ISSN (O): 2581-8740 | ISSN (P): 2581-8732
Anjali *et al.*, 2021
DOI: 10.21276/SSR-IIJLS.2021.7.4.2

Research Article

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Design and Discovery of Novel Curcumin-pyrazole-triazine Conjugates and its Evaluation against Multi-drug Resistant Microbes

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Received: 13 Jan 2021/ Revised: 28 Mar 2021/ Accepted: 21 Jun 2021

ABSTRACT

Background: In this work, unique curcumin pyrazole triazine conjugates were produced by combining curcumin with a nitrogen-containing heterocyclic compound 2, 4, 6-trichloro-1, 3, 5-triazine to create a physiologically relevant hybrid molecule capable of overcoming multi-drug resistance with improved pharmacological activity.
Methods: Synthesized drugs were characterized by Melting point, FTIR, ^{1}H NMR, ^{13}C NMR spectroscopy and Mass spectrometry. The conjugates were also evaluated for antibacterial (gram-positive and gram-negative bacteria) and antifungal (yeast and mould) activity with the help of the agar well diffusion method, the minimum inhibitory concentration via resazurin based micro-broth dilution method and minimum bactericidal concentration.
Results: The molecules containing halogens in their structure such as A5, A8, A3, and A4 have shown the best antibacterial with MIC ranging between 32-128µg/ml. Furthermore, the bioconjugate A5 had the most potent antibacterial action against Gram-positive bacteria *Staphylococcus aureus* and Gram-negative bacterial *Pseudomonas aeruginosa*, as well as antifungal activity against *Candida albicans*.
Conclusion: Pharmaceutically important hybrid molecules can be used in future to cure bacterial and fungal diseases.

Key-words: Antibacterial activity, Antifungal activity, Curcumin conjugates, Multidrug resistance, Triazine conjugates

INTRODUCTION

Multidrug resistance (MDR) is a global health concern as drug-resistant pathogens "Superbugs" are continuously acquiring new strategies to become resistant [1]. According to the WHO, these resistant microbes (Bacteria, Fungi) can withstand antimicrobial drug attacks, resulting in inadequate medication, tolerance, and infection spread.

How to cite this article
Anjali, Singh UP, Malairajan P, Lawrence R, Jeyakumar E. Design and Discovery of Novel Curcumin-pyrazole-triazine Conjugates and its Evaluation against Multi-drug Resistant Microbes. SSR Inst. Int. J. Life Sci., 2021; 7(4): 2834-2843.

Access this article online
https://iijls.com/

Exposure to these medications creates the requisite selective pressure for the emergence and dissemination of resistant pathogens [2].

Despite renewed attempts to produce novel antibiotics, the number of medication approvals has been steadily declining recently due to their major ineffectiveness towards MDRs [3-5]. As a result of these concerns, there has increased interest in research into alternative antimicrobials. Measurable studies highlighted the role of natural compounds and their extracts in reducing antibiotic dependence in bacterial treatment, thus avoiding the development of antibiotic resistance [6]. These cost-effective natural compounds are now understood to have various biological, chemical, and physical functions, and those plants such as turmeric

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(curcumin), clove, allspice, cinnamon, thyme, and garlic contain numerous antimicrobial compounds [7-9].

Distinct molecular diversity of these natural products can be used to enhance their intrinsic biological activity or drug-like properties by designing combinatorial libraries. Even after their effective tolerability with less or negligible toxicity, their limitations in physicochemical properties making them barely dissolved in gastrointestinal aqueous fluid [10,11]. This can be accomplished by semi-synthetic modification of the parent molecule after critical structural elements necessary for biological activity are identified in libraries and validated using computing methods [12]. This approach of conjugation with other materials enhanced the biological activity of natural compounds such as ADMET (Adsorption, Distribution, Metabolism, Excretion and Transport), the physicochemical properties once administered in the human's body [13].

With the simplicity of 2, 4, 6-trichloro-1, 3, 5-triazine conjugates with its ability to promote biological activities against multidrug-resistant bacteria, research on these (s-triazine) heterocyclic compounds has proven to be an efficient lead compound. The s-triazine compound may be a good economically viable starting material for developing a new antibiotic conjugating form [14]. It has been widely studied that s-triazine has high antibacterial and antifungal efficacy, which is primarily produced by a nucleophilic substitution reaction [15]. And for the first time, this economically feasible starting material was conjugated to an ingredient (curcumin) of the prestigious natural product Turmeric in the hope of improving synergistic anti-MDR activity and to some extent good physicochemical properties.

MATERIALS AND METHODS

Chemicals used for research works were procured from Rankem Chemical, India. Whattmann Filter paper no. 1 and 2,4,6-trichloro1,3,5-triazine (Sigma Aldrich). A thin layer chromatography plate was prepared by using silica gel-G on a glass plate. The spots were examined in an iodine chamber. Melting points of products as well as intermediates was examined through the Gallenkamp melting point apparatus (HICON, India) and were uncorrected. The FTIR spectra (in 2.0 cm^{-1}, flat, smooth, abex) were recorded on the Perkin Elmer-Spectrum RX-I spectrophotometer. The ^{1}H NMR and ^{13}C NMR spectra were recorded on Bruker Advance II 400 and 100 NMR spectrophotometer. A mass spectrum was recorded on THERMO-TSQ 8000. Varied multidrug-resistant gram-positive bacteria *S. aureus* (MCCB0017), *S. pyogenes* (MCCB0093), *Bacillus cereus* (MCCB0143), *B. subtilis* (MCCB0189), *C. perfringens* (MCCBR040), *L. monocytogenes* (MCCB0028) and gram negative bacteria *E. coli* (MCCB0016), *Salmonella typhi* (MCCB0127), *S. dysenteriae* (MCCB0128), *V. cholera* (MCCB0047), *C. jejuni* (MCCB0303), *H. pylori* (MCCB0301, *P. aeruginosa* (MCCB0035) were selected for biological evaluation of drugs. Pathogenic fungi such as *C. albicans* (MCCB0290) and *A. fumigatus* (MCCB0206) were also selected for biological evaluation.

