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Synthesis, Biological Activity of 2-phenylindolizine Acetamide Derivatives as Potential Antibacterial Agents

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ABSTRACT

The discovery of camptothecin and its analogs was the major breakthrough through which ''indolizine moiety'' came into limelight. A novel compounds, 2-phenylindolizine acetamide derivatives (8a-8k) has been synthesized by the reaction of 2-oxo-2-(2-phenylindolizin-3-yl)acetyl chloride (6) with different aromatic compounds (7a-7k) in presence of triethylamine in Dichloromethane (DCM). The synthesis and screened for their antimicrobial activities of novel class of 2-phenylindolizin acetamide scaffolds are described by variation in therapeutic effects of parent molecule. Result revealed that the target compounds 8b, 8a and 8f exhibited a remarkable increase of antimicrobial activity against than reference compound ampicillin, fluconazole against in three medically relevant organisms like Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa is observed. Further, 8j and 8d showed better activity against antifungal strains Candida albicans, Aspergillus flavus and Aspergillus fumigates.

Keywords: Indolizines, Antimicrobial activity

INTRODUCTION

Strategic introduction of functional substituents at various positions of core chemical skeletons at will is very important in medicinal chemistry with respect to drug discovery. For instance, as illustrated in Figure 1, several indolizines were reported to exhibit different biological activities with therapeutic potential depending on the substitution patterns of the core structure [1-3]. Therefore, development of new synthetic methods to install diverse functional groups at suitable positions around an indolizines [4,5] core should further extend versatility of this scaffold in many different medicinal areas. Indolizines are aromatic organic compounds containing condensed five and six-membered rings with bridging nitrogen (isomer of indole) [6]. Heterocycles, possessing indolizine core have also found numerous biological and pharmacological activities, such as anti-inflammatory [7,8], antiviral [9], aromatase inhibitory [10], analgesic [11], antitumor activities [12,13].

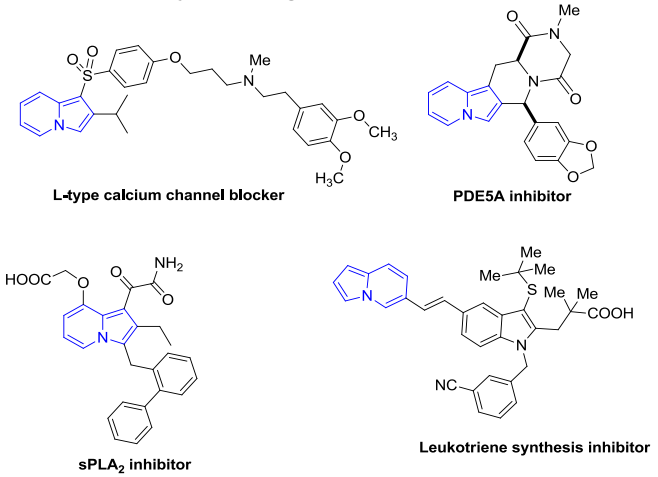


Figure 1: Several indolizines were reported to exhibit different biological activities

Most reviews on indolizines describe the chemistry and synthesis of indolizines derivatives. In a recent review by Singh and Mmatli, authors focused mainly on the progress in synthesis, and a few biological activities of indolizine derivatives from 2000-2010 were mentioned [14]. In a recent review by Vemula et al. [15], few activities of indolizine derivatives were discussed. However, these reviews lacked a detailed biological activity analysis of indolizine derivatives. Considering the importance of indolizine nucleus and the lack of any significant review on its biological activities, this review highlights the biological activity of representative indolizine derivatives, important inherent observations of the leading studies, mechanism of action, and current and future prospects related to indolizine derivatives. As part of our research interest on nitrogen-fused bicycles, we have recently reported mild and facile syntheses of indolizines and indolizinones, employing a strategy where activation of alkene allows for subsequent intermolecular ring closure by nucleophilic attack on 2nd position of indolizine ring is a highly efficient manner.

EXPERIMENTAL SECTION

Media and chemicals

Nutrient broth, nutrient agar and 5 mm diameter antibiotic assay were obtained from Hi-Media Laboratories Limited, India. Barium chloride dehydrate GR, concentrated sulphuric acid GR, Dimethyl Sulphoxide (DMSO) GR, Sodium chloride AR and Potassium dichromate were obtained from Ranbaxy Laboratories Ltd, Chemical Division, India. The standard bacterial and fungal strains were procured from National Centre from Cell Science (NCCS), Pune, India. The bacterial included two Gram-positive bacterial isolates *Staphylococcus aureus* (NCCS 2079) and *Bacillus cereus* NCCS 2106 and two Gram-negative bacterial isolates *Escherichia coli* (NCCS 2065) and *Pseudomonas aeruginosa* (NCCS 2200). The fungicidal organisms included were *Aspergillus niger* (NCCS 1196) and *Candida albicans* (NCCS 3471). The bacteria were grown and maintained on nutrient agar (Hi-Media, Mumbai) and were subculture when needed.

Glass wares and apparatus

Glass petridish, glass tubes, beakers, erlenmeyer flasks, bacterial loop and measuring cylinder were used. All the glass wares were of borosilicate grade. Digital electronics balance (Shankar Scientific supplies, India), Yorco Horizontal Laminar air flow bench (Yorco sales Pvt. Ltd, New Delhi, India), Ausco incubator, Zone reader (Cintex industrial Corporation, India), hot air oven, autoclave and UV/Visible spectrophotometer (Shimadzu corporation, Japan).

Laboratory chemicals were provided by Rankem India Ltd. and Ficher Scientific Ltd. Melting points were determined by the open tube capillary method. Reactions were monitored by using Thin Layer Chromatography (TLC) in the solvent system Ethyl acetate–Hexane. The spots were observed by exposure to iodine Vapors or by UV light or P-anisaldehyde stain solution. The purity of the compounds was determined by High-Performance Liquid Chromatography (HPLC). The IR spectra were received by Perkine Elmer 1720 FT-IR spectrometer (KBr pellets). The ^{1}H-NMR and ^{13}C-NMR spectra were obtained by Bruker Advance II 300 spectrometer using Tetramethylsilane (TMS) because the internal standard in Cadmium Chloride ($CDCl_3$). Commercial chemicals were distilled from CaH_2 and degassed (freeze and thaw) three times prior to use, THF, ethyl acetate, hexanes distilled from Na/benzophenone.

General procedure for synthesis of target compounds (8a-8k)

2-(2-methylpyridin-1(2H)-yl)-1-phenylethanone (3): To a solution of 2-bromo-1-phenylethanone (30.0 g, 0.241 mol) in CH_2Cl_2 (300 ml) was cooled to 0°C and was added 2-methylpyridine (6 ml), Et_3N and $(Boc)_2O$ (63 ml, 0.29 mol) and stirred for 6 h to give 2-(2-methylpyridin-1(2*H*)-µl)-1-phenylethanone. Reaction completion was observed by TLC. Water was added to the reaction mixture and separated the organic layer (2 × 300 ml). The combined organic layer was washed with brine solution and was dried over anhydrous Na_2SO_4. Organic layer was concentrated under reduced pressure. The crude material was purified by column chromatograph, yield 50.0 g, 93%.

