

Original Research Article

Brine shrimp lethality assay test, *Insilico*-molecular Docking studies as a preliminary screening models of some newly synthesized 2-substituted 4,6 dichloro 1,3,5, triazine chalcone hybrids for potential cytotoxic activity

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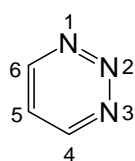
Abstract: An attempt was made in the present study to test for in vivo Brine Shrimp Lethality Assay (BSLT) and molecular docking studies of some newly synthesized 2-substituted 4,6 di chloro 1,3 triazine-chalcone hybrids as potential cytotoxic agents. Cytotoxicity was evaluated in terms of LC₅₀ (lethality concentration). Thirty nauplii were added into three replicates of each concentration of the synthesized compounds. After 24 hours the surviving brine shrimp larvae were counted and LC₅₀ was assessed. Schrodinger Mastero docker was used for insilico molecular docking studies on selected cancer protein Targets. The results drawn from the preliminary studies showed that the compounds TCH-9, 12 and 20, 24 were potent against the brine shrimp with minimum LC₅₀ values of 3.125, and 6.25µg/mL respectively. These findings suggest that the synthesized 1,3,5 triazine chalcone hybrids possess potential cytotoxic activity.

Keywords: Brine shrimp lethality assay, LC₅₀, molecular docking, Triazine chalcone hybrids

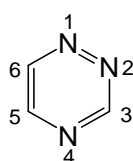
INTRODUCTION

Triazines are a class of organic nitrogen-containing six-member heterocyclic compounds known for a long period of time. They can structurally be existing as three isomers varied with their position of nitrogen atoms on the benzene ring, and are referred to

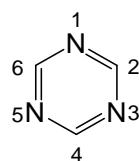
as 1,2,3-triazine (1), 1,2,4-triazine (2) and 1,3,5-triazine (3). In particular, considerable attention has been devoted to the development of 1, 3, 5-triazine derivatives in comparison with 1, 2, 3-triazine and 1,2,4-triazine derivatives, due to their variety of applications in different fields [1,2].



(1)



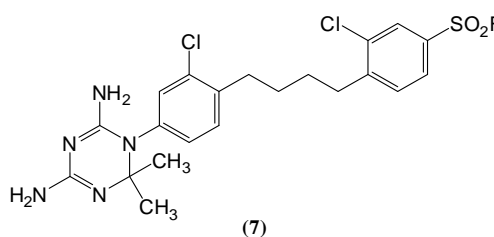
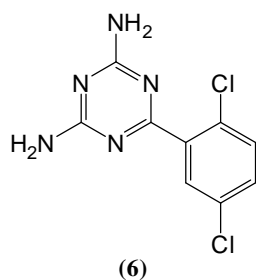
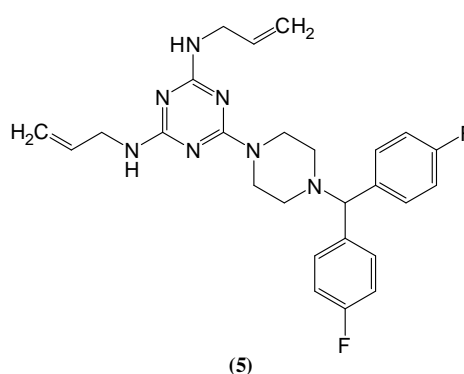
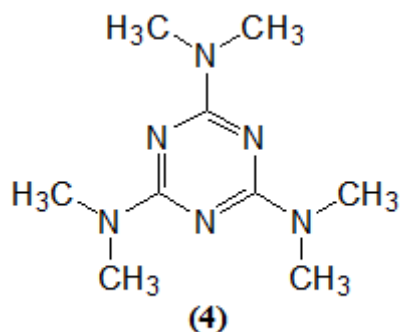
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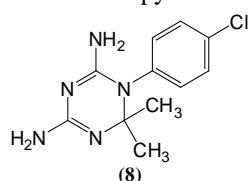
(3)