General procedure for synthesis of curcumin conjugates and its characterization

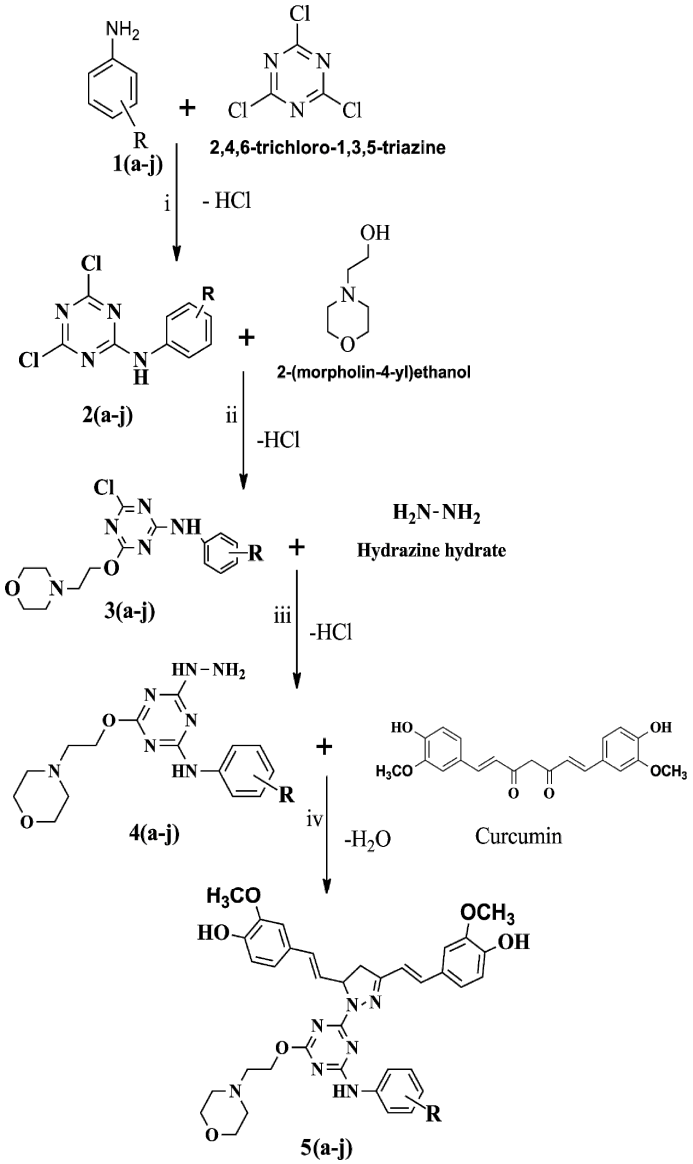

Fig. 1: Scheme of Curcumin pyrazole triazine conjugates synthesis

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Where (a-j) : a=CN.SO_4,b= CN, c = 3-F, d = 4-Br, e = 3-Cl, f = 3-NO_2, g = 2-NO_2, h = 2-Cl, i = 4-NO_2, j = H

Reagents and Reaction conditions-
(i)= Acetone medium, Sodium bicarbonate at 0-5^{0}C.
(ii)= Acetone medium, Sodium hydroxide at 45-50^{0}C
(iii)= Acetone medium, sodium hydroxide at 60^{0}C
(iv)= Glacial acetic acid, reflux temperature below 100°C

Synthesis of N substituted phenyl-1, 3, 5-triazine-2-amine derivatives as compound (2a-j)- Reaction as shown in Fig. 1 between 2, 4, 6-trichloro-1, 3, 5-triazine (0.01M) and 1(a-j) (0.01M) was carried out by stirring in presence of (20 ml) acetone at 0-5°C. Meanwhile, (0.1M) $NaHCO_3$ used to neutralize liberated HCl. Completion of the reaction was verified by TLC (acetone: benzene, 1:1). The addition of crushed ice resulted in the precipitate and then filtered. The purity of the compound was checked by using a melting point. The product was air-dried [16].

Synthesis of 4-chloro-6-(2-morpholinoethoxy)-N-R-phenyl-1,3,5-triazin-2-amine derivatives as compound (3a-j)- Equimolar amount (0.01M) of product 2(a-j) and 2-(moroholin-4-yl) ethanol as shown in Fig. 1 was taken in a three-necked round bottom flask fitted with the water condenser. The mixture was refluxed at 45-50°C in presence of acetone. Here, (0.1M) NaOH used for the neutralization of liberated acid. Completion of the reaction was confirmed by TLC (acetone: benzene, 2:1) and the visualization of the compound was carried out in an iodine chamber, after that re-crystallization done by ethanol only in the case of 4-aminobenzonitrile containing derivatives. Purity of compounds determined by the measuring melting point, after filtration, the product was vacuum dried [17].

Synthesis of N substituted phenyl-4-hydrazinyl-6-(2-morpholinoethoxy)-1, 3, 5-triazin-2-amine derivatives as compound (4a-j).

In this step the intermediate product 3 (a-j) (0.01M) refluxed with hydrazine hydrate (0.01M) as shown in Fig. 1 in presence of acetone (20 ml) was taken in a three-necked round bottom flask fitted with the water condenser. Basicity was maintained (0.1M) NaOH. Completion of the reaction was monitored by TLC with appropriate solvent systems. The visualization of the compound was carried out in an iodine chamber. The product was purified by re-crystallization by using ethanol in the case of 4-aminobenzonitrile containing triazine. Thereafter compound was filtered and vacuum dried.

Synthesis of of N-phenylamino -1, 3, 5-triazin-2-yl)-4, 5-dihydro-1H-pyrazol-3-yl) vinyl)-2-methoxyphenol derivatives as compound 5(a-j)- In the final step as shown in Fig. 1 above product 4(a-j) (0.01M) was conjugated to 1,7-bis(4-hydroxy-3-methoxyphenyl) hepta-1,6-diene-3,5-dione (curcumin, 0.01M) at reflux temperature in presence of glacial acetic acid (30 ml). Completion of the reaction was monitored by the TLC (acetone: benzene, 1:1). The visualization of the compound was carried out in an iodine chamber. The product was re-crystallized with ethanol only in the case of 4-aminobenzonitrile containing triazine. After filtration of the compound, it was dried in a vacuum. The purity of the compound was checked by determining the melting point [18].