2-oxo-2-(2-phenylindolizin-3-yl)acetyl chloride (6): Compound 2-phenylindolizine (4) (5 g, 0.18 mol), oxalylchloride (5) (6 ml, 0.15 mol) reaction in the presence of THF (1 ml, 0.04 mol) and toluene as a solvent to give desired product 6 in good yield.

Synthesis of target compounds (8a-8k)

To a solution of 2-oxo-2-(2-phenylindolizin-3-yl)acetyl chloride (6) (3.0 g, 0.18 mol) in CH_2Cl_2 (30 ml) was cooled to 15°C and was added 3-aminobenzoate (7a-7k) (6 ml), Et_3N and (6 ml, 0.10 mol) and stirred for 2 h to give Methyl-3-(2-oxo-2-(2-phenylindolizin-3-yl)acetamido)benzoate (8a). Reaction completion was observed by TLC. Water was added to the reaction mixture and separated the organic layer (2 × 300 ml). The combined organic layer was washed with brine solution and was dried over anhydrous Na_2SO_4. Organic layer was concentrated under reduced pressure. The crude material was purified by column chromatography, yield 50.0 g, 57.1%.

Methyl-3-(2-oxo-2-(2-phenylindolizin-3-yl)acetamido)benzoate (8a): M.p. 142°C; HPLC conditions: Column: Zorbax SB C-18 (4.6 × 250) mm, λ_{max}: 210 nm, Mobile phase: A: 0.01 M NaH_2PO_4 B: Acetonitrile (30: 70), Flow rate: 1.0 ml/min, Retention time: 5.24 min, HPLC Purity: 97.8%; To an ice-cold solution of 2-oxo-2-(2-phenylindolizin-3-yl)acetyl chloride (6) (0.5 g, 1.76 mmol) and methyl 3-aminobenzoate (0.3 g, 1.93 mmol), in DCM (10 ml) was added triethylamine (1 ml, 7.03 mmol),. The reaction mixture was stirred at retention time for 2 h. The solvent was removed under vacuum to give the crude compound. The crude compound obtained was purified by silica gel column chromatography using ethyl acetate–hexane (1:4) as eluent to yield (8a); Yield: 0.4 g (57.1%),

Methyl-3-(2-oxo-2-(2-phenylindolizin-3-yl)acetamido)benzoate (8a): IR (KBR): 3450 cm^{-1}, 3259 cm^{-1}, 3084 cm^{-1}, 1718 cm^{-1}, (C=O) 1681 cm^{-1}, 1658 cm^{-1}, (C=C) 1596 cm^{-1}, 1572 cm^{-1}, 1452 cm^{-1}, 1422 cm^{-1}, 1336 cm^{-1}, 1287 cm^{-1}, 1243 cm^{-1} 1172.51 cm^{-1}. ^{1}H-NMR, $CDCl_3$, δ_{ppm}=9.77 (d, *J*=7.5, 1H, Ar-H), 8.25 (s, 1H, N-H), 7.75 (m, 2H, Ar-H), 7.61 (dd, *J*=7.0, 1H, Ar-H), 7.52-7.41 (m, 3H, Ar-H), 7.22-7.33 (m, 5H, Ar-H), 7.0 (t, *J*=8.0, 1H, Ar-H), 6.65 (1H, s, Ar-H), 3.91 (3H, s, OCH_3); ^{13}C-NMR, δ=165.94, 157.44, 153.21, 145.21, 138.24, 132.68, 130.63, 129.92, 129.85, 123.85, 121.58, 120.77, 117.28, 114.12, 100.83, 55.19. LC-MS (m/z): 398 (M^+), 399.2 (M+H).

Butyl-4-(2-oxo-2-(2-phenylindolizin-3-yl)acetamido)benzoate (8b): Physical state: Solid, Color: Yellow, M.p. 123°C; HPLC conditions: Column: Kromasil 100 C-18 (4.6 × 250) mm, λ_{max}: 265 nm, Mobile phase: 0.01 M NaH_2PO_4 (pH: 3.0): Acetonitrile (20:80), Flow rate: 1.0 ml/min, Retention time: 7.63 min, HPLC Purity: (94.2%) Please refer to the attached chromatogram, TLC system: Ethyl acetate–Hexane (1:1) Rf value: 0.63; To an ice-cold solution of 2-oxo-2-(2-phenylindolizin-3-yl)acetyl chloride (6) (0.3 g, 1.05 mmol), and n-butyl-4-aminobenzoate (0.22 g, 1.16 mmol), in DCM (5 ml) was added triethylamine (3 ml, 2.11 mmol), The reaction mixture was stirred at retention time for 1 h. The solvent was evaporated in vacuum to give the crude compound.

The crude compound obtained was purified by silica gel column chromatography using ethyl acetate–hexane (1:4) as eluent to yield (8b); Yield: 0.25 g (62.5%).

IR (KBR): 3476 cm^{-1}, 3249 cm^{-1}, 3185 cm^{-1}, 3109 cm^{-1}, 2958 cm^{-1}, 1705 cm^{-1}, 1688 cm^{-1}, (C=O) 1601 cm^{-1}, 1571 cm^{-1}, (C=C) 1540 cm^{-1}, 1455 cm^{-1}, 1423 cm^{-1}, 1314 cm^{-1}, 1280 cm^{-1}, 1175.4 cm^{-1}. ^{1}H-NMR, $CDCl_3$) δ_{ppm}=9.67 (d, J=7.5, 1H, Ar-H), 8.23 (s, 1H, N-H), 7.94 (dd, J=7.6, 2H, Ar-H), 7.84 (dd, J=7.6, 1H, Ar-H), 7.60 (dd, J=8.0, 1H, Ar-H), 7.42 (m, 2H, Ar-H), 7.22-7.35 (m, 5H, Ar-H), 7.01 (t, J=7.5, 1H, Ar-H), 6.52 (1H, s, Ar-H), 4.32 (2H, s, OCH_2), 1.72 (2H, p, CH_2), 1.45 (2H, J=7.5, q, CH_2), 1.10 (3H, J=7.0, t, CH_3); ^{13}C-NMR, δ=164.21, 158.12, 152.20, 144.45, 139.78, 130.60, 132.12, 130.78, 128.45, 122.78, 120.60, 118.45, 113.78, 102.78, 54.12. LC-MS (m/z): 440 (M^+), 441.2 (M+H).