1,3,5-Triazines can also be called as symmetric or *s*-triazines. The chemistry of this group of compounds has been studied intensively since past two centuries due to their wide spread applications in the pharmaceutical, textile, plastic and rubber industries and are used as pesticides, dyestuffs, optical bleaches, explosives and surface active agents. In recent times, several studies have been carried out on the antitumor

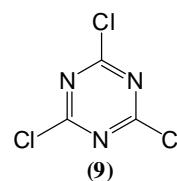
activity of 1,3,5-triazines. Some of these analogues, hexamethylmelamine (4), almitrine (5) and irsogladine (6) are clinically used as anticancer agents. Baker triazines (4, 6-Diamino-2,2-dimethyl-1,2-dihydro-1,3,5-triazine based analogs) are becoming increasingly important as pharmaceuticals. Baker triazine antifol (7) had been undergoing clinical trials as a drug candidate in cancer chemotherapy [3-7].



Although 1,3,5-triazines are well known in the context of anticancer drugs, this ring is also found in the drug used in the chemotherapy of malaria, as seen in



case of cycloguanil (8) [8]. Recently, 2, 4, 6-trisubstituted-1, 3, 5-triazine scaffolds were discovered as a potent inhibitors of *M. tuberculosis* H37Rv [9].



All 1, 3, 5-triazine derivatives that have wide practical applications are 2,4,6-mono, di- or tri-substituted, symmetrical and nonsymmetrical compounds bearing different substituents. The most important reagent for obtaining these synthetic molecule transformations is cyanuric chloride (9), due to the reactivity of the chlorine atoms towards nucleophiles [10]. Chalcones are an important group of natural products that consist of two aromatic rings joined by an $\alpha\beta$ -unsaturated carbonyl system. The $\alpha\beta$ -unsaturated carbonyl system enables chalcones and their heteroanalogs to undergo conjugated addition reactions in the presence of Lewis acid and basic catalysts. [11] Literature has indicated that this reaction has been exploited to obtain heterocyclic compounds of biological significance [12]. This Research has focused on the characterization and bioactivity study of some new fully unsaturated 2-substituted 4,6-dichloro-1,3,5-triazines-chalcone hybrids. Thirty Five libraries of 4,6 dichloro -1,3,5-triazines were proposed to synthesize for this study. Earlier at our research laboratory we synthesized 35 compounds and were characterized by FT-IR, ^1H , ^{13}C and Mass spectra [13].

Earlier in our research laboratory *In vitro* Cytotoxic activity using MTT assay method and antimicrobial studies were performed and

communicated. In this research paper we extend our work and aim for *insilico* studies and brine shrimp assay. The reasons for the selection were derived from literature reviews on previous works, from which pharmacological activity of 4,6 dichloro -1,3,5-triazines –Chalcones for Cytotoxicity and *insilico* molecular docking studies on protein targets was not demonstrated ever. The compounds were screened for cytotoxic activity using Brine shrimp lethality assay and *insilico* molecular docking studies were performed on five protein targets.

