, H-2a), 4.32 (s, 1H, H,2b), 3.33 (s, 3H, OMe).

2.3.6. 3-(4-Chlorophenyl)-2H-benzo[b] [1,4]thiazine (VI)

Light orange amorphous solid; Yield: 70 %; M.P.: 156–158 °C; EI-MS *m/z* (% rel. abund.): 259 [M^+, 39], 261 [M+2, 13], 258 (100), 223 (29), 121 (4); ^{1}H NMR ($CDCl_3$, 400 MHz): δ_H 7.61 (dd, $J_{8,7} = 7.9$ Hz, $J_{8,6} = 0.9$ Hz, 1H, H-8), 7.52 (d, $J_{2',3'/6'.5'} = 8.6$ Hz, 2H, H-2′, H-6′), 7.34 (m, 1H, H-7), 7.25 (d, $J_{3',2'/5',6'} = 6.0$ Hz, 2H, H-3′, H-5′), 7.14 (m, 1H, H-6), 6.92 (dd, $J_{5,6} = 7.7$ Hz, $J_{5,7} = 1$ Hz, 1H, H-5), 4.03 (s, 1H, H-2′).

2.3.7. 3-(3,4-Dichlorophenyl)-2H-benzo[b] [1,4]thiazine (VII)

White powder; Yield: 70 %; M.P.: 179–180 °C; EI-MS *m/z* (% rel. abund.): 293 [M^+, 79], 295 [M+2, 54], 297 [M+4, 10], 292 (100), 257 (28.5), 223 (27), 121 (9); HR-EIMS Calcd for $C_{14}H_9Cl_2NS$: 292.9824, Found 292.9833; ^{1}H NMR ($CDCl_3$, 400 MHz): δ_H 7.43 (ovp, 3H, H-8, H-5′, H-2′), 7.37 (m, 1H, H-7), 7.30 (ovp, 2H, H-6, H-6′), 7.26 (dd, $J_{5,6} = 7.6$ Hz, $J_{5,7} = 1.2$ Hz, 1H, H-5), 3.96 (s, 2H, H-2); ^{13}C NMR (75 MHz, DMSO-d_6): 153.9, 142.2, 136.4, 135.3, 132.8, 130.1, 129.1, 128.3, 128.1, 128.0, 127.5, 126.4, 119.1, 33.79.

2.3.8. 3-(3-Methoxyphenyl)-2H-benzo[b] [1,4]thiazine (VIII)

White amorphous solid; Yield: 84 %; M.P.: 159–160 °C; EI-MS *m/z* (% rel. abund.): 255 [M^+, 34], 254 (100), 239 (6), 223 (10), 210 (9).^{1}H NMR (DMSO-d_6, 400 MHz): δ_H 7.59 (dd, $J_{6',4'} = 7.9$ Hz, $J_{6',2'} = 1$Hz, 1H, H-6′), 7.35 (m, 1H, H-7), 7.19 (ovp, 3H, H-8, H-2′, H-5′),

7.12 (m, 1H, H-6), 7.01 (m, 1H, H-4′), 6.85 (dd, $J_{5,6}$ = 7.7 Hz, $J_{5,7}$ = 1.1 Hz, 1H, H-5), 4.34 (s, 2H, H-2), 3.68 (s, 3H, OMe).

2.3.9. 3-(2,4-Dichlorophenyl)-2H-benzo[b] [1,4]thiazine (IX)

Off-white amorphous solid; Yield: 69 %; M.P.: 190–191 °C; EI-MS *m/z* (% rel. abund.): 293 [M^+, 6], 295 [M+2, 4], 297 [M+4, 1), 280 (100), 282 (68), 284 (16) 223 (10), 209 (8), 136 (8), 109 (21); HR-EIMS Calcd for $C_{14}H_9Cl_2NS$: 292.9824, Found 292.9833; ^{1}H NMR (DMSO-d_6, 400 MHz): δ_H 7.76 (d, $J_{3',5'/6',2'}$ = 1.9 Hz, 1H, H-3′), 7.68 (d, $J_{6',5'}$ = 8.3 Hz, 1H, H-6′), 7.56 (dd, $J_{5',6'}$ = 8.3 Hz, $J_{5',3'}$ = 2.0 Hz, 1H, H-5′), 7.49 (dd, $J_{8,7}$ = 7.1Hz, $J_{8,6}$ = 1.4 Hz, 1H, H-8), 7.46 (dd, $J_{5,6}$ = 7.4 Hz, $J_{5,7}$ = 2.1 Hz, 1H, H-5), 7.34 (ovp, 2H, H-6, H-7), 6.79 (s, 1H, H-2a), 5.69 (d, 1H, H-2b); ^{13}C NMR (125 MHz, DMSO-d_6): 154.3, 140.5, 136.7, 134.72, 132.4, 129.5, 128.6, 128.0, 127.7, 127.6, 126.1, 120.1, 64.1.

2.3.10. 3-([1,1′-Biphenyl]-4-yl)-2H-benzo[b] [1,4]thiazine (X)

Light brown amorphous solid; Yield: 74 %; 233–234 °C; EI-MS *m/z* (% rel. abund.); 301 (M^+, 92), 300 (100), 299 (19), 288 (18), 287 (34), 296 (12), 248 (21), 125 (35), 124 (79); H NMR: (DMSO-d_6, 400 MHz): δ_H 7.63 (m, 2H, H-2′, H-6′), 7.51 (d, $J_{3',2'/5',6'}$ = 8.0 Hz, 2H, H-3′, H-5′), 7.43 (t, $J_{3''(2'',4'')}$ = 7.6 Hz, $J_{5'',6''}$, $J_{5''4''}$ = 7.6 Hz, 2H, H-3″, H-5″), 7.33 (t, $J_{7(6,8)}$ = 7.2 Hz, 1H, H-7), 6.89 (t, $J_{4'',(3'',5'')}$ = 7.60 Hz, 1H, H-4″), 6.83 (ovp, 4H, H-5, H-8, H-2″, H-6″), 6.54 (t, $J_{6,(5,7)}$ = 7.2 Hz, 1H, H-6), 4.99 (s, 1H, H-2).

2.3.11. 4-(2H-Benzo[b] [1,4]thiazin-3-yl)phenol (XI)

Pale yellow amorphous solid; Yield: 86 %; M.P.: 280–281 °C; EI-MS *m/z* (% rel. abund.): 241 [M^+, 100], 240 (77), 227 (12), 209 (19), 121 (11); HR-EIMS Calcd for $C_{14}H_{11}NOS$: 241.0558, Found 241.0561; ^{1}H NMR (DMSO-d_6, 400 MHz): δ_H 10.04 (s, 1H, OH), 7.53 (dd, $J_{8,7}$ = 7.9 Hz, $J_{8,6}$ = 0.9 Hz, 1H, H-8), 7.42 (d, $J_{2',3'/6',5'}$ = 8.8 Hz, 2H, H-2′, H-6′), 7.36 (m, 1H, H-7), 7.14 (m, 1H, H-6), 6.98 (dd, $J_{5,6}$ = 7.7 Hz, $J_{5,7}$ = 1.0 Hz, 1H, H-5), 6.64 (d, $J_{3',2/5',6'}$ = 8.8 Hz, 1H, H-3′, H-5′), 4.19 (s, 2H, H-2); ^{13}C NMR (125 MHz, DMSO-d_6): 160.0, 154.7, 142.6, 129.6, 128.1, 128.0, 126.9126.8, 125.9119.5, 115.0, 30.20.