N-(2,4-dimethoxyphenyl)-2-oxo-2-(2-phenylindolizin-3-yl)acetamide (8c): Physical state: Solid, Color: Yellow, M.p. 132°C; HPLC conditions: Column: Zorbax SB C18 (4.6 × 250) mm, λ_{max}: 210 nm, Mobile phase: 0.01 M NaH_2PO_4: Acetonitrile (30:70), Flow rate: 1.0 ml/min, Retention time: 6.46 min, HPLC Purity: (99.6%) Please refer to the attached chromatogram, TLC system: Ethyl acetate–Hexane (3:7) R_f value: 0.42; To an ice-cold solution of 2-oxo-2-(2-phenylindolizin-3-yl)acetyl chloride (6) (0.3 g, 1.05 mmol) and 2,4-dimethoxyaniline (0.18 g, 1.16 mmol), in DCM (10 ml) was added triethylamine (0.6 ml, 4.24 mmol),. The reaction mixture was stirred at retention time for 2 h. The solvent was evaporated under vacuum to give the crude compound. The crude compound was purified by silica gel column chromatography using ethyl acetate–hexane (3: 22) as eluent to yield (8c); Yield: 0.35 g (82.7%).

IR (KBR): 3389 cm^{-1}, 3061 cm^{-1}, 2999 cm^{-1}, 2931 cm^{-1}, 2835 cm^{-1}, 1682 cm^{-1}, 1603 cm^{-1}, (C=O) 1587 cm^{-1}, (C=C) 1529 cm^{-1}, 1495 cm^{-1}, 1454 cm^{-1}, 1411 cm^{-1}, 1341 cm^{-1}, 1158 cm^{-1}. ^{1}H-NMR, 400 MHz, $CDCl_3$) δ_{ppm}=9.82 (d, J=7.5, 1H, Ar-H), 8.61 (s, 1H, N-H), 7.52-7.62 (m, 2H, Ar-H), 7.42-7.47 (m, 2H, Ar-H), 7.54 (m, 5H, Ar-H), 7.98 (t, J=7.5, 1H, Ar-H), 6.64 (1H, s, Ar-H), 6.46 (d, J=8.0, 1H, Ar-H), 6.32 (dd, J=7.5, 1H, Ar-H), 3.72 (3H, s, OCH_3), 3.84 (3H, s, OCH_3); ^{13}C-NMR (75 MHz, $CDCl_3$), δ_{ppm}=162.78, 159.47, 151.72, 145.12, 140.78, 135.62, 131.22, 130.80, 128.40, 123.80, 121.58, 118.40, 115.80, 103.80, 59.85. LC-MS (m/z): 400 (M^+), 401.2 $(M+H)^+$.

2-oxo-2-(2-phenylindolizin-3-yl)-N-(pyridin-3-yl)acetamide (8d): Physical state: Solid, Color: Green, M.p. 120°C; HPLC conditions, Column: Kromasil 100 C18 (4.6 × 250) mm, λ_{max}: 235 nm, Mobile phase: 0.01 M NaH_2PO_4 (pH: 3.0): Acetonitrile (30:70), Flow rate: 1.0 ml/min, Retention time: 4.9 min, HPLC purity (98.4%) Please refer to the attached chromatogram, TLC system: Ethyl acetate: Hexane (1:1) Rf value: 0.43; To an ice-cold solution of 2-oxo-2-(2-phenylindolizin-3-yl)acetyl chloride (6) (1 g, 3.52 mmol) and 3-aminopyridine (0.32 g, 3.39 mmol), in dichloromethane (15 ml) was added triethylamine (1.0 m;, 7.17 mmol). The reaction mixture was stirred at ambient temperature for 2 h. The solvent was evaporated under vacuum to give the crude compound 2-oxo-2-(2-phenylindolizin-3-yl)-N-(pyridin-3-yl)acetamide (8d). The crude compound obtained was purified by silica gel column chromatography using ethyl acetate-hexane (1:4) as eluent to yield (8d). Yield 0.180 g (15%).

IR (KBR): 3173 cm^{-1}, 3028 cm^{-1}, 1685 cm^{-1}, 1583 cm^{-1} (C=O), 1529 cm^{-1}, (C=C) 1413 cm^{-1}, 1343 cm^{-1}, 1301 cm^{-1}, 1242 cm^{-1}. ^{1}H-NMR (300 MHz, $CDCl_3$), δ_{ppm}=9.80 (d, J=7.5, 1H, Ar-H), 8.82 (s, 1H, N-H), 8.41 (dd, J=7.5, 1H, Ar-H), 8.26 (dd, J=7.0, 2H, Ar-H), 7.82 (t, J=8.0, 1H, Ar-H), 7.56 (m, 3H, Ar-H), 7.42 (m, 2H, Ar-H), 7.30 (t, J=8.0, 1H, Ar-H), 7.22 (m, 2H, Ar-H), 7.14 (t, J=7.8, 1H, Ar-H), 7.02 (m, 2H, Ar-H), 6.62 (s, 1H, Ar-H). ^{13}C-NMR (75 MHz, $CDCl_3$), δ_{ppm}=167.94, 159.44, 153.21, 149.42, 146.98, 136.38, 136.03, 135.83, 134.11, 132.68, 130.63, 129.92, 129.85, 123.85, 121.58, 120.77, 114.12, 113.83, 50.78. LC-MS (m/z): 341 (M^+), 342 (M+H).

2-oxo-2-(2-phenylindolizin-3-yl)-N-(pyridin-4-yl)acetamide (8e): M.p. 126°C; Physical state: Solid, Color: Yellow, Column: Kromasil 100 C18 (4.6 × 250) mm, λ_{max}: 235 nm, Mobile phase: 0.01 M NaH_2PO_4 (pH: 3.0): Acetonitrile (50:50), Flow rate: 1.0 ml/min, Retention time: 5.6 min, (99.8%) Please refer to the attached chromatogram; TLC system: Methanol–Chloroform (1:9) R_f value: 0.57; To an ice-cold solution of 2-oxo-2-(2-phenylindolizin-3-yl)acetyl chloride (6) (0.5 g, 1.76 mmol) and 4-aminopyridine (0.18 g, 1.93 mmol), in dichloromethane (6 ml) was added triethylamine(0.5 ml, 3.52 mmol). The reaction mixture was stirred at ambient temperature for 1 h. The solvent was evaporated under vacuum to give the crude compound 2-oxo-2-(2-phenylindolizin-3-yl)-N-(pyridin-4-yl)acetamide (8e). The crude compound obtained was purified by silica gel column chromatography using methanol–chloroform as eluent to yield (8e). Yield: 0.450 g (75%).

IR (KBR): 3478 cm^{-1}, 3031 cm^{-1}, 1673 cm^{-1}, 1591 cm^{-1} (C=O), 1554 cm^{-1}, (C=C) 1486 cm^{-1}, 1451 cm^{-1}, 1418 cm^{-1}, 1337 cm^{-1}, 1305 cm^{-1}, 1242 cm^{-1}. (^{1}H-NMR, $CDCl_3$), δ_{ppm}=9.78 (d, J=7.5, 1H, Ar-H), 8.31-8.40 (m, 3H, N-H, Ar-H), 7.58-7.70 (m, 2H, Ar-H), 7.42 (m, 2H, Ar-H), 7.24-7.36 (m, 5H, Ar-H), 7.16(t, J=7.6, 1H, Ar-H), 7.02 (t, J=8.0, 1H, Ar-H), ^{13}C-NMR, δ=167.94, 159.42, 153.20, 149.40, 145.78, 135.42, 136.25, 134.24, 132.89, 130.34, 129.24, 127.26, 123.80, 121.60, 120.70, 114.22, 113.45, 51.28. LC-MS (m/z): 341 (M^+), 342 (M+H).