Antibacterial activity- Different conjugates were screened for antibacterial activity according to the guidelines of the Clinical Laboratory Standard Institute (CLSI). Different pathogenic strains of gram-negative bacteria and gram-positive bacteria were used for screening. The experiment was conducted using nutrient broth media. The nutrient broth culture with loopful bacterial strains was incubated at 37±1°C for 16–18 hrs, and the microbial culture was adjusted to the McFarland standard. Diluting the bacterial suspension with sterile solution yielded a final concentration of 1.5x10^8 CFU/mL. The plates of nutrient agar media were prepared. With the help of stainless steel cork, 5-mm diameter wells were made into swabbed agar plates. To make a neat solution, testing compounds were dissolved in dimethyl sulfoxide (DMSO). Following that, the wells were loaded with 30 µl of testing samples and incubated at 37±1°C for 16–18 hrs and it was performed in triplicate. Their activity was assessed by measuring the zone of inhibition against bacterial pathogens using a zone reader (Himedia zone scale) [19].

Minimum inhibitory concentration (MIC) and Minimum bactericidal concentration (MBC)- Different bacterial strains were screened for MIC. Each well of a 96-well microplate was filled with 75 µl nutrient broth and for testing drugs of many concentrations in 2-fold dilution as 1024, 512, 256, 128, 64, 32, 16 µg/ml. Then 75 µl of each test organism was inserted in each well. The remaining

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two wells in each row of microplates were then allowed to go negative control (i.e., the extract was substituted with 50 µl of 10% DMSO) and positive control as no extract but 50 µl antibiotic. Finally, each well-received 50 µl of resazurin solution. The experiments were conducted in triplets and bacterial isolates were incubated for 24 h at 37±1°C. The last volume of a drug capable of penetrating visible microbial growth of microorganisms or no change in resazurin dye colour was then reported as the MIC value [20]. To assess MBC, a loopful of the mixture of each well that did not exhibit microbial growth was sub-cultured by streaking on nutrient agar plates and incubated for 24 h at 37±1°C. MBC was recorded as the lowest concentrations of extract that did not display any established colony [21].

Antifungal activity- This method was performed according to the guidelines of CLSI. Sabouraud dextrose agar/broth media for *C. albicans* and potato dextrose agar for *A. fumigatus* were used to experiment. Broth culture with a loopful fungal strain was incubated at 37±1°C for 16–18 hrs for yeast and 28±1°C for 72 hrs mold and microbial culture was adjusted to McFarland standard for yeast 1.5 x 10^8 and spore suspension adjusted for mold at 5x10^4 spores. Total 20 ml media were poured then solidifying plates were swabbed using a sterilized cotton swab with 100 µl. With the help of a cork borer of 5 mm diameter wells were made. Testing compounds were dissolved in DMSO to make a neat solution. After that the wells were loaded with 30 µl of testing samples and allowed to incubate at 37±1°C for 16–18 hrs for yeast and 28±1°C for 72 hrs mold. Their activity was evaluated by measuring the zone of inhibition. The procedure was performed in triplicate for the pathogen. The compound was tested against the test organism in triplicate [19].

Statistical Analysis- The antibacterial activity of different curcumin conjugates synthesized in the present study was analyzed using two-way Analysis of Variance (ANOVA) followed by F-test and the significance was tested at 5%, 1% and 0.1% and results interpreted accordingly.

RESULTS

Chemistry and characterization of synthesized compounds- The designed library of target compounds and respective intermediates were synthesized as outlined in Fig. 1. Aromatic amines (4-aminobenzonitrile, 4-nitroaniline, 3-nitroaniline, 3-floroaniline, 4-bromoaniline, 3-chloroaniline, 2-niroaniline, 2-chloroaniline, and aniline) and 2, 4, 6, - trichloro-s-triazine are the reactants involved in the first step in the scheme for the synthesis of curcumin conjugates. The formation of mono substituted triazine occurred as a result of a nucleophilic aromatic substitution reaction with the hydrolysis of one of the chloro groups in the form of HCl in the presence of acetone as a solvent and sodium bicarbonate ($NaHCO_3$) as a neutralizing agent. In the second step an intermediate 2(a-j) reacted with 2-(morpholin-4-yl) ethanol, where OH group of morpholine hydrolyzed with one of the chloro group via nucleophilic substitution reaction of s-triazine to form di-substituted s-triazine derivative. This reaction took place in the presence of acetone and liberated acid (HCl), which was neutralized by sodium hydroxide (NaOH). The second intermediate 3(a-j) of the next step, then reacted with hydrazine hydrate to form tri substituted 1, 3, 5-triazine with HCl liberation. In the last step, curcumin conjugated to 4(a-j) intermediate that resulted in the cyclization of diketone moiety of curcumin by hydrazine hydrate with the liberation of a water molecule to form final product as N-phenyl-4-hydrazinyl-6-)2- morpholinoethoxy)-1,3,5-triazine-2-amine derivatives. The final compounds were characterized by various spectroscopic data's such as FT-IR, ^{1}H NMR, ^{13}C NMR and Mass spectra.