2.3.12. 3-(4-Fluorophenyl)-2H-benzo[b] [1,4]thiazine (XII)

Pale orange amorphous solid; Yield: 80 %; M.P.: 187–188 °C; EI-MS *m/z* (% rel. abund.): 243 [M^+, 69], 242 (100), 229 (6), 211 (12), 122 (10), 121 (32), 77 (12); ^{1}H NMR (DMSO-d_6, 400 MHz): δ_H 7.67 (m, 2H, H-2′, H-6′), 7.58 (dd, $J_{2',3'/6',5'}$ = 8.8 Hz, $J_{2',4'/6',4'}$ = 5.5 Hz, 2H, H-2′, H-6′), 7.38 (m, 1H, H-7), 7.17 (m, 1H, H-6), 7.12 (t, $J_{3'(2',4')}$ = 8.71 Hz, $J_{5'(4',6)'}$ = 8.7 Hz, 2H, H-3′, H-5′), 6.19 (dd, $J_{5,6}$ = 7.6 Hz, $J_{5,7}$ = 0.8 Hz, 1H, H-5), 4.37 (s, 1H, H-2).

2.3.13. 3-(Naphthalen-2-yl)-2H-benzo[b] [1,4]thiazine (XIII)

White amorphous solid; Yield: 75 %; M.P.: 288–289 °C; EI-MS *m/z* (% rel. abund.): 275 [M^+, 100], 273 (27), 261 (16), 243 (18), 241 (17), 153 (10); .^{1}H NMR (DMSO-d_6, 400 MHz): δ_H 8.26 (dd, 1H, H-3′), 7.94 (d, $J_{4,3}$ = 8.8 Hz 1H, H-4′), 7.74 (ovp, 2H, H-8, H-1′), 7.57 (ovp, 4H, H-5′, H- 6′, H-7′, H-8′), 7.47 (t, $J_{7(6,8)}$ = 7.6 Hz, 1H, H-7), 7.06 (t, $J_{6(5,7)}$ = 7.62, 1H, H-6), 6.79 (d, $J_{5,6}$ = 6.8 Hz 1H, H-5), 4.66 (s, 2H, H-2).

2.3.14. 3-(4-Methoxyphenyl)-2H-benzo[b] [1,4]thiazine (XIV)

Pale yellow amorphous solid; Yield: 70 %; M.P.: 171–172 °C; EI-MS *m/z* (% rel. abund.): 255 [M^+, 53], 254 (100), 240 (14), 223 (18), 210 (10), 121 (3); ^{1}H NMR (DMSO-d_6, 400 MHz): δ_H 7.54 (ovp, 3H, H-8, H-2′, H-5′), 7.37 (t, $J_{7(6,8)}$ = 7.8 Hz, 1H, H-7), 7.16 (t, $J_{6(7,5)}$ = 7.5 Hz, 1H, H-6), 6.95 (d, $J_{5,6}$ = 7.4 Hz, 1H, H-5), 6.82 (d, $J_{3',2'/5',6'}$ = 8.7 Hz, 2H, H-3′ H-5′), 4.26 (s, 2H, H-2), 3.82 (s, 3H, OMe).

2.3.15. 2-(2H-Benzo[b] [1,4]thiazin-3-yl)phenol (XV)

Bright yellow amorphous solid; Yield: 76 %; M.P.: 280–281 °C; EI-MS *m/z* (% rel. abund.); 241 (M^+, 100), 227 (20), 208 (96), 181 (10), 180 (39), 117 (10); H NMR: (DMSO-d_6, 400 MHz) δ_H 14.64 (s, 1H, OH), 7.92 (d, 1H, $J_{8,7}$ = 7.7 Hz, H-8), 7.41 (ovp, 3H, H-5, H-6, H-7), 7.27 (t, 1H, $J_{4'(5',3)'}$ = 7.60 Hz, H-4′), 7.20 (t, 1H, $J_{5'(4',6')}$ = 7.4 Hz, H-5′), 6.94 (m, 2H, H-6′, H-3′), 4.03 (s, 2H, H-2).

2.3.16. 2-(2H-Benzo[b] [1,4]thiazin-3-yl)-4-chlorophenol (XVI)

Bright orange crystals; Yield: 70 %; M.P.: 245–246 °C; EI-MS *m/z* (% rel. abund.): 275 [M^+, 100], 277 [M+2, 60], 244 (34), 242 (97), 214 (21), 207 (56), 179 (60), 121 (7). HR-EIMS Calcd for $C_{14}H_{11}ONClS$: 275.0164, Found 275.0172; ^{1}H NMR ($CDCl_3$, 400 MHz): δ_H 14.65 (s, 1H, OH), 7.99 (d, $J_{3',5'}$ = 2.4 Hz, 1H, H-3′), 7.43 (ovp, 3H, H-7, H-8, H-5′), 7.28 (t, $J_{6(5,7)}$ = 7.4 Hz, 1H, H-6), 7.22 (m, 1H, H-5), 6.99 (d, $J_{6',5'}$ = 8.8 Hz, 1H, H-6′), 4.03 (s, 2H, H-2); ^{13}C NMR (100 MHz, DMSO-d_6): 160.8, 160.5, 140.0, 133.2, 128.6, 127.3, 126.9, 124.4, 122.3, 119.5, 118.7, 21.69.

2.3.17. 3-(4-Nitrophenyl)-2H-benzo[b] [1,4]thiazine (XVII)

Yellow amorphous solid; Yield: 65 %; M.P.: 188–189 °C; EI-MS *m/z* (% relative abund.); 270 (M^+, 76), 269 (100), 258 (2), 240 (12), 223 (50), 121 (6); H NMR: (DMSO-d_6, 400 MHz): δ_H 7.67 (m, 2H, H-2′, H-6′), 7.58 (dd, $J_{2',3'/6',5'}$ = 8.8 Hz, $J_{2',4'/6',4'}$ = 5.56 Hz, 2H, H-2′, H-6′), 7.38 (m, 1H, H-7), 7.17 (m, 1H, H-6), 7.12 (t, $J_{3'(2',4')}$ = 8.70 Hz, $J_{5'(4'/6')}$ = 8.7 Hz, 2H, H-3′, H-5′), 6.19 (dd, $J_{5,6}$ = 7.6 Hz, $J_{5,7}$ = 0.8 Hz, 1H, H-5), 4.37 (s, 1H, H-2).

2.4. Culture and maintenance of Acanthamoeba castellanii

T4 genotype *Acanthamoeba castellanii* (ATCC 50492) isolated from an AK patient was used in the present study. *Acanthamoeba* was

subcultured in 10 mL of the growth medium, i.e., proteose peptone, yeast extract, and glucose (PYG), in a 75 cm^2 tissue culture flask and incubated for 48 h at 37 °C [26]. PBS was used to wash the cells, and new PYG media was introduced 12 h before the evaluation. After incubation, the cells were removed by putting the flasks on ice for 20 min, carefully tapping, and finally being used for further study.

2.5. Anti-amoebic evaluation

The amoebicidal assay was performed to find the effects of tested benzothiazine derivatives (**I-XVII**) on the viability of *Acanthamoeba castellanii*. The assay was performed using the previously described method [27]. Briefly, trophozoites of *A. castellanii* were detached using 10 mL of PBS and placed the flask on ice for 20 min, followed by gently tapping the flask. The suspended cells were centrifuged at 3000 rpm for 10 min. After centrifugation, the pellet was re-suspended in 1 mL of RPMI-1640. The number of trophozoites was counted using a haemocytometer. The amoebicidal effects of each compound were determined using 5×10^5 trophozoites of *Acanthamoeba castellanii*. The cells were treated with two different concentrations (50 and 100 μM) of the benzothiazine derivatives in 24-well plates. Untreated cells were used as a negative control, while chlorhexidine (100 μM) was used as a positive control. DMSO was used as a solvent control. After treatment, the cells were incubated for 24 h, maintaining the temperature at 30 °C. The vitality of trophozoites was assessed after incubation using the trypan blue exclusion test. Each well had 0.1 % trypan blue added to it, and the viability of the cells was enumerated by using a haemocytometer. The images of each well were recorded using a magnification of 200× on an inverted microscope.