N-(6-methoxypyridin-3-yl)-2-oxo-2-(2-phenylindolizin-3-yl)acetamide (8f): M.p. 122°C; Physical state: Solid, Color: Yellow, Column: Zorbax SB C18 (4.6 × 250) mm, λ_{max}: 210 nm, Mobile phase: H_2O: Acetonitrile (40:60), Flow rate: 1.0 ml/min, Retention time: 5.8 min, Purity (99.1%) Please refer to the attached chromatogram, TLC system: Ethyl acetate–Hexane (1:1) Rf value: 0.46; To an ice-cold solution of 2-oxo-2-(2-phenylindolizin-3-yl)acetyl chloride (6) (0.5 g, 1.76 mmol) and 5-amino-2-methoxypyridine (0.2 g, 1.61 mmol), in DCM (10 mL) was added triethylamine (0.5 ml, 3.58 mmol). The reaction mixture was stirred at retention time for 1 h. The solvent was evaporated under vacuum to give the crude compound N-(6-methoxypyridin-3-yl)-2-oxo-2-(2-phenylindolizin-3-yl)acetamide (8f). The crude compound obtained was purified by silica gel column chromatography using ethyl acetate–hexane (3:7) as eluent to yield (8f). Yield: 0.150 g (22.93%). IR (KBR): 3262 cm^{-1}, 3117 cm^{-1}, 3087 cm^{-1}, 2944 cm^{-1}, 1649 cm^{-1}, 1588 cm^{-1} (C=O), 1550 cm^{-1}, (C=C) 1488 cm^{-1}, 1416 cm^{-1}, 1383 cm^{-1}, 1335 cm^{-1}, 1304 cm^{-1}, 1269 cm^{-1}, 1240 cm^{-1}. (^{1}H-NMR, $CDCl_3$), δ_{ppm}=9.68 (d, J=7.6, 1H, Ar-H), 8.14 (s, 1H, N-H), 7.94 (d, J=7.6, 1H, Ar-H), 7.61 (dd, J=7.6, 1H, Ar-H), 7.40-7.51 (m, 3H, Ar-H), 7.26-7.32 (m, 4H, Ar-H), 7.01 (t, J=8.0, 1H, Ar-H), 6.61 (m, 2H, Ar-H), 3.82 (s, 3H, OCH_3); LC-MS (m/z): 371 (M^+), 372 (M+H).

Methyl 5-(2-oxo-2-(2-phenylindolizin-3-yl)acetamido)thiophene-3-carboxylate (8g): M.p. 110°C; Physical state: Solid, Color: Yellow, Column: Zorbax SB C18 (4.6 × 250) mm, λ_{max}: 220 nm, Mobile phase: H_2O: Acetonitrile (30:70), Flow rate: 1.0 ml/min, Retention time: 4.97 min, Purity (96.2%) Please refer to the attached chromatogram, TLC system: Ethyl acetate: Hexane (1:1) Rf value: 0.51; To an ice-cold solution of 2-oxo-2-(2-phenylindolizin-3-yl)acetyl chloride (6) (0.5 g, 1.76 mmol) and 5-Aminothiophene-3-carboxylic acid methyl ester (0.3 g, 1.9 mmol) in DCM (10 ml) was added triethylamine (1 ml, 7.03 mmol). The reaction mixture was stirred at retention time for 2 h. The solvent was evaporated under vacuum to give the crude compound methyl 5-(2-oxo-2-(2-phenylindolizin-3-yl)acetamido)thiophene-3-carboxylate (8 g). The crude compound obtained was purified by silica gel column chromatography using Ethyl acetate: Hexane (1: 3) as eluent to yield 0.20 g (28.16%) (8 g).

IR (KBR): 3290 cm^{-1}, 3100 cm^{-1}, 3026 cm^{-1}, 2926 cm^{-1}, 1710 cm^{-1}, 1671 cm^{-1}, 1628 cm^{-1}, 1598 cm^{-1} (C=O), 1556 cm^{-1}, 1511 cm^{-1}, (C=C) 1416 cm^{-1}, 1343 cm^{-1}, 1311 cm^{-1}, 1247 cm^{-1}, 1235 cm^{-1}. (^{1}H-NMR, $CDCl_3$) δ_{ppm}=9.78 (d, J=7.5, 1H, Ar-H), 9.01 (s, 1H, N-H), 7.64 (m, 2H, Ar-H), 7.41-7.52 (m, 2H, Ar-H), 7.30-7.41 (m, 2H, Ar-H), 7.02 (m, 2H, Ar-H), 6.84 (s, 1H, Ar-H), 3.82 (s, 3H, OCH_3); LC-MS (m/z): 404 (M^+), 405 (M+H).

2-oxo-2-(2-phenylindolizin-3-yl)-N-(3,4,5-trimethoxyphenyl)acetamide (8h): Physical state: Solid, Color: Yellow, M.p. 128°C; HPLC conditions: Column: Zorbax SB C18 (4.6 × 250) mm, λ_{max}: 210 nm, Mobile phase: 0.01 M NaH_2PO_4: Acetonitrile (30:70), Flow rate: 1.0 ml/min, Retention time: 6.46 min, HPLC Purity: (99.5%) Please refer to the attached chromatogram, TLC system: Ethyl acetate–Hexane (3:7) R_f value: 0.44; To an ice-cold solution of 2-oxo-2-(2-phenylindolizin-3-yl)acetyl chloride (6) (0.2 g, 1.15 mmol) and 3,4,5-trimethoxyaniline (0.2 g, 1.12 mmol), in DCM (15 ml) was added triethylamine (0.5 ml, 4.20 mmol),. The reaction mixture was stirred at retention time for 2 h. The solvent was evaporated under vacuum to give the crude compound 2-oxo-2-(2-phenylindolizin-3-yl)-N-(3,4,5-trimethoxyphenyl)acetamide (8h). The crude compound was purified by silica gel column chromatography using ethyl acetate–hexane (3: 22) as eluent to yield (8h); Yield: 0.35 g (83.8%).

IR (KBR): 3428 cm^{-1}, 3072 cm^{-1}, 2986 cm^{-1}, 2924 cm^{-1}, 2835 cm^{-1}, 1680 cm^{-1}, 1613 cm^{-1}, (C=O) 1567 cm^{-1}, (C=C) 1530 cm^{-1}, 1490 cm^{-1}, 1452 cm^{-1}, 1415 cm^{-1}, 1352 cm^{-1}, 1160 cm^{-1}. ^{1}H-NMR, $CDCl_3$), δ_{ppm}=9.81 (d, J=6.5, 1H, Ar-H), 8.60 (s, 1H, N-H), 7.56 (m, 2H, Ar-H), 7.40-7.48 (m, 2H, Ar-H), 7.35-7.30 (m, 5H, Ar-H), 7.22 (t, J=7.5, 1H, Ar-H), 6.64 (1H, s, Ar-H), 6.45 (d, J=8.0, 1H, Ar-H), 6.30 (dd, J=7.6, 1H, Ar-H), 3.70 (9H, s, OCH_3); LC-MS (m/z): 430 (M^+), 431.1 (M+H).