4-((4-(3,5-bis(4-hydroxy-3-methoxyphenethyl)-4,5-dihydro-1Hpyrazol-1-yl)-6 (2morpholinoethoxy)-1,3,5-triazin-2-yl)amino)benzonitrile sulfate hydrate. A2- Yellow amorphous solid; Yield: 40%; M.P.:210^{0}C FTIR (vmax; cm^{-1} KBr): 3196.38 (O-H stretching), 3080.37 (N-H stretching), 2923.38 (Aromatic C-H stretching), 2230.42 (OCH_3 stretching), 1738.28 (C=O stretching), 1683.31 (C=N stretching), 1586.20 (CH_2 bending), 1551.21 (C=C stretching), 1367.27 (N-N stretching), 779.42; ^{1}H NMR (400MHz, DMSO-d6, TMS) δ ppm: 10.84 (s, 2H, Ar-OHx2), 7.46 (d, 2H, J=0.01 Hz, Ar-H), 7.32 (d, 2H, J=0.71 Hz, Ar-H), 6.81 (d, 1H, J=0.78 Hz, pyrazole-H), 6.77 (d, 2H, J=0.01 Hz, Ar-H), 6.73 (d, 2H, J=0.78 Hz, Ar-H), 6.57 (d, 2H, J=0.72 Hz, Ar-H), 3.83 (s, 1H, NH), 3.68 (s, 6H, OCH_3x2), 3.60 (t, 2H, J=3.47 Hz, CH_2, methylene), 3.58 (d, 4H, J=0.14 Hz, morpholine-H), 2.51-1.91 (m, 4H, aliphatic CHx4), 2.49 (t, 2H, J=3.60 Hz, CH_2, methylene), 2.50 (d, 4H, J=3.60 Hz, morpholine-H); ^{13}C NMR (100MHz, DMSO-d6) δ ppm:172.07, 165.52, 164.39, 161.36, 148.19, 133.07, 132.73, 124.07, 120.57, 119.04, 113.28, 108.87,

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108.73, 107.86, 104.95, 40.03, 39.82, 39.62, 39.41, 39.20, 38.99, 38.78, 21.40, 21.04; GC-MS: 281.0 (Triazine-Morpholine-M), 326.5 (Triazine- morpholine and amine, M+H).

4-((4-(3,5-bis(4-hydroxy-3-methoxyphenethyl)-4,5-dihydro-1Hpyrazol-1-yl)-6-(2-morpholinoethoxy)-1,3,5-triazin-2-yl)amino)benzonitrile. A2- Dark brown amorphous solid; Yield: 44%; M.P.:180^{0}C; FTIR (vmax; cm^{-1} KBr): 3196.38 (O-H stretching), 3080.37 (N-H stretching), 2923.38 (Aromatic C-H stretching), 2230.42 (OCH_3 stretching), 1738.28 (C=O stretching), 1683.31 (C=N stretching), 1586.20 (CH_2 bending), 1551.21 (C=C stretching), 1367.27 (N-N stretching), 779.42; ^{1}H NMR (400MHz, DMSO-d6, TMS) δ ppm: 10.84 (s, 2H, Ar-OHx2), 7.46 (d, 2H, J=0.01 Hz,Ar-H), 7.32 (d, 2H, J=0.71 Hz, Ar-H), 6.81 (d, 1H, J=0.78 Hz, pyrazole-H), 6.77 (d, 2H, J=0.01 Hz, Ar-H), 6.73 (d, 2H, J=0.78 Hz, Ar-H), 6.57 (d, 2H, J=0.72 Hz, Ar-H), 3.83 (s, 1H, NH), 3.68 (s, 6H, OCH_3x2), 3.60 (t, 2H, J=3.47 Hz, CH_2, methylene), 3.58 (d, 4H, J=0.14 Hz, morpholine-H), 2.51-1.91 (m, 4H, aliphatic CHx4), 2.49 (t, 2H, J=3.60 Hz, CH_2, methylene), 2.50 (d, 4H, J=3.60 Hz, morpholine-H); ^{13}C NMR (100MHz, DMSO-d6) δ ppm:172.07, 165.52, 164.39, 161.36, 148.19, 133.07, 132.73, 124.07, 120.57, 119.04, 113.28, 108.87, 108.73, 107.86, 104.95, 40.03, 39.82, 39.62, 39.41, 39.20, 38.99, 38.78, 21.40, 21.04; GC-MS: 281.0 (Triazine-Morpholine-M), 326.5 (Triazine-morpholine and amine, M+H).

4-(2-(1-(4-((4-fluorophenyl)amino)-6-(2-morpholinoethoxy)-1,3,5-triazin-2-yl)-3(4-hydroxy-3-methoxystyryl)-4,5-dihydro-1Hpyrazol-5-yl)ethyl)-2-methoxyphenol. A3- Dark brown amorphous solid; Yield: 67.05%; M.P.:58^{0}C; FTIR (vmax; cm^{-1} KBr): 3367.80 (O-H stretching), 1579.51 (C=N stretching), 1506.50 (CH_2 bending), 1395.27 (N-N stretching), 1262.78, 1124.04 (C-F stretching), 906.25, 608.10; ^{1}H NMR (400MHz, DMSO-d6, TMS) δ ppm: 8.08 (s, 2H, Ar-OHx2), 7.63 (d, 2H, J=1.43 Hz,Ar-H), 7.46 (d, 2H, J=1.55 Hz, Ar-H), 7.14 (d, 1H, J=1.42 Hz, Ar-H), 7.04 (d, 2H, J=0.72 Hz, Ar-H), 7.00 (d, 2H, J=0.72 Hz, Ar-H), 6.97 (t, 2H, J=3.69 Hz, CHx-2), 6.95 (t, 2H, J=0.72 Hz, CHx-2), 6.70 (s, 1H,pyrazole-H), 4.08 (t, 2H, J=3.86 Hz, CH_2, methylene), 3.83 (s, 1H, NH), 3.81 (s, 6H, OCH_3x2), 3.57-2.50 (m, 8H, morpholine-H), 2.53 (t, 2H, J=15.53 Hz, CH_2, methylene); ^{13}C NMR (100MHz, DMSO-d6) δ ppm:78.90, 78.11, 78.57, 78.24, 66.11, 60.91, 60.47, 57.99, 56.46, 53.53, 53.32, 40.12, 39.91, 39.70, 39.49, 39.28, 39.07, 38.86, 21.40, 20.61; GC-MS: 281.0 (Triazine-Morpholine-M), 355.1 (Triazine-morpholine and amine, M+2H).