Assessment of the phonotypic alteration of *Acanthamoeba castellanii* after treatment with 1,4-benzothiazines.

As previously described, the phenotypic modified assay of *A. castellanii* was performed using encystation and excystation.

2.5.1. Encystation assays

The encystation assay was carried out to assess the benzothiazine derivatives' ability to hinder the phenotypic transformation of trophozoites into cysts. The assay was carried out as previously reported [28]. Concisely, a total of 5×10^5 *Acanthamoeba castellanii* trophozoites were enumerated and subjected to 50 and 100 μM concentrations of benzothiazine derivatives in a 24-well plate containing an encystation medium (5 mM $MgCl_2$ and 8 % glucose) in PBS. In addition, 100 μM chlorhexidine was a positive control, while DMSO (less than 1 %) was used as a solvent control. The cells were then incubated for 72 h at 30 °C. After incubation, trophozoites and immature cysts were denatured in 0.25 % sodium dodecyl sulfate (SDS). Only mature double-walled cysts were counted using a haemocytometer. The images of each well were recorded after 72 h of incubation at a magnification of 200× under an inverted microscope.

2.6. Cysts preparation

The cysts of *A. castellanii* were prepared as described earlier [28]. Briefly, 5×10^5 trophozoites were inoculated on a non-nutrient agar plate. The plates were incubated at 30 °C for 14 days. The trophozoites converted into mature cysts following incubation. The cysts were scraped using 5 mL of PBS, and the suspension was centrifuged for 10 min at 10000 rpm. The supernatant was discarded, and the pellet was re-suspended in 1 mL of PBS. A haemocytometer was used to enumerate the cysts.

2.7. Excystation assays

The excystation assay assessed the effects of the benzothiazine derivatives on the transformation of *Acanthamoeba* cysts to the trophozoite stage. The assay was conducted according to the previously described procedure [28]. Concisely, 1×10^6 *Acanthamoeba castellanii* cysts were enumerated and treated with 50 and 100 μM of benzothiazine derivatives in a PYG medium in a 24-well plate. The cysts were incubated for 72 h and maintained at 30 °C. Cysts that had not been treated served as a negative control, whereas 100 μM chlorhexidine served as a positive control. Cells treated with less than 1 % DMSO were used as a solvent control. After incubation, the emerging trophozoites were only enumerated using a haemocytometer. The effects of compounds on the excystation were monitored for up to 72 h under an inverted microscope. In addition, the images of each well were recorded at a magnification of 200× after 72 h of incubation.

2.7.1. Culture and maintenance of human keratinocyte cells (HaCaT)

Human keratinocyte cells (HaCaT) were cultured in 10 mL supplemented RPMI-1640. The growth medium was supplemented with vitamins, streptomycin 100 μg/mL, penicillin 100 units/mL, fetal bovine serum 10 %, Nu-serum 10 %, pyruvate 1 mM, glutamine 2 mM, and non-essential amino acids. The cells were grown at 37 °C in a 5 % CO_2 incubator until 80–90 % confluent. The monolayer was washed and detached by adding trypsin (2 mL/flask). The cells were centrifuged at 1600×*g* for 5 min at 24 °C. The pellet was resuspended in supplemented RPMI-1640. Then, cells were seeded in 96-well plates and kept in a standard CO_2 incubator at 37 °C for 24 h. After incubation, the monolayer of cells was used for the toxicity assay.

2.7.2. Cell cytotoxicity assays

The toxicity assay assessed benzothiazine derivatives' possible toxicity to the host cells. The assay was performed as per the previous protocol [27]. The toxicity of compounds was determined against human keratinocyte (HaCaT) cells using a lactate dehydrogenase (LDH) assay kit. Briefly, a 96-well plate was loaded with cells and incubated at 37 °C for 24 h to form a monolayer. The

monolayer was then challenged with 50 and 100 μM concentrations of benzothiazines in 200 μL of RPMI-1640. The cells were kept in a standard CO_2 incubator at 37 °C for 24 h. Untreated cells in RPMI-1640 alone were used as a negative control, while Triton X-100 was treated as a positive control. After incubation, the quantity of LDH released by injured or dead cells was used to evaluate the degree of cell death. The extent of LDH was enumerated using an LDH assay kit (Invitrogen, Illinois, USA). The reading was recorded using a microplate reader at 490 nm. The formula ((Sample absorbance - negative control absorbance)/(Positive control absorbance - negative control absorbance) × 100) was used to determine the percentage of chemical toxicity.

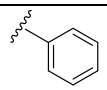
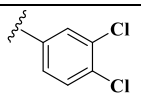
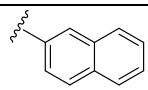
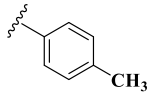
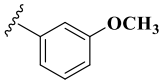
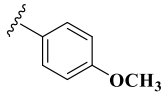
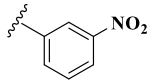
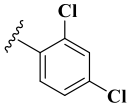
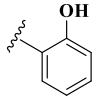
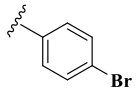
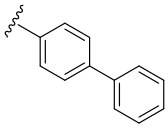
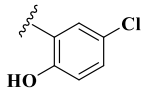
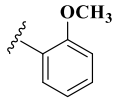
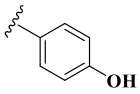
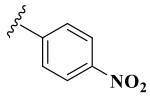
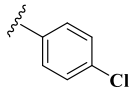
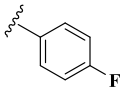
2.8. Statistical analysis

The data is presented as the mean and standard error, with $n = \geq 4$. Using a two-paired distribution, the student t-test was applied to compare the two groups' significant differences. A statistical difference of at least $^*P < 0.05$ is based on the different values.

3. Results and discussion

3.1. Chemistry

A total of seventeen derivatives of 1,4-benzothiazine were synthesized. Four out of seventeen are new derivatives (**VII**, **IX**, **XI**, and **XVI**), while the rest are known structurally [29–31]. The syntheses of the 3-phenyl-1,4-benzothiazines (**I-XVII)** involved a one-pot reaction of 2-amino thiophenol with substituted bromoacetophenones in the presence of triethylamine (TEA) as a base (Scheme-1).

Comp.	R	Comp.	R	Comp.	R
I		**VII**		**XIII**	
II		**VIII**		**XIV**	
III		**IX**		**XV**	
IV		**X**		**XVI**	
V		**XI**		**XVII**	
VI		**XII**			

In a typical reaction mechanism, the formation of 1,4-benzothiazine derivatives involves condensation of α-halo-ketones and aminobenzothiol in a base (triethyl amine) catalyzed reaction. The sulfur atom, as a soft nucleophile, attacks the α-carbon (soft electrophilic centre) of α-halo-ketones rather than the adjacent carbonyl group (hard electrophilic centre), followed by the departure of Br (an excellent leaving group). In the following steps, the amino (NH_2) group attacks the carbonyl carbon of ketone, and cyclization occurs. As a result of base-catalyzed cyclization, the 1,4-thiazine ring was formed after the water molecule's removal, forming the N═C bond (Fig. 1).