Methyl-6-(2-oxo-2-(2-phenylindolizin-3-yl)acetamido)nicotinate (8i): M.p. 118°C; physical state: Solid, Color: Light Yellow, Column: Kromasil 100 C18 (4.6 × 250) mm, λ_{max}: 235 nm, Mobile phase: 0.01 M NaH_2PO_4 (pH: 3.0): Acetonitrile (50:50), Flow rate: 1.0 ml/min, Retention time: 5.9 min, (99.6%) Please refer to the attached chromatogram, TLC system: Methanol–Chloroform (1:9) R_f value: 0.59; To an ice-cold solution of 2-oxo-2-(2-phenylindolizin-3-yl)acetyl chloride (6) (0.5 g, 1.86 mmol) and methyl-6-aminonicotinate (0.14 g, 1.90 mmol), in dichloromethane (10 ml) was added triethylamine (0.6 ml, 3.51 mmol). The reaction mixture was stirred at ambient temperature for 2 h. The solvent was evaporated under vacuum to give the crude compound Methyl-6-(2-oxo-2-(2-phenylindolizin-3-yl)acetamido)nicotinate (8i). The crude compound obtained was purified by silica gel column chromatography using methanol–chloroform as eluent to yield (8i). Yield: 0.450 g (75%).

IR (KBR): 3470 cm^{-1}, 3039 cm^{-1}, 2984 cm^{-1}, 2894 cm^{-1}, 1670 cm^{-1}, 1591 cm^{-1} (C=O), 1550 cm^{-1}, (C=C) 1482 cm^{-1}, 1445 cm^{-1}, 1415 cm^{-1}, 1330 cm^{-1}, 1289 cm^{-1}; (^{1}H-NMR, $CDCl_3$), δ_{ppm}=9.80 (d, J=8.0, 1H, Ar-H), 8.28-8.35 (m, 3H, N-H, Ar-H), 7.55-7.68 (m, 2H, Ar-H), 7.40 (m, 2H, Ar-H), 7.22-7.34 (m, 5H, Ar-H), 7.14 (t, J=7.5, 1H, Ar-H), 7.08 (t, J=7.6, 1H, Ar-H), 3.68 (3H, s, OCH_3); LC-MS (m/z): 399 (M^+), 400 (M+H).

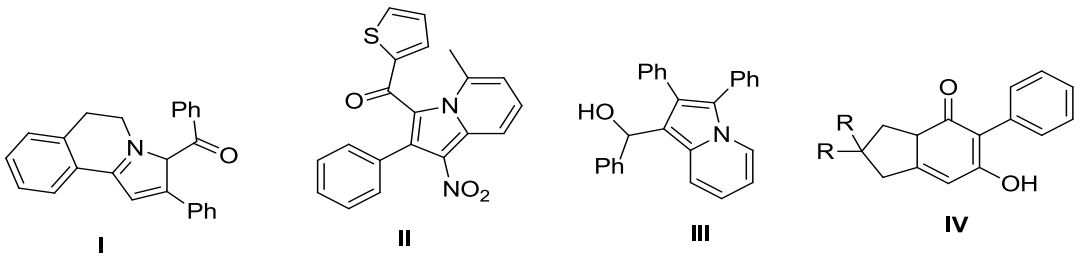
Ethyl-3-(2-oxo-2-(2-phenylindolizin-3-yl)acetamido)benzoate (8j): Physical state: Solid, Color: Yellow, M.p. 129°C; HPLC conditions: Column: Kromasil 100 C18 (4.6 × 250) mm, λ_{max}=265 nm, Mobile phase: 0.01 M NaH_2PO_4 (pH: 3.0): Acetonitrile (20:80), Flow rate: 1.0 ml/min, Retention time: 7.63 min, HPLC Purity: (94.2%) Please refer to the attached chromatogram, TLC system: Ethyl acetate–Hexane (1:1) Rf value: 0.63; To an ice-cold solution of 2-oxo-2-(2-phenylindolizin-3-yl)acetyl chloride (6) (0.3 g, 1.05 mmol) and ethyl 3-aminobenzoate (0.20 g, 1.16 mmol), in DCM (5 ml) was added triethylamine (3 ml, 2.11 mmol). The reaction mixture was stirred at retention time for 1 h. The solvent was evaporated in vacuum to give the crude compound. The crude compound obtained was purified by silica gel column chromatography using ethyl acetate–hexane (1:4) as eluent to yield (8j) Yield: 0.25 g (62.5%),

IR (KBR): 3476 cm^{-1}, 3249 cm^{-1}, 3185 cm^{-1}, 3109 cm^{-1}, 2958 cm^{-1}, 1705 cm^{-1}, 1688 cm^{-1}, (C=O) 1601 cm^{-1}, 1571 cm^{-1}, (C=C) 1540 cm^{-1}, 1455 cm^{-1}, 1423 cm^{-1}, 1314 cm^{-1}, 1280 cm^{-1}, 1175.4 cm^{-1}. ^{1}H-NMR, $CDCl_3$) δ_{ppm}=9.67 (d, J=7.5, 1H, Ar-H), 8.23 (s, 1H, N-H), 7.94 (dd, J=7.6, 2H, Ar-H), 7.84 (dd, J=7.6, 1H, Ar-H), 7.60 (dd, J=8.0, 1H, Ar-H), 7.42 (m, 2H, Ar-H), 7.22-7.35 (m, 5H, Ar-H), 7.01 (t, J=7.5, 1H, Ar-H), 6.52 (1H, s, Ar-H), 4.32 (2H, s, OCH_2), 1.72 (2H, p, CH_2), 1.45 (2H, J=7.5, q, CH_2), 1.10 (3H, J=7.0, t, CH_3); ^{13}C-NMR, δ=164.21, 158.12, 152.20, 144.45, 139.78, 130.60, 132.12, 130.78, 128.45, 122.78, 120.60, 118.45, 113.78, 102.78, 54.12. LC-MS (m/z): 412 (M^+), 413.1 (M+H).

2-oxo-2-(2-phenylindolizin-3-yl)-N-(pyridin-2-yl)acetamide (8k): M.p. 128°C; Physical state: Solid, Color: Yellow, Column: Kromasil 100 C18 (4.6 × 250) mm, λ_{max}: 235 nm, Mobile phase: 0.01 M NaH_2PO_4 (pH: 3.0): Acetonitrile (50:50), Flow rate: 1.0 ml/min, Retention time: 5.6 min, TLC system: Methanol–Chloroform (1:9) R_f value: 0.57, Purity (99.8%) Please refer to the attached chromatogram, To an ice-cold solution of 2-oxo-2-(2-phenylindolizin-3-yl)acetyl chloride **(6)** (0.5 g, 1.76 mmol), and pyridin-2-amine (0.18 g, 1.93 mmol), in dichloromethane (6 ml) was added triethylamine (0.5 ml, 3.52 mmol). The reaction mixture was stirred at ambient temperature for 1 h. The solvent was evaporated under vacuum to give the crude compound 2-oxo-2-(2-phenylindolizin-3-yl)-N-(pyridin-2-yl)acetamide (8k). The crude compound obtained was purified by silica gel column chromatography using methanol–chloroform as eluent to yield (8k). Yield: 0.450 g (75%).