4-(2-(1-(4-((4-bromophenyl)amino)-6-(2-morpholinoethoxy)-1,3,5-triazin-2-yl)-3-(4-hydroxy-3-methoxystyryl)-4,5-dihydro-1Hpyrazol-5-yl)ethyl)-2-methoxyphenol. A4- Dark brown amorphous solid; Yield: 66.07%; M.P.:84^{0}C; FTIR (vmax; cm^{-1} KBr): 3321.49 (O-H stretching), 1567.89 (C=N stretching), 1510.78 (CH_2 bending), 1396.32 (C-Br stretching), 1256.03, 1019.69, 905.06; ^{1}H NMR (400MHz, DMSO-d6, TMS) δ ppm: 8.19 (s, 2H, Ar-OHx2), 7.44 (d, 2H, J=0.32 Hz,Ar-H), 7.40 (d, 2H, J=0.34 Hz, Ar-H), 7.38 (d, 1H, J=0.70 Hz, Ar-H), 6.82 (d, 2H, J=0.34 Hz, Ar-H), 6.80 (d, 2H, J=0.05 Hz, Ar-H), 6.77 (t, 2H, J=0.32 Hz, CHx-2), 6.74 (t, 2H, J=0.34 Hz, CHx-2), 6.68 (s, 1H,pyrazole-H), 4.10 (t, 2H, J=3.86 Hz, CH_2, methylene), 3.92 (s, 1H, NH), 3.82 (s, 6H, OCH_3x2), 3.58-2.51 (m, 8H, morpholine-H), 2.54 (t, 2H, J=3.01 Hz, CH_2, methylene); ^{13}C NMR (100MHz, DMSO-d6) δ ppm:79.06, 78.73, 78.41, 66.05, 60.49, 57.99, 53.55, 40.17, 39.96, 39.75, 39.51, 39.33, 39.13, 38.92, 21.25; GC-MS: 281.0 (Triazine-Morpholine-M), 417.3 (Triazine-morpholine and amine, M+3H), 647.5 (Curcumin-Para bromoaniline M+Cl).

4-(2-(1-(4-((3-chlorophenyl)amino)-6-(2-morpholinoethoxy)-1,3,5-triazin-2-yl)-3-(4-hydroxy-3-methoxystyryl)-4,5-dihydro-1Hpyrazol-5-yl)ethyl)-2-methoxyphenol. A5- Dark brown amorphous solid; Yield: 32.67%; M.P.:80^{0}C; FTIR (vmax; cm^{-1} KBr): 3308 (O-H streching), 2924 (C-H streching), 1643 (C=C streching), 1395 (CH_3), 1395 (CH_3 vibration), 1340 (C-H alkane stretching), 1127, 1021 (C-O streching), 1095 (C-O-C), 905 (CH= CH_2), 671 (C-Cl), 538; ^{1}H NMR (400MHz, DMSO, TMS) δ ppm: 8.19 (s, 2H, Ar-OHx2), 8.04 (s, 1H, Ar-NH), 7.25 (d, 2H, J=1.64 Hz, Ar-H), 7.23 (d, 1H, J=2.7 Hz, Ar-H), 7.22 (d, 1H, J=2.3 Hz, Ar-H), 7.04 (d, 1H, J=1.7 Hz, Ar-H), 7.03 (d, 1H, J=4.6 Hz, Ar-H), 7.01 (d, 1H, J=1.4 Hz, Ar-H), 6.97 (d, 1H, J=4.6 Hz, Ar-H), 6.95 (d, 1H, pyrazole-H), 6.78 (d, 1H, J=1.5 Hz, Ar-H), 6.66 (d, 2H, J=4.6 Hz, Ar-H), 6.63 (d, 1H, J=1.2 Hz, Ar-H), 4.18 (s, 2H, CH_2, Methylene), 3.85-3.76 (s, 6H,2xO CH_3), 2.72 (s, 2H, CH_2, Methylene), 3.57-2.51 (m, 8H, 4x CH_2, Morpholine-H), 2.53 (s, 2H, CH_2, Methylene); 13C NMR (400MHz, DMSO) δ ppm:79.36, 78.93, 78.73, 78.43, 66.14, 59.13, 53.62, 43.12, 39.96, 39.75, 39.55, 39.34,

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39.13, 38.92; GC-MS: 131.0 (Morpholine-M), 253 (Triazine- amine, M+H), 377 (Curcumin, M+Cl).

4-(2-(5-(4-hydroxy-3-methoxyphenethyl)-1-(4-(2-morpholinoethoxy)-6-((3nitrophenyl)amino)-1,3,5-triazin-2-yl)-4,5-dihydro-1H-pyrazol-3-yl)vinyl)-2-methoxyphenol. A6- Dark brown amorphous solid; Yield: 93%; M.P.:80^{0}C; FTIR (vmax; cm^{-1} KBr): 3372.90 (O-H stretching), 1580.12 (C=N stretching), 1519.89 (NO2 stretching), 1396.14 (CH_3 vibration), 1342.84 (N-N stretching), 1256.92, 1093.49, 905.63; ^{1}H NMR (400MHz, DMSO-d6, TMS) δ ppm: 8.19 (s, 2H, Ar-OHx2), 7.51 (d, 1H, J=1.00 Hz,Ar-H), 7.13 (d, 1H, J=1.17 Hz, Ar-H), 7.12 (d, 1H, J=1.16 Hz, Ar-H), 7.10 (d, 1H, J=2.58 Hz, Ar-H), pyrazole-H), 6.79 (d, 2H, J=11.18 Hz, Ar-H), 6.63 (d, 2H, J=0.86 Hz, Ar-H), 6.54 (d, 2H, J=1.16 Hz, Ar-H), 6.53 (t, 4H, J=2.58 Hz, CHx-4), 6.51 (s, 1H,pyrazole-H), 4.11 (t, 2H, J=12.07 Hz, CH_2), 3.99 (s, 1H, NH), 3.83 (s, 6H, O CH_3x2), 3.56-2.52 (m, 8H, morpholine-H), 2.55 (t, 2H, J=3.01 Hz, CH_2); ^{13}C NMR (100MHz, DMSO-d6) δ ppm:79.06, 78.93, 78.72, 78.04, 66.12, 66.07, 60.50, 58.01, 53.55, 53.34, 40.16, 39.95, 39.74, 39.53, 39.33, 39.12, 38.91, 21.07; GC-MS: 281.0 (Triazine-Morpholine-M), 346.2 (Triazine-morpholine and amine, M+H), 617.3 (Curcumin-meta nitroaniline M+Cl).