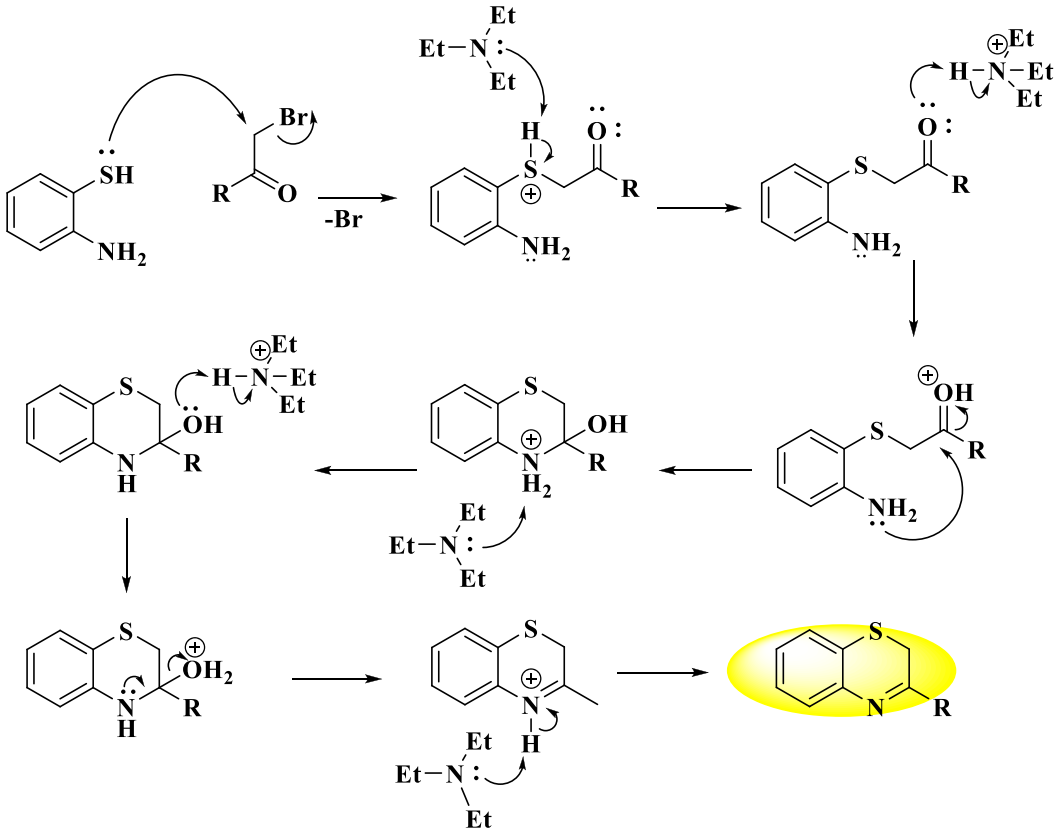


Fig. 1. A plausible mechanism for the formation of a 1,4-benzothiazine ring.

3.2. Characteristic spectral features of the representative compound XVI

3.2.1. Electron impact ionization mass spectrometry (EI-MS)

The EI-MS spectrum of the representative compound **XVI** displayed a $[M]^+$ peak (the molecular ion peak) at *m/z* 275 and a [M+2] peak corresponding to chlorine isotopes at *m/z* 277. The parent molecule yielded a fragment at *m/z* 258 due to the loss of hydroxyl radical. The parent molecule's fragmentation also generated a fragment at *m/z* 240 owing to the neutral loss of a chloro group.

^{1}H NMR Spectroscopy.

The ^{1}H NMR spectrum of compound **XVI** was recorded in DMSO-d_6 using a Bruker Avance Neo 400 MHz spectrometer. Compound **XVI** has 10 protons; OH appeared as the most downfield proton at δ_H 14.65 ppm. Among the 3 aryl protons, the H-3′ resonated as a doublet at δ_H 7.99 with a *meta* coupling with H-5 ($J = 2.0$ Hz). At δ_H 7.43, the H-5′ of the aryl ring, H-7, and H-8 of the benzo ring of benzothiazine appeared as an overlapped signal. H-6, H-7, and H-5 gave a triplet at δ_H 7.28 with a coupling value of $J = 7.4$ Hz. The H-5 of the benzo ring appeared at δ_H 7.22 with the overlapped signal. H-6′ at δ_H 6.99 ppm appeared as a doublet, exhibiting *ortho* coupling with proton 5, giving a *J* value of 8.84 Hz. The most upfield signal was for CH_2 of the thiazine ring, which appeared as a singlet for 2 protons at δ_H 4.37 (Fig. 2).

3.3. Anti-amoebic evaluation

All 1,4-benzothiazine derivatives (**I-XVII**) were evaluated for the first time against *A. castellanii*. In addition, encystation and excystation inhibition assays were also performed. All derivatives revealed anti-amoebic potential against *A. castellanii and* inhibited the encystation and excystation of *A. castellanii*. The details are given in the following section.

3.4. Benzothiazine derivatives significantly decreased the viability of A. castellanii trophozoites

The anti-amoebic assessment examined the potential of 1,4-benzothiazine derivatives (**I-XVII**) against *Acanthamoeba castellanii* belonging to the T4 genotype. The results shown here were obtained after 24 h of incubation of *Acanthamoeba* trophozoites with 50 and 100 μM concentrations of compounds. The assay results exhibited that all compounds showed significant ($p < 0.05$, *t*-test, two-tail distribution) activity against the viability of trophozoites. The histograms revealed that the number of cells was maintained in

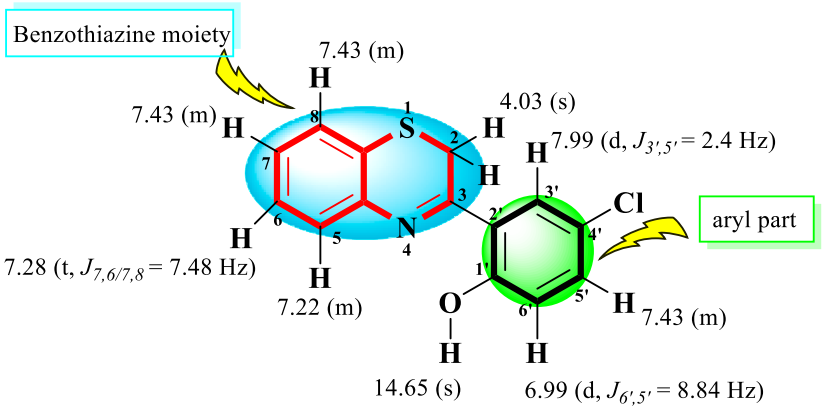


Fig. 2. ^{1}H NMR chemical shifts of representative compound XVI.

negative and solvent control in RPMI-1640. However, compared to solvent control (DMSO), there is considerable activity in a dose-dependent manner for all the tested compounds. The most potent anti-amoebic activity was recorded for compound **IX**, followed by **XVI** and **X**, reducing the viability of trophozoites to 5.37 × 10^4, 2.00 × 10^5, and 2.48 × 10^5, respectively (Fig. 3A). The illustrative images of the 24-well plate are shown in Fig. 3B.

The precise mechanism of action of these compounds against *A. castellanii* is unknown. However, 1,4-benzothiazine derivatives are known to induce apoptosis in mouse thymocytes *in vitro* through a complex signaling pathway [32]. It includes activation of caspase-3, cytochrome-c release, loss of mitochondrial membrane potential, etc. [33]. Thus, the mode of action of these compounds against *Acanthamoeba* should be identified in future studies.

3.5. Benzothiazine derivatives inhibited the encystation and excystation of A. castellanii

Benzothiazine compounds were further assessed to examine their ability to hinder phenotypic alterations. The results displayed here are obtained after 72 h of incubation of *Acanthamoeba* (trophozoites and cysts) with 50 and 100 μM concentrations of compounds.

3.6. Benzothiazine significantly inhibited encystation

The encystation assay was performed to assess the ability of compounds (**I** –**XVII**) to stop the transformation of trophozoites to a dormant and resistant cyst stage. All compounds inhibited the transformation dose-dependently. However, the analysis revealed that only five compounds significantly ($p < 0.05$, *t*-test, two-tail distribution) inhibited encystation at higher tested concentrations, i.e., 100 μM. Compared to solvent control, the most potent activity was recorded for three compounds, IX, XVI, and X. As shown in Fig. 4A, it is important to note that the number of cysts decreased to 5.62 10^4, 9.87 10^4, and 1.15 10^4 with these compounds respectively. The representative images of the 24-well plate are shown in Fig. 4B.