MATERIALS AND METHODS

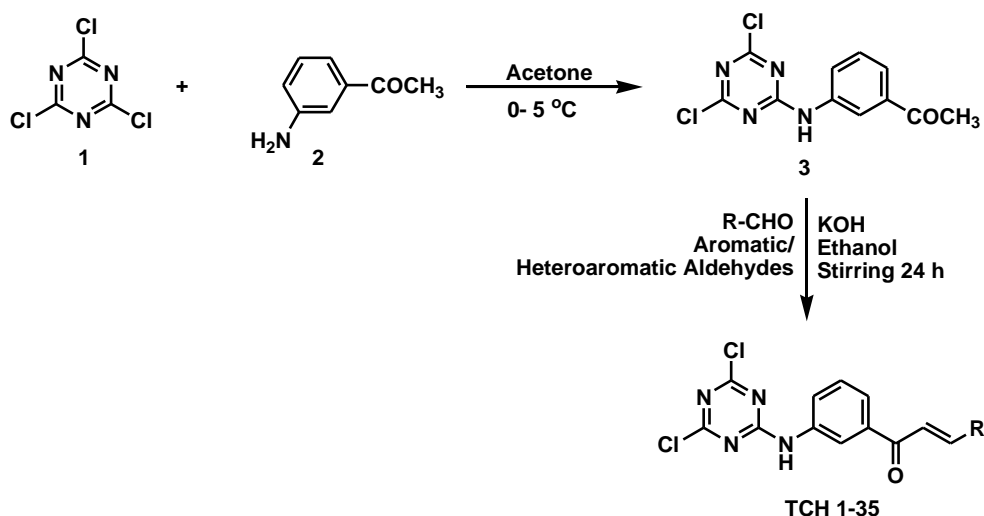
Melting points were determined on a open capillary tubes in an EZ-MELT automated digital melting point apparatus and are uncorrected. Infrared spectra were recorded neat on a Perkin Elmer spectrophotometer. And 2D-NMR spectra were recorded on a Bruker Avance DPX 400 MHz NMR spectrometer in CDCl_3 (or acetone- d_6) with TMS as an internal standard at room temperature. Electron impact (EI) High resolution mass spectra (HR-MS) were carried out on LC-MS 6100Q Mass Spectrometer (Agilent Technologies) ionisation energy 70 eV, at the Laila Impex R& D Center, Vijayawada. All reactions were monitored by TLC, which was carried out on 0.25 mm layer of Merck silica gel 60 F254 pre-coated on

aluminium sheets. Laboratory grade chemicals and solvents available commercially in high purity were used. All the prepared compounds were identified by physical properties, IR, HRMS and NMR data. Yields reported are isolated yields unless indicated otherwise.

Chemistry General

The reaction sequence intended for the preparation of title compounds (TCH 1-35) is shown in Scheme, and their physical properties are depicted. The chief intermediate in the present study 1-(3-(4,6-dichloro-1,3,5-triazin-2-ylamino)phenyl)ethanone (3) was prepared by reaction between cyanuric chloride

i.e. 2,4,6-trichloro-1,3,5-triazine (1) and 3-aminoacetophenone (2). Further, successive base catalyzed Claisen-Schmidt condensation of the compound 3 with appropriate substituted aromatic/ hetero aromatic aldehydes in the presence of 100% potassium hydroxide solution in ethanol afforded a series of 1-(3-(4,6-dichloro-1,3,5-triazin-2-ylamino)phenyl)-3-(substituted)-2-propen-1-ones (TCH 1-35) in good yield [14]. All the newly synthesized compounds were characterized by CHN elemental analysis and spectroscopic methods such as FT-IR, ^1H NMR, and mass spectral analysis. Eventually all the spectra of the new products (TCH 1-35) are in keeping with the predictable structures.



Scheme-1. Chemical synthesis of 1, 3, 5-triazine-chalcone hybrid molecules TCH 1-35

Experimental section

Synthesis of 1-(3-(4, 6-dichloro-1, 3, 5-triazin-2-ylamino)phenyl) ethanone (3)

To a solution of 2,4,6-trichloro-1,3,5-triazine (1) (0.01 M) dissolved in 20 mL of acetone, 3-aminoacetophenone (2) (0.01 M) was added slowly by delivering through a spatula in small quantities and the resulting mixture was stirred at 0-5°C temperature for 3h. The crude 1-(3-(4,6-dichloro-1,3,5-triazin-2-ylamino) phenyl) ethanone (3) was washed on the vacuum filter with cold methanol and then recrystallized from ethanol.