IR (KBR): 3478 cm^{-1}, 3031 cm^{-1}, 1673 cm^{-1}, 1591 cm^{-1} (C=O), 1554 cm^{-1}, (C=C) 1486 cm^{-1}, 1451 cm^{-1}, 1418 cm^{-1}, 1337 cm^{-1}, 1305 cm^{-1}, 1242 cm^{-1}. (^{1}H-NMR, $CDCl_3$), δ_{ppm}=9.78 (d, J=7.5, 1H, Ar-H), 8.31-8.40 (m, 3H, N-H, Ar-H), 7.58-7.70 (m, 2H, Ar-H), 7.42 (m, 2H, Ar-H), 7.24-7.36 (m, 5H, Ar-H), 7.16 (t, J=7.6, 1H, Ar-H), 7.02 (t, J=8.0, 1H, Ar-H), ^{13}C-NMR, δ=167.94, 159.42, 153.20, 149.40, 145.78, 135.42, 136.25, 134.24, 132.89, 130.34, 129.24, 127.26, 123.80, 121.60, 120.70, 114.22, 113.45, 51.28. LC-MS (m/z): 341 (M^+), 342 (M+H).

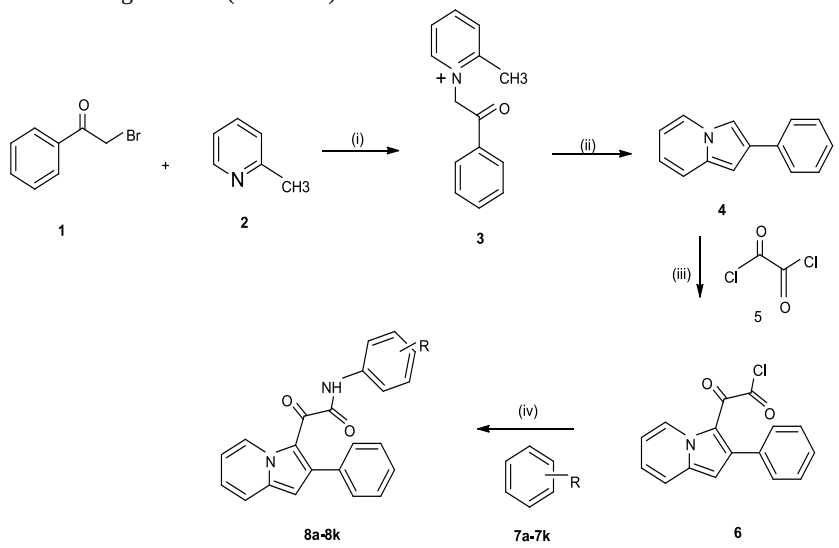
Synthesized and examined newly synthesized indolizine derivatives for their antimicrobial activity against thirteen bacterial and three fungal strains [16]. Compound I exhibited dual antibacterial and antifungal activity with Minimum Inhibitory Concentration (MIC) values in the range of 500-1,000 µg/ml against fungal strains *A. niger, C. albicans* and *C. tropicalis*, while for bacterial strains MIC values were in the range of 32-500 µg/ml (Figure 2). As per the study, the authors concluded that the phenyl moiety in compound II might be responsible for its antimicrobial properties against a Gram-negative bacterium (*E. coli*), a Gram-positive bacterium (*S. aureus*), while their antifungal potential was assessed against *C. albicans* and *A. flavus* [17] (Figure 2). In order to explore the antimycobacterial potential of indolizine [18] synthesized and tested 1-substituted indolizine derivatives for their activity against *Mycobacterium tuberculosis* H_{37}Rv. Interestingly, the authors claimed compound III as the first anti mycobacterial indolizine with MIC value=6.25 µg/ml. Among the synthesized compounds, compound IV showed significant activity (MIC: 16 µg/ml) against mycobacterium tuberculosis (Figure 2) [19].



I II III IV

Figure 2: Compounds against *Mycobacterium tuberculosis* (I-IV)

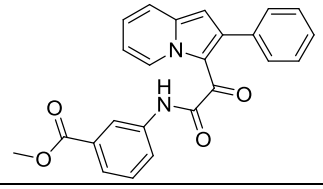
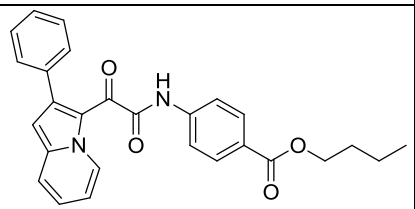
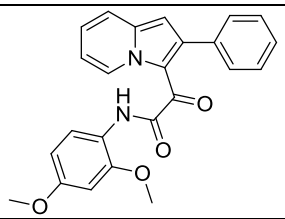
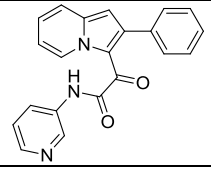
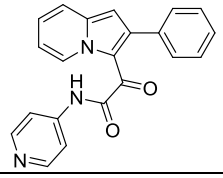
Despite being an important medicinal moiety, a detailed review on the biologically active potential of indolizine derivative is unavailable. To the best of our knowledge, previous reviews on indolizine derivatives mainly focused on their chemistry and synthesis. The present work discusses the versatile nature of indolizine derivatives and their possible mechanism of action. Important SAR points are discussed with each study to highlight the rationale behind the study. Furthermore, the present study also provides information about current/future prospects of the topic and different indolizine derivatives in clinical trials and synthesized various 2-Phenylindolizine acetamide derivatives carried out the antimicrobial activity of newly synthesized targets 8a-8h (Scheme 1).

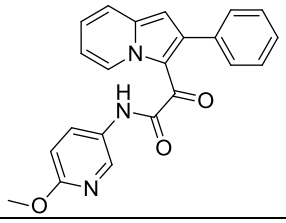
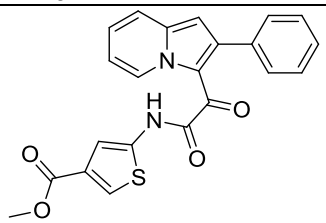
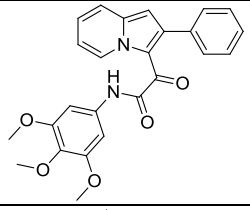
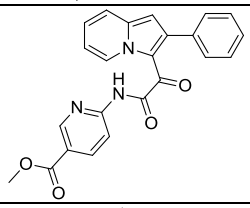
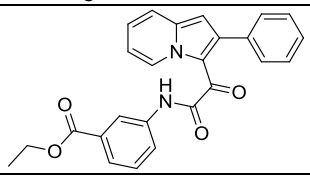
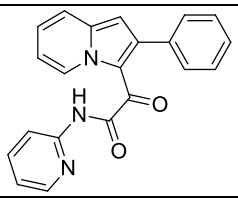