3.6.1. Benzothiazine significantly inhibited excystation

The excystation assay was performed to determine whether benzothiazines (**I-XVII**) can inhibit the revival of pathogenic trophozoites from a dormant cyst stage. The histogram showed that all compounds inhibited the transformation dose-dependently. However, the statistical analysis revealed that most of the compounds significantly ($p < 0.05$, *t*-test, two-tail distribution) inhibited the phenotypic conversion at higher concentrations, i.e., 100 μM. The most effective compound was found to be **IX**, followed by **XI** and **XVI**. However, at 100 μM concentration, compound **XI** exhibited results equivalent to chlorhexidine (positive control) (Fig. 5A). These compounds inhibited the number of emerging trophozoites to 1.87 × 10^4, 1.15 × 10^4, 7.37 × 10^4, and 1.83 × 10^4, respectively. The images of the 24-well plate are shown in Fig. 5B.

Acanthamoeba exists in two stages: trophozoites and cysts. The trophozoites are converted into a dormant and resistant cyst stage under harsh conditions (in the presence of therapeutic agents, lack of nutrition, extreme pH, etc.). However, when favourable conditions, the cyst can convert back to the pathogenic trophozoite stage [8]. Most used drugs exhibit activity against the trophozoites stage of *Acanthamoeba* only; hence, the chances of recurrence of infections due to the cyst are high [3]. The present study identified that benzothiazine derivatives significantly inhibited the phenotypic alteration and, therefore, should be considered for developing novel alternative therapeutic agents.

3.6.2. Most benzothiazine derivatives showed low cytotoxicity against normal human cell lines

The cytotoxicity assay was conducted to assess the host cells' toxicity. Benzothiazine derivatives were tested at 50 and 100 μM concentrations against the human keratinocyte (HaCaT) cell line using the LDH assay kit. The histogram revealed that most compounds showed minimum toxicity (i.e., less than 10 %), while some showed moderate toxicity, i.e., less than 30 %, against human

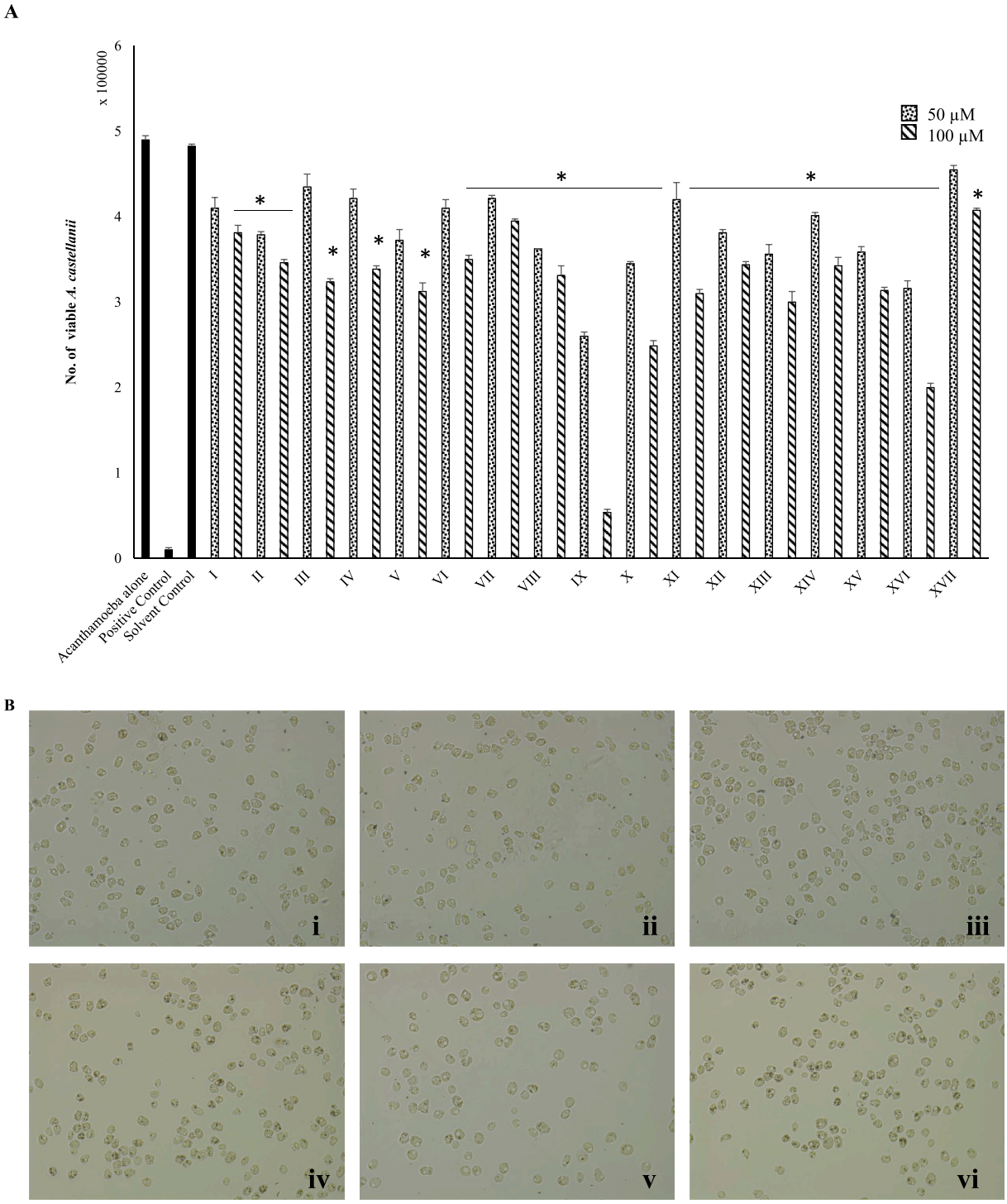


Fig. 3. Anti-amoebic assay. (A) Number of viable *A. castellanii* upon treatment with 1,4-benzothiazine derivatives. (B) Representative images of the 24-well plate were recorded using 200X on an inverted microscope.