4-((4-(3,5-bis(4-hydroxy-3-methoxyphenethyl)-4,5-dihydro-1Hpyrazol-1-yl)-6-(2 morpholinoethoxy)-1,3,5-triazin-2-yl)amino)benzonitrile sulfate hydrate. A7- Yellow amorphous solid; Yield: 40%; M.P.:210^{0}C FTIR (vmax; cm^{-1} KBr): 3196.38 (O-H stretching), 3080.37 (N-H stretching), 2923.38 (Aromatic C-H stretching), 2230.42 (OCH_3 stretching), 1738.28 (C=O stretching), 1683.31 (C=N stretching), 1586.20 (CH_2 bending), 1551.21 (C=C stretching), 1367.27 (N-N stretching), 779.42; ^{1}H NMR (400MHz, DMSO-d6, TMS) δ ppm: 10.84 (s, 2H, Ar-OHx2), 7.46 (d, 2H, J=0.01 Hz, Ar-H), 7.32 (d, 2H, J=0.71 Hz, Ar-H), 6.81 (d, 1H, J=0.78 Hz, pyrazole-H), 6.77 (d, 2H, J=0.01 Hz, Ar-H), 6.73 (d, 2H, J=0.78 Hz, Ar-H), 6.57 (d, 2H, J=0.72 Hz, Ar-H), 3.83 (s, 1H, NH), 3.68 (s, 6H, OCH_3x2), 3.60 (t, 2H, J=3.47 Hz, CH_2, methylene), 3.58 (d, 4H, J=0.14 Hz, morpholine-H), 2.51-1.91 (m, 4H, aliphatic CHx4), 2.49 (t, 2H, J=3.60 Hz, CH_2, methylene), 2.50 (d, 4H, J=3.60 Hz, morpholine-H); ^{13}C NMR (100MHz, DMSO-d6) δ ppm:172.07, 165.52, 164.39, 161.36, 148.19, 133.07, 132.73, 124.07, 120.57, 119.04, 113.28, 108.87, 108.73, 107.86, 104.95, 40.03, 39.82, 39.62, 39.41, 39.20, 38.99, 38.78, 21.40, 21.04; GC-MS: 281.0 (Triazine-Morpholine-M), 326.5 (Triazine- morpholine and amine, M+H).

4-(2-(1-(4-((2-chlorophenyl)amino)-6-(2-morpholino ethoxy)-1,3,5-triazin-2-yl)-3-(4-hydroxy-3-metho xystyryl)-4,5-dihydro-1Hpyrazol-5-yl)ethyl)-2-methoxy phenol. A8- Dark brown amorphous solid; Yield: 81.15 %; M.P.:150^{0}C; FTIR (vmax; cm^{-1} KBr): 3371.84 (O-H stretching), 1562.62 (C=N stretching), 1393.29 (CH_3 vibration), 1260.51 (N-N stretching), 1125.11, 1092.48, 749.94 (C-Cl stretching); ^{1}H NMR (400MHz, DMSO-d6, TMS) δ ppm: 8.17 (s, 2H, Ar-OHx2), 7.44 (d, 1H, J=0.76 Hz,Ar-H), 7.42 (d, 1H, J=0.89 Hz, Ar-H), 7.32 (d, 1H, J=1.36 Hz, Ar-H), 7.31 (d, 1H, J=0.89 Hz, Ar-H), 7.16 (d, 2H, J=5.19 Hz, Ar-H), 7.11 (d, 2H, J=1.36 Hz, Ar-H), 6.79 (d, 2H, J=0.89 Hz, Ar-H), 6.76-6.67 (t, 4H, J=5.19 Hz, CHx-4), 6.63 (s, 1H,pyrazole-H), 4.11 (t, 2H, J=5.24 Hz, CH_2), 3.86 (s, 1H, NH), 3.82 (s, 6H, OCH_3x2), 3.57-2.52 (m, 8H, morpholine-H), 2.56 (t, 2H, J=11.38 Hz, CH_2); ^{13}C NMR (100MHz, DMSO-d6) δ ppm: 129.07, 127.05, 79.02, 78.89, 78.69, 78.36, 66.07, 60.50, 58.00, 53.55, 53.34, 40.17, 39.96, 39.75, 39.54, 39.33, 39.12, 38.92, 21.42; GC-MS: 282.7 (Triazine-Morpholine-M+H), 601.2 (Curcumin-Para chloroaniline M+Cl), 697.2 (M).

4-(2-(5-(4-hydroxy-3-methoxyphenethyl)-1-(4-(2-morpholinoethoxy)-6-((4 nitrophenyl)amino)-1,3,5-triazin-2-yl)-4,5-dihydro-1H-pyrazol-3-yl)vinyl)-2-methoxyphenol. A9- Dark brown amorphous solid; Yield: 71%; M.P.:60^{0}C; FTIR (vmax; cm^{-1} KBr): 3246.17 (O-H stretching), 2933.11 (C-H stretching), 1729.71 (C=O stretching), 1568.63 (C=N stretching), 1498.61 (NO2 stretching), 1378.73 (CH_3 vibration), 1323.84 (N-N stretching), 1253.52, 1023.08, 905.28; ^{1}H NMR (400MHz, DMSO-d6, TMS) δ ppm: 8.17 (s, 2H, Ar-OHx2), 8.06 (d, 2H, J=4.91 Hz,Ar-H), 7.53 (d, 2H, J=1.27 Hz, Ar-H), 7.18 (d, 2H, J=2.62 Hz, Ar-H), 7.09 (d, 2H, J=2.61 Hz, Ar-H), 7.00 (d, 2H, J=1.27 Hz, Ar-H), 6.97 (t, 2H, J=4.25 Hz, CHx2), 6.95 (t, 2H, J=2.62 Hz, CHx2), 6.67 (s, 1H,pyrazole-H), 4.34 (t, 2H, J=2.21 Hz, CH_2), 3.92 (s, 1H, NH), 3.84 (s, 6H, OCH_3x2), 3.70-2.55 (m, 8H, morpholine-H), 2.44 (t, 2H, J=3.85 Hz, CH_2); ^{13}C NMR (100MHz, DMSO-d6) δ ppm:78.81, 78.48, 78.16, 39.87, 39.67, 39.46; GC-MS: 281.1 (Triazine-Morpholine-M), 345.2 (Triazine-morpholine and amine, M).