Scheme 1: Synthesis of 2-phenylindolizin acetamide derivatives (8a-8k)

Reagent and conditions: (i) MeOH, Reflux, 2 h, (ii) aq $NaHCO_3$, Reflux, 3 h, (iii) Toluene, THF, 4 h, (iv) DCM, Et_3N, 2 h, 25°C, yield 28-83%

Table 1: 2-phenylindolizine acetamide derivatives (8a-8k)

Entry	Compound	Melting point	Physical state/Color	M. W	% of yield	Molecular formula
8a		142	Yellowish green solid	399.2	57.1%	$C_{24}H_{18}N_2O_4$
8b		123	Yellow solid	441.2	62.5%	$C_{27}H_{24}N_2O_4$
8c		132	Yellow solid	401.2	82.7%	$C_{24}H_{20}N_2O_4$
8d		120	Green solid	342	15%	$C_{21}H_{15}N_3O_2$
8e		126	Yellow solid	342	75%	$C_{21}H_{15}N_3O_2$

8f		122	Yellow solid	372	22.93%	$C_{22}H_{17}N_3O_3$
8g		110	Yellow solid	405	28.16%	$C_{22}H_{16}N_2O_4S$
8h		128	Green solid	431.1	83.8%	$C_{25}H_{22}N_2O_5$
8i		118	Yellow solid	400	75%	$C_{23}H_{17}N_3O_4$
8j		129	White color solid	413.1	62.5%	$C_{25}H_20N_2O_4$
8k		128	Yellow solid	342	75%	$C_{21}H_{15}N_3O_2$

RESULTS AND DISCUSSION

Chemistry

All the inhibitors were synthesized starting with 2-bromo-1-phenylethanone (1) (Scheme 1). 1 was reacted with 2-methylpyridine (2) in presence of MeOH to give 2-(2-methylpyridin-1(2H)-yl)-1-phenylethanone (3) in excellent yield. Subsequently the 2-phenylindolizin (4) was prepared by using 3, $NaHCO_3$. Further, the compound 6 was prepared by using 2-phenylindolizine (4), oxalyl dichloride (5) reaction in the presence of Tetrahydrofuran (THF) and toluene as a solvent to give desired product 6 in good yield, followed by condensation of different aromatic amines (7a-7k) in presence of DCM, $Et_3N$to get the target compounds (8a-8k) in good yields (Scheme 1 and Table 1). The structures were characterized by spectral techniques. In general, in Fourier Transform Infra-Red (FTIR) spectra, characteristic NH stretching peaks near 3476-3410 cm^{-1} and C=O stretching peaks near 1735-1680 cm^{-1} are shown. The Proton Nuclear Magnetic Resonance (^{1}H-NMR) of synthesized derivatives showed multiple signals corresponding to resonance of aromatic protons, multiplet in the region of δ=7.52-6.65. The proton of the CH_3 group exhibited a singlet in the region of δ=3.65-3.91. The proton of N–Exhibited a singlet in the region of δ=8.25. All the compounds were characterized by mass spectral analysis. Compounds show that M+peak corresponds to the molecular weight.

Biological activity

The results of biological studies of newly synthesized compounds (8a-8k) reveal that the compounds possess significant antibacterial and antifungal activities. Additionally the series of compounds were tested to ascertain their antibacterial, antifungal capacity and are summarized in Table 2 and Figure 3. From the assay it was evident that, some compounds from the series were found to be associated with promising antibacterial, antifungal properties. From antibacterial and antifungal activity screening results, it has been observed that compounds 8b, 8a, 8f and 8j possess excellent activity. In the series compounds 8b, 8a and 8f exhibited best antibacterial activity against *S. aureus* with mm values at 23, 22 and 22, respectively. Further, compound 8a, 8b showed very good antibacterial activity against *P. aeruginosa* with mm values of 23, 22 mm. Furthermore compounds 8c, 8e, 8g and 8k showed moderate *in vitro* antibacterial activity against Gram-positive, Gram-negative bacteria (*S. aureus, E. coli, P. aeruginosa*). Compounds 8j and 8d in the series show excellent antifungal activity against organism's *C. albicans*, *A. flavus* with mm values 20, 10 and 11, 10 and 8a, 8i, 8j active against *A. fumigatus* with 11 mm respectively.

Antibacterial activity

The antibacterial activity of synthesized compounds was studied by the disc diffusion method [20,21] against the following pathogenic organisms. The gram-positive bacterial screened were *S. aureus* and *B. cereus*. The gram negative bacterial screened were *E. coli* and *P. aeruginosa*. The synthesized compounds were used at the concentration of 250 µg/ml and 500 µg/ml using DMSO as a solvent. The amoxicillin 10 µg/disc and Streptomycin 30 µg/disc were used as a standard (Himedia laboratories limited, Mumbai).

Disc diffusion method

A suspension of *S. aureus* was added to sterile nutrient agar at 45°C. The mixture was transferred to sterile petridishes to give a depth of 3-4 mm and allowed to solidify. Precautions were observed to reduce uniform layer of medium on the plate. Sterile discs 5 mm in diameter (made from Whatman filter paper) were immersed in the solutions of synthesized compounds (250 µg/ml) and maintain an untreated control sample for comparison. Leave the plates to stand for 1 h at room temperature as a period of pre-incubation diffusion to minimize the effects of variations in different time. Then the plates were incubated at 37°C for 24 h and observed for antibacterial activity. The diameter of the zone of inhibition was measured for each plate in which the zone of inhibition was observed. The average zone of inhibition was calculated and compared with that of standard. A similar procedure was adopted for studying the antibacterial activity against the other organisms.

Antifungal activity

The antifungal activity of synthesized compounds were studied by disc diffusion method [22,23] against the organisms of *A. niger* and *C. albicans*. Compounds were treated at the concentrations of 250 µg/ml using DMSO as a solvent. The standard used was ketoconazole 50 µg/ml and griseofulvin 50 µg/ml against both the organisms.

Disc diffusion method

A suspension of *A. niger* was added to a sterile Sabouraud dextrose agar at 45°C. The mixture was transferred to sterile petridishes and allowed to solidify. Sterile discs 5 mm in diameter (made from Whatman filter paper) immersed in the solutions of synthesized compounds and control were placed on the surface of agar medium with forceps and pressed gently to ensure even contact. Leave the plates to stand for 1 h at room temperature as a period of preincubation diffusion to minimize the effects of variation at 37°C for 13 h and observed for antibacterial activity. The diameters of the zone of inhibition were measured for the plates in which the zone of inhibition was observed. The average zone of inhibition was calculated with that of standard.