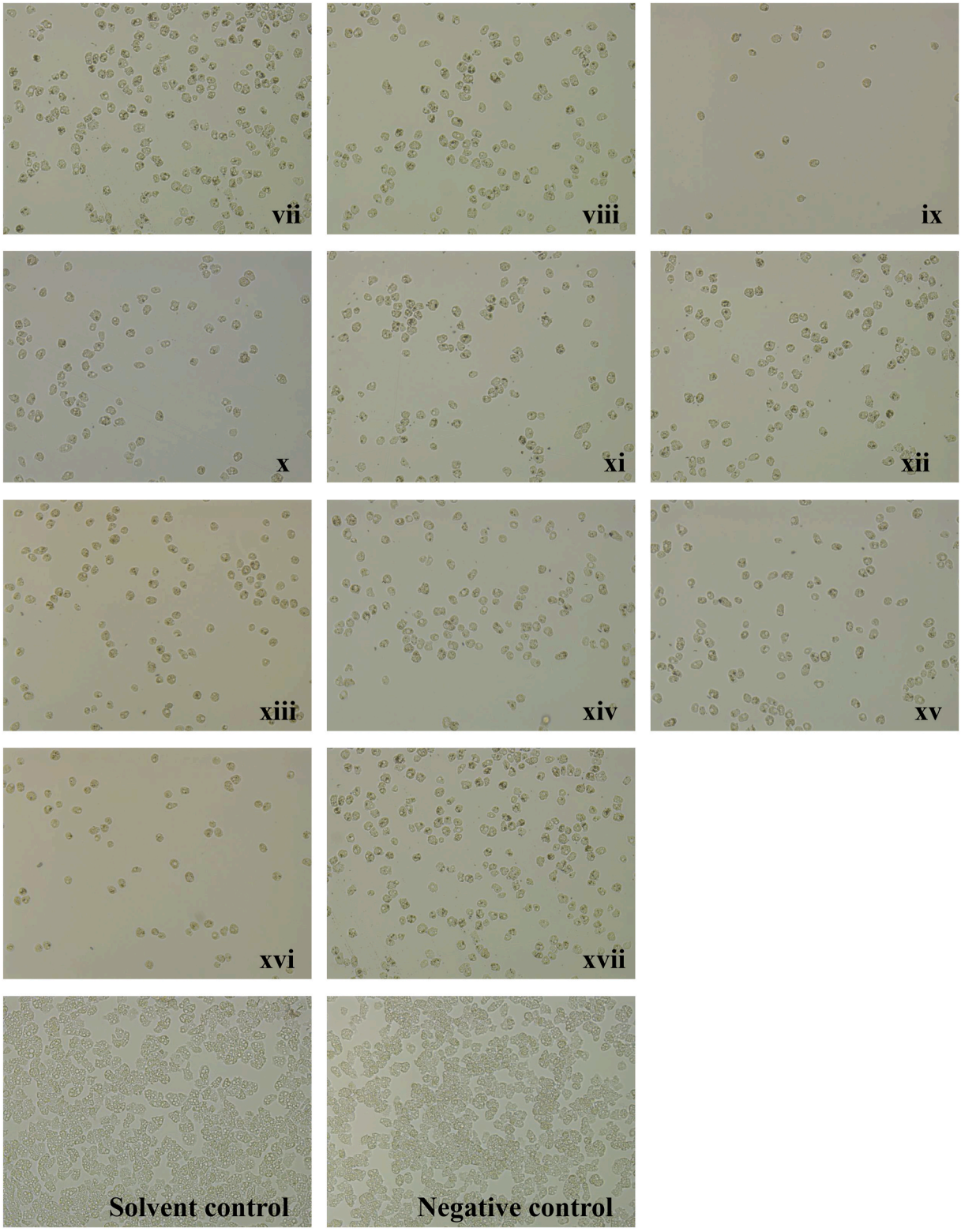


Fig. 3. (*continued*).

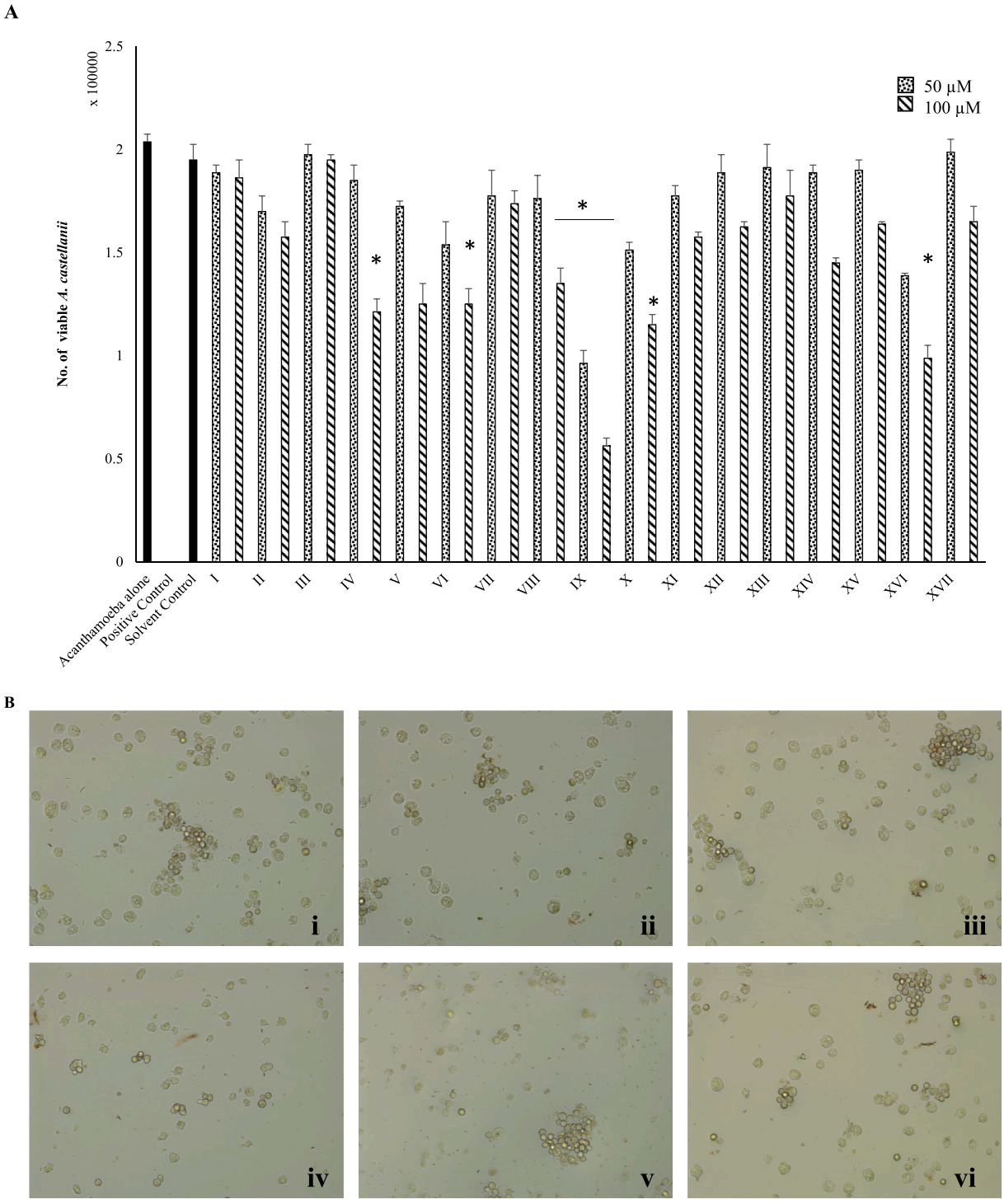


Fig. 4. Encystation assay. (A) Number of viable *A. castellanii* cysts upon treatment with 1,4-benzothiazine derivatives. (B) Representative images of the 24-well plate were recorded using 200X on an inverted microscope.