ISSN (O): 2581-8740 | ISSN (P): 2581-8732
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4-(2-(5-(4-hydroxy-3-methoxyphenethyl)-1-(4-(2-morpholinoethoxy)-6-(phenylamino)-1,3,5-triazin-2-yl)-4,5-dihydro-1H-pyrazol-3-yl)vinyl)-2-methoxyphenol.

A10- Dark brown amorphous solid; Yield: 68.49%; M.P.:160^{0}C;FTIR (vmax; cm^{-1} KBr): 3032.76 (C-H stretching), 1720.60 (C=O stretching), 1551.29 (C=N stretching), 1389.86 (CH_3 vibration), 1324.82 (N-N stretching), 1264.18, 1022.77, 958.75; ^{1}H NMR (400MHz, DMSO-d6, TMS) δ ppm: 9.65 (s, 2H, Ar-OHx2), 7.62 (d, 2H, J=1.10 Hz,Ar-H), 7.31 (d, 2H, J=4.65 Hz, Ar-H), 7.14 (d, 2H, J=1.87 Hz, Ar-H), 7.10 (d, 2H, J=5.70 Hz, Ar-H), 7.07 (d, 1H, J=5.06 Hz, Ar-H), 6.87 (d, 2H, J=2.26 Hz, Ar-H), 6.86 (t, 2H, J=2.17 Hz, CHx2), 6.84 (t, 2H, J=1.09 Hz, CHx2), 6.68 (s, 1H, pyrazole-H), 5.87 (t, 2H, J=1.87 Hz, CH_2), 3.84 (s, 1H, NH), 3.35 (s, 6H, OCH_3x2), 3.56-2.49 (m, 8H, morpholine-H), 2.51 (t, 2H, J=1.45 Hz, CH_2); ^{13}C NMR (100MHz, DMSO-d6) δ ppm:183.17, 165.57, 164.42, 161.40, 154.21, 149.33, 148.23, 147.96, 140.66, 137.28, 128.74, 126.30, 124.11, 121.17, 121.06, 115.67, 111.32, 107.93, 55.67, 40.12, 39.91, 39.08, 38.87, 21.48; GC-MS: 281.1 (Triazine-Morpholine-M), 537.0 (Curcumin-triazine-aniline M+ ^{3}H).

Antibacterial activity of synthesized curcumin derivatives- The antibacterial activity of ten curcumin pyrazole triazine conjugates was evaluated and the screening results are reported in Table 1. It was evident from the assay that compound 5 and 8 both have chlorine atom in their aniline moiety enhanced their antimicrobial activity. In the case of the most potent drug A5 it has been reported that among gram-positive bacteria *B. subtilis, S. aureus* and *Clostridium perfringens* were found to have the highest zone of inhibition ranged 27. Total 26 mm and 25 mm, among gram-negative bacteria drug was found most potent against *P. aeruginosa* with a zone of inhibition of 28 mm. The overall efficacy of different compounds can be found as 5>8>3>4>6>7>10>1>2>9.

Table 1: Antibacterial activity of curcumin pyrazole triazine conjugates

S. No.	Test organisms	Zone of inhibition (mm)									
		A1	A2	A3	A4	A5	A6	A7	A8	A9	A10
1	*L. monocytogenes*	5.66	7.33	14.66	14.66	13.66	15.00	6.33	14.00	7	8.33
2	*B. cereus*	8.66	8.33	13.33	11.33	12.33	10.33	11.33	20.33	7.33	10.33
3	*B. subtilis*	8.33	10.33	15.66	27.66	26.33	22.66	17.66	26.67	7.33	9.00
4	*C. perfringens*	11.00	10	19.66	16.66	24.66	20.66	11.33	15.67	9.66	11.67
5	*S. aureus*	7.00	8.33	31.66	27.66	26.33	23.66	13.66	31.33	7.66	10.33
6	*S. pyogenes*	5.66	6.33	6.66	0	11.33	0	8.00	13.33	0	6.33
7	*E. coli*	6.00	6	20.00	18.66	17.66	16.66	11.66	14.67	6.33	6.00
8	*P. aeruginosa*	0	0	22.66	26.66	27.66	25.66	11.66	25.67	0	7.00
9	*S. typhi*	6.33	6.33	14.33	16.66	14.66	17.00	11.33	17.67	6	6.00
10	*S. dysenteriae*	6.33	6.66	15.66	16.00	15.33	16.33	11.66	17.33	7.66	6.66
11	*V. cholera*	6.33	6.66	15.33	15.00	15.66	11.66	11.66	17.67	7.66	6.66
12	*C. jejuni*	6.66	6.33	12.33	12.33	12.33	11.33	0	16.00	0	6.00
13	*H. pylori*	6.33	0	11.33	11.66	14.66	12.00	6.00	13.33	7.00	10.33

* includes well size of 5 mm diameter

A1=4-aminobenzonitrile, A 2= 4-aminobenzonitrile, A3= 3-floroaniline, A 4= 4-bromoaniline, A5= 3-cloroaniline, A6= 3-nitroaniline, A7 = 2-nitroaniline, A8 = 2-cloroaniline, A9= 4-nitroaniline, 10 = Aniline

ISSN (O): 2581-8740 | ISSN (P): 2581-8732
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DOI: 10.21276/SSR-IIJLS.2021.7.4.2

Minimum inhibitory concentration and Minimum Bactericidal concentration- The minimum inhibitory concentration of synthesized Curcumin pyrazole triazine conjugate A5 having a best antimicrobial activity was determined as depicted in Table 2 by resazurin based micro broth dilution method. The drugs were taken in the concentration ranges as 512, 256, 128, 64 and 32 µg/ml. Among gram-negative bacteria, the best MIC was observed in the case of *P. aeruginosa* 32 µg/ml as compared to *H. pylori* 64 µg/ml and *E. coli, S. dysentriae* with MIC value 128 µg/ml. Among Gram-positive bacteria*, S. aureus* was found to have the lowest MIC that is 32 µg/ml than *B. subtilis* and *L. monocytogenes* showed MIC at 64 µg/ml and the highest value 128 µg/ml was observed in the case of *C. perfringens.* Both gram-negative bacteria *P. aeruginosa* and gram-positive bacteria *S. aureus* were found to have good minimum bactericidal activity at 128 µg/ml concentration. Except *for E. coli* with an MBC value of 512 µg/ml all microbes such as *B. subtilis, S. dysentriae, L. monocytogenes* and *H. pylori* reported with highest MBC value of concentration 256 µg/ml.