A number of 2-phenylindolizin acetamide derivatives (8a-8k) were synthesized and evaluated for gram positive and gram negative bacteria and the compound with different p-substituted on the phenyl group there group evaluated for *in vitro* antibacterial activities, and this compound QSAR studies through Hansch analysis showed a linear correlation of the activity with electronic distribution along with Sterimol parameters. Small electron-donor groups with hydrophilic properties increase the *in vitro* activity against Gram-positive, Gram-negative bacteria. Further the target compounds 8a-8k were also exhibit good activity against fungal species like *C. albicans, A. flavus and A. fumigatus.*

Antimicrobial evaluation of novel compounds 8(a-k)

Table 2: Antimicrobial activity and antifungal activity of synthesized compounds 8(a-k)

Compounds	Zone of inhibition in mm					
	Antibacterial activity			Antifungal activity		
	S. aureus	***E. coli***	***P. aeruginosa***	***C. albicans***	***A. flavus***	***A. fumigatus***
8a	22	22	23	12	10	11
8b	23	21	22	11	9	10
8c	21	18	19	10	9	10
8d	ND	ND	17	11	10	11
8e	20	17	18	10	9	10
8f	22	20	21	10	9	10
8g	16	15	19	13	ND	9
8h	ND	18	ND	ND	11	8
8i	22	20	21	10	10	11
8j	21	18	19	20	10	11
8k	17	16	20	14	ND	10
Ampicillin	20	21	22	21	ND	ND
Fluconazole	22	20	23	22	ND	ND

ND: No zone of inhibition

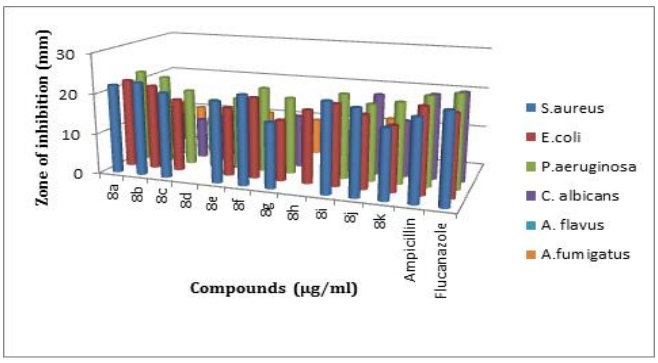


Figure 3: Antimicrobial activity and antifungal activity of target compounds (8a-8k)

CONCLUSION

The present study describes a simple, inexpensive, and easy method for synthesis of target compounds (8a-8k) in a stipulated time, without using any drastic conditions. The yield of all 2-phenylindolizine acetamide derivatives were found to be in the range of 15-83%. The assigned structure was further established by IR, ^{1}H-NMR, ^{13}C-NMR and MS spectral studies. All compounds studied in this work were screened for their *in vitro* antimicrobial activities against the standard strains. From the present study, it can be concluded that the compound 8b, 8a and 8f exhibited and potentially be developed into useful antibacterial agents *S. aureus* organism. Further, the synthesized compound 8j and 8d show excellent antifungal activity against organism's *C. albicans, A. flavus* and *A. fumigates* when compared to reference compounds (Ampicillin, Flucanazole), which can prompt future researchers to synthesize a series of 2-phenylindolizin acetamide derivatives containing a wide variety of substituent's, with the aim of producing a novel heterocyclic system, with enhanced antibacterial, antifungal activity.

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REFERENCES

[1] J. Gubin, J. Lucchetti, J. Mahaux, D. Nisato, G. Rosseels, M. Clinet, P. Polster, P. Chatelain, *J. Med. Chem*., **1992**, 35, 981.
[2] J.H. Hutchinson, M. Therien, R. Frenette, Patent-EP-535924, A1-19930407.
[3] S. Hagishita, M. Yamada, K. Shirahase, T. Okada, Y. Murakami, Y. Ito, T. Matsuura, M. Wada, T. Kato, M. Ueno, Y. Chikazawa, K. Yamada, T. Ono, I. Teshirogi, M. Ohtani, *J. Med. Chem*., **1996**, 39, 3636.
[4] W. Flitsch, In Comprehensive Heterocyclic Chemistry, A.R. Katritzky, C.W. Rees, Eds.; Pergamon Press: Oxford., **1984**, 4, 443.
[5] G.S. Singh, E.E. Mmatli, *Eur. J. Med. Chem*., **2011**, 46, 5237.
[6] E. Georgescu, F. Dumitrascu, F. Georgescu, C. Draghici, L. Barbu, *J. Heterocycl. Chem*., **2013**, 50, 78-82.
[7] H. Malonne, J. Hanuise, J. Fontaine, *Pharm. Pharmacol. Commun*., **1998**, 4, 241-242.
[8] K. Kitadokoro, S. Hagishita, T. Sato, M. Ohtani, K. Miki, *J. Biochem*., **1998**, 123, 619-623.
[9] L.D. Bolle, G. Andrei, R. Snoeck, Y. Zhang, A.V. Lommel, M. Otto, A. Bousseau, C. Roy, E.D. Clercq, L. Naesens, *Biochem. Pharmacol*., **2004**, 67, 325-336.
[10] P. Sonnet, P. Dallemagne, J. Guillon, C. Engueard, S. Stiebing, J. Tangue, B. Bureau, S. Rault, P. Auvray, S. Moslemi, *Bioorg. Med. Chem*., **2000**, 8, 945-955.
[11] F. Campagna, A. Carotti, G. Casini, M. Macripo, *Heterocycles*., **1990**, 31, 97-107.
[12] M. Bols, V.H. Lillelund, H.H. Jensen, X. Liang, *Chem. Rev*., **2002**, 102, 515-553.
[13] N. Asano, R.J. Nash, R.J. Molyneux, G.W.J. Fleet, *Tetrahedron: Asymmetry*., **2000**, 11, 1645-1680.
[14] G.S. Singh, E.E. Mmatli, *Eur. J. Med. Chem*., **2011**, 46(11), 5237-5257.
[15] V. Vemula, S. Vurukonda, C. Bairi, *Int. J. Pharm. Sci. Rev. Res*., **2011**, 11, 159-163
[16] A. Hazra, S. Mondal, A. Maity, S. Naskar, P. Saha, R. Paira, K.B. Sahu, P. Paira, S. Ghosh, C. Sinha, A. Samanta, S. Banerjee, N.B. Mondal, *Eur. J. Med. Chem*., **2011**, 46(6), 2132-2140
[17] E.S. Darwish, *Molecules*., **2008**, 13(5), 1066-1078.
[18] L.L. Gundersen, A.H. Negussie, F. Rise, O.B. Ostby, *Arch. Pharm. Pharm. Med. Chem*., 336(3), 191-195.
[19] G. Dannhardt, W. Meindl, S. Gussmann, S. Ajili, T. Kappe, *Eur. J. Med. Chem*., **1987**, 22(6), 505-510.
[20] F. Kavanagh, Analytical Microbiology 2nd Volume, Academic Press, New York and London, **1972**, 11-23.
[21] A.A. Miles, S.S. Misra, *J. Hyg*., **1938**, 38, 732.
[22] S. Satish, K.A. Raveesha, G.R. Janardhana, *Lett. Appl. Microbiol*., **2002**, 22, 145.
[23] S. Senthil Kumar, M. Kamaraj, American-Eurasian, *J. Agric. Environ. Sci*., **2010**, 7, 176.