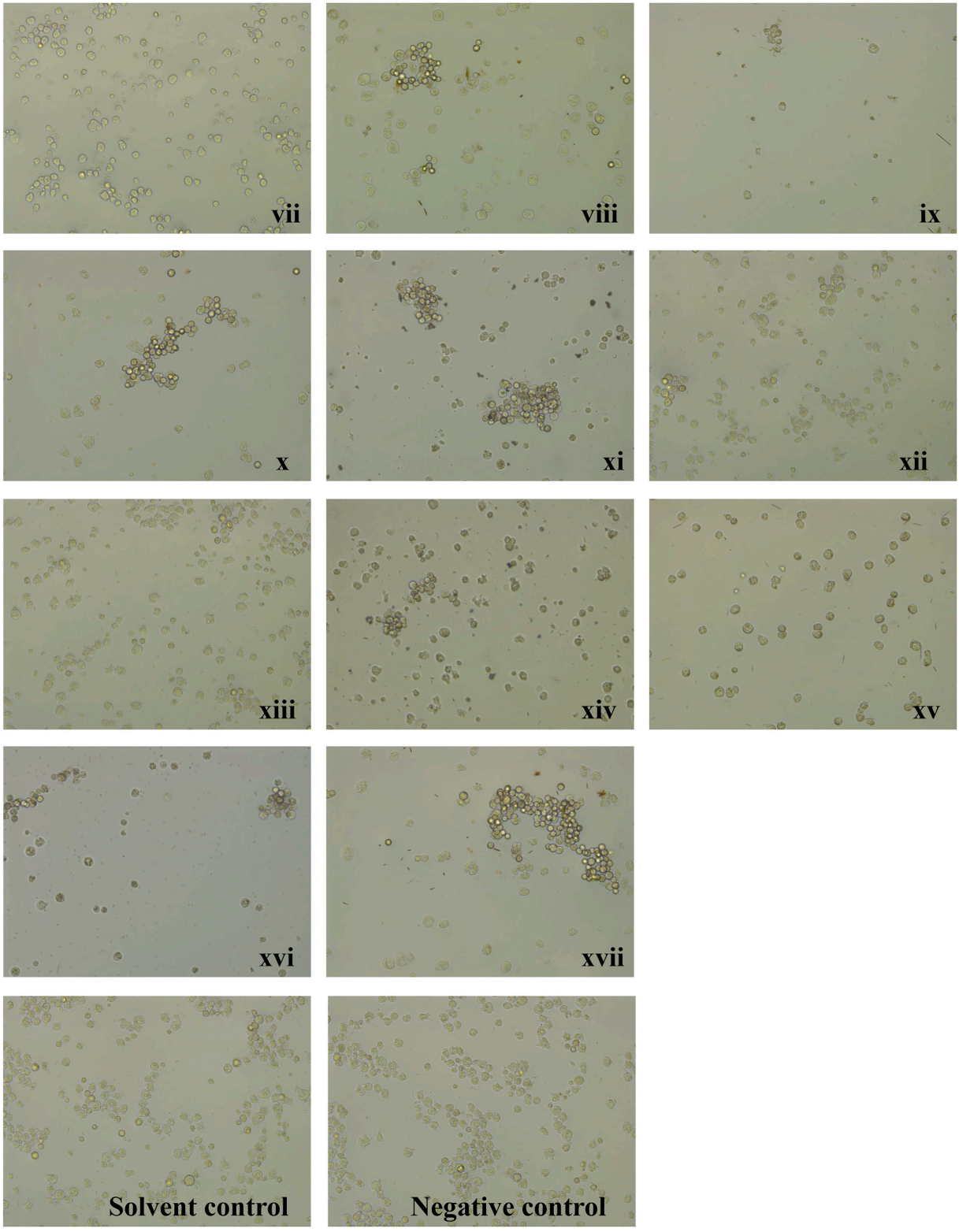


Fig. 4. (*continued*).

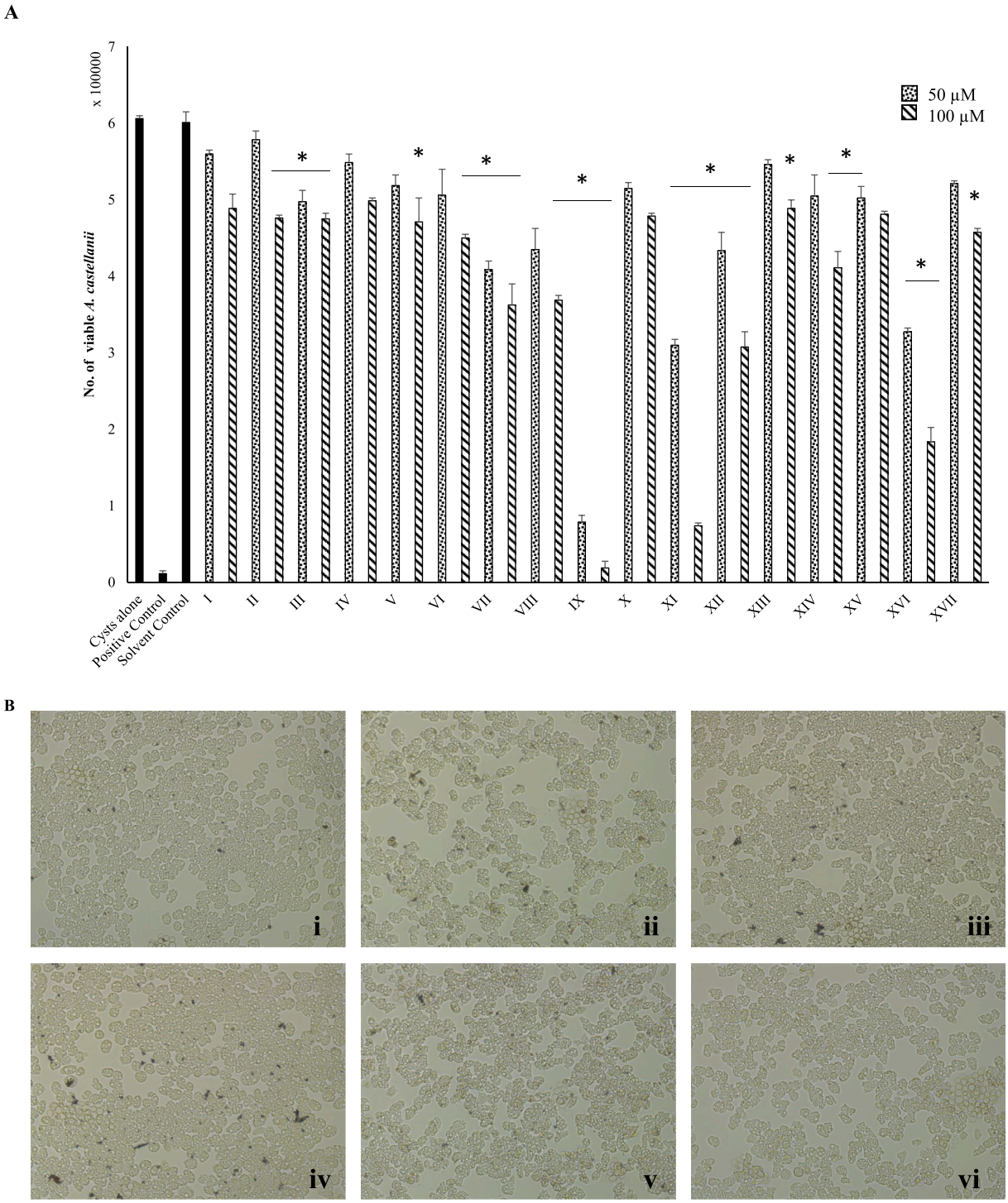


Fig. 5. Excystation assay. (A) Number of viable *A. castellanii* trophozoites upon treatment with 1,4-benzothiazine derivatives. (B) Representative images of the 24-well plate were recorded using 200X on an inverted microscope.