Table 2: MIC and MBC of bacterial pathogens

S.No.	Organisms	MIC (µg/ml)	MBC (µg/ml)
1	*E. coli*	128	512
2	*S. aureus*	32	128
3	*B. subtilis*	64	256
4	*S. dysenteriae*	128	256
5	*C. perfringens*	128	256
6	*L. monocytogenes*	64	256
7	*H. pylori*	64	256
8	*P. aeruginosa*	32	128

Antifungal activity of synthesized curcumin derivatives- Varied curcumin pyrazole triazine conjugates were tested for antifungal activity using the agar well diffusion process, shown in Table 3 in accordance with CLSI. All synthesized compounds were found to be resistant to the pathogenic mold *A. fumigatus*. Only four drugs displayed antifungal efficacy against pathogenic yeast *Candida albicans* with compound A5 having the most potent zone of inhibition (21 mm) and compound A4 having the least potent zone of inhibition (14 mm). As a consequence of the findings, chlorine-containing aniline derivatives were discovered to be the most potent antifungal agent. The presence of a chlorine atom in meta-position increased its antifungal effect. In comparison to these halogens, fluorine at ortho-position and bromine at para-position yielded important effects, but not as much as the chlorine-containing aniline derivatives of curcumin pyrazole triazine conjugates.

Table 3: Antifungal activity of curcumin pyrazole triazine conjugates

Compounds	Zone of inhibition (mm) *	
	C. albicans	*A. fumigatus*
A1	0	0
A2	0	0
A3	15	0
A4	14	0
A5	21	0
A6	0	0
A7	0	0
A8	17	0
A9	0	0
A10	0	0

* includes the well size of 5 mm diameter
A1=4-aminobenzonitrile, A 2= 4-aminobenzonitrile, A3= 3-floroaniline, A 4 = 4-bromoaniline, A5 = 3-cloroaniline, A6= 3-nitroaniline, A7 = 2-nitroaniline, A8 = 2-cloroaniline, A9= 4-nitroaniline, 10 = Aniline

DISCUSSION

Concerned about the ongoing emergence of multidrug-resistant bacterial and fungal disease, there is an urgent need for the development of new antimicrobial agents [22,23]. Even newly developed antibiotics are ineffective against these MDRs [24]. In this regard, we proposed unique curcumin pyrazole triazine conjugates that have been found to have antibacterial and antifungal action. Nitrogen-containing heterocyclic compounds with higher binding affinities to biological receptors have piqued the interest of many researchers as compounds to use as conjugating moieties [25]. As a result of its increased importance in biological studies, particularly for antibacterial [26] and antifungal activity [19,27]. A nitrogen-containing heterocyclic compound with good biological activities known as 2, 4, 6-trichloro-1, 3, 5-triazine has been used as a conjugating agent with curcumin. For verification of synthesized curcumin pyrazole triazine conjugates, their structures were characterized by different spectroscopic methods such as FTIR, FT NMR (^{1}H NMR and ^{13}C NMR) and Mass Spectrometry. Thereafter synthesized curcumin pyrazole triazine conjugates were evaluated for the antibacterial and

ISSN (O): 2581-8740 | ISSN (P): 2581-8732
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DOI: 10.21276/SSR-IIJLS.2021.7.4.2

antifungal activity that resulted in significant outcomes against both fungal and bacterial pathogens.

Against both types of pathogens compound A5 containing 3-chloroaniline moiety was found to possess the most potent antimicrobial activity. Among six gram-positive bacteria only four *L. monocytogenes, S. aureus, B. subtilis* and *C. perfringens* were found to be most susceptible to compound A5 having a zone of inhibition 14, 26, 27, and 25 mm and MIC values of 64, 32, 64 and 128 µg/ml. Among seven gram-negative bacterial *E. coli*, *S. dysenteriae*, *H. pylori, P. aeruginosa* was reported with the best zone of inhibition 18, 16, 15, and 28 mm and MIC values with 128, 128, 64 and 32µg/ml. Regarding antifungal agent similar compound A5 was found to be the most potent against *C. albicans* with zone of inhibition of 21 mm. All synthesized compounds were found to be resistant to the pathogenic *A. fumigatus*. According to these findings, curcumin pyrazole triazine conjugates containing halogen derived anilines such as Cl, Br, and F can bear a biologically significant hybrid molecule, which could address a lead compound for various targets and may provide the possibility of reducing multidrug resistance.

CONCLUSIONS

Novel Curcumin pyrazole triazine conjugates were synthesized bearing covalent linkage with suitable ligands to enhance its biological activity and characterized by the FTIR ^{1}H NMR, ^{13}C NMR and Mass spectrometry. The investigation of antibacterial and antifungal screening data revealed that all the Curcumin derivatives bearing halogen moiety showed moderate to good bacterial and fungal inhibition. Significant results were obtained in the case of both gram-positive bacteria *Bacillus subtilis* with the zone of inhibition 28 mm and MIC 32 µg/ml and gram-negative bacteria *Pseudomonas aeruginosa* with the zone of inhibition 28 mm and MIC 32 µg/ml. Among pathogenic fungi, *Candida albicans* was found to be most susceptible to compound A5.

The pharmaceutically important hybrid molecules can be used in future to cure bacterial and fungal diseases.

ACKNOWLEDGMENTS

The authors are thankful to the Department of Pharmaceutical Sciences and the Department of Industrial Microbiology, Sam Higginbottom University of Agriculture, Technology and Sciences for providing necessary research facilities to carrying out experiments.

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