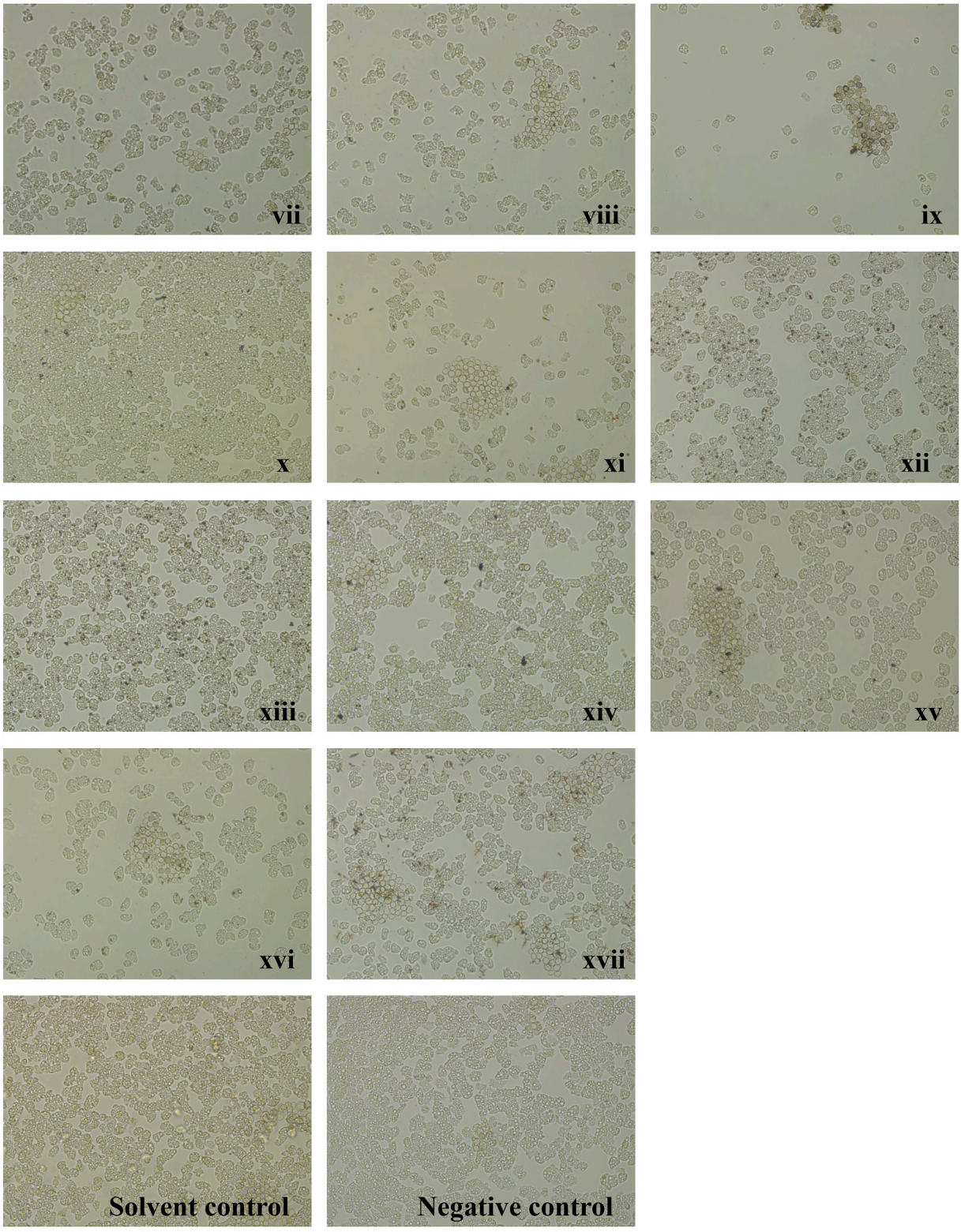


Fig. 5. (*continued*).

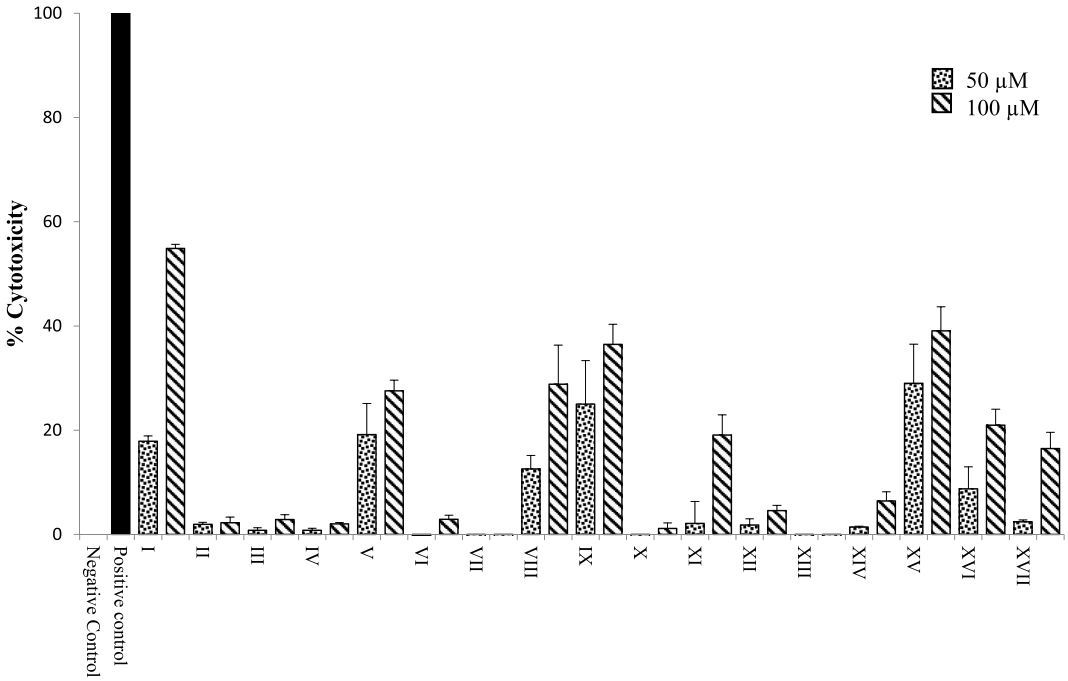


Fig. 6. Percent cytotoxicity, determined upon treatment with 1,4-benzothiazine derivatives.

keratinocyte cells. However, all compounds showed toxicity to host cells dose-dependently. Fig. 6 showed that HaCaT cells showed maximum sensitivity against compounds **I** (55 %), **IX** (36 %), and **XV** (39 %) while showing minimum sensitivity to compound **X** (2 %) at the highest tested concentration, i.e., 100 µM (Fig. 6).

The present study found that most of these compounds showed low or moderate toxicity against the HaCaT cell line, which is promising. Thus, these could be considered safe for developing new drugs against *Acanthamoeba* infection. However, HaCaT cells showed sensitivity to compound **IX** (36 %) and should be used to develop surface disinfectant. Owing to the anti-amoebic properties of these benzothiazine derivatives presented in this work, we aimed to synthesize more structure variants of 1,4-benzothiazine for further evaluation against different Acanthamoeba species to establish detailed structure-activity relationships for lead optimization. Furthermore, 1,4-benzothiaznines should be evaluated for their interactions with key *Acanthamoeba* enzymes *via in silico* studies, and gene expression analysis should be carried out to understand their mechanism of anti-amoebic activities.

4. Conclusion

A library of seventeen (17) synthetic benzothiazine derivatives showed amoebicidal potential *in vitro* against *A. castellanii,* especially compounds **IX**, **X**, and **XVI**. Moreover, these compounds also prevented the excystation and encystation of *A. castellanii*. The cytotoxicity assessment against HaCaT cells showed that compounds **X** and **XVI** also showed minimum toxicity, which can be studied further for a mode of action and *in vivo* studies. Based on these findings, it is suggested that compounds **X** and **XVI** should be tested further as potential therapeutic agents for the treatment of *Acanthamoeba* infection, while compound **IX** can be employed as a disinfectant for household surfaces and contact lenses.

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Ethical approval

Not required.

Data availability statement

Data will be provided upon request on case-to-case basis.

CRediT authorship contribution statement

Alishba: Data curation, Investigation, Writing - original draft. **Usman Ahmed:** Data curation, Formal analysis, Methodology, Writing - original draft. **Muhammad Taha:** Investigation, Methodology, Resources. **Naveed Ahmed Khan:** Formal analysis, Supervision, Validation, Writing - review & editing. **Uzma Salar:** Formal analysis, Methodology, Visualization, Writing - review & editing. **Khalid Mohammed Khan:** Conceptualization, Resources, Supervision, Validation, Writing - review & editing. **Ayaz Anwar:** Conceptualization, Formal analysis, Funding acquisition, Supervision, Validation, Writing - original draft, Writing - review & editing. **Ruqaiyyah Siddiqui:** Conceptualization, Formal analysis, Funding acquisition, Resources, Writing - review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e23258.

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