Synthesis of 1, 3, 5-triazine-chalcone hybrid molecules (TCH 1-35)

To a solution of 1-(3-(4,6-dichloro-1,3,5-triazin-2-ylamino)phenyl)ethanone (3) (0.005 M) and suitably substituted aldehydes (0.005 M) in ethanol (10 ml), aqueous solution of potassium hydroxide (100%) was added drop wise with continuous stirring at room temperature over a period of 10 min. The reaction mixture was then kept at room temperature for about 48 h with occasional shaking. After 48 h it was poured into ice-cold water, and then neutralized to pH 2 using 5N hydrochloric acid. The light yellow precipitate obtained was filtered, washed, dried, and recrystallized from

ethanol. The 1, 3, 5-triazine-chalcone hybrid molecules TCH 1-35 were obtained in good yield. All the synthesized compounds as mentioned in were characterized by spectroscopic methods such as FTIR, ^1H NMR, ^{13}C NMR and mass spectral analysis and presented separately under each compound.

(E)-1-(3-(4,6-dichloro-1,3,5-triazin-2-ylamino)phenyl)-3-(phenyl)-2-propen-1-one (TCH-1): Light yellow crystals. FT-IR (KBr, ν_{max} , cm^{-1}): 3155 (N-H), 3031 (C-H, aromatic), 2884 (C-H, aliphatic), 1688 (C=O), 1645 (C=C, aliphatic), 1513 (C=C, aromatic), 689 (C-Cl). ^1H NMR (400 MHz, DMSO- d_6 , δ , ppm): 7.13-7.74 (m, 9H, Ar-H), 7.78 (d, $J = 15.2$ Hz, 1H, HC=CH (H- α)), 8.01 (d, $J = 15.2$ Hz, 1H, HC=CH (H- β)), 9.74 (s, 1H, NH). ESI-MS (m/z): 372 [M+H] $^+$. Molecular formula, $\text{C}_{18}\text{H}_{12}\text{Cl}_2\text{N}_4\text{O}$, Relative mol.mass- 371, M.P 123°C, % Yield 60, Anal. Calcd : C, 58.24; H, 3.26; N, 15.09; Found: C, 58.21; H, 3.21; N, 15.05%.

(E)-1-(3-(4,6-dichloro-1,3,5-triazin-2-ylamino)phenyl)-3-(2-methylphenyl)-2-propen-1-one (TCH-2): Light yellow crystals. FT-IR (KBr, ν_{max} , cm^{-1}): 3152 (N-H), 3022 (C-H, aromatic), 2881 (C-H, aliphatic), 1689 (C=O), 1623 (C=C, aliphatic), 1501 (C=C, aromatic), 688 (C-Cl). ^1H NMR (400 MHz, DMSO- d_6 ,

δ , ppm): 2.32 (s, 3H, CH₃), 7.43-8.04 (m, 8H, Ar-H), 7.78 (d, J = 15.2 Hz, 1H, HC=CH (H- α)), 8.01 (d, J = 15.2 Hz, 1H, HC=CH (H- β)), 9.74 (s, 1H, NH). ESI-MS (m/z): 386 [M+H]⁺. Molecular formula, C₁₉H₁₄Cl₂N₄O, Relative mol.mass- 385, M.P 135^oC, % Yield 51. *Anal.* Calcd : C, 59.24; H, 3.66; N, 14.54; Found: C, 59.22; H,3.62; N, 14.52%.

(*E*)-1-(3-(4,6-dichloro-1,3,5-triazin-2-ylamino)phenyl)-3-(3-methylphenyl)-2-propen-1-one (TCH-3): Light yellow crystals. FT-IR (KBr, ν_{\max} , cm⁻¹): 3127 (N-H), 3027 (C-H, aromatic), 2777 (C-H, aliphatic), 1703 (C=O), 1603 (C=C, aliphatic), 1450 (C=C, aromatic), 688 (C-Cl). ¹H NMR (400 MHz, DMSO-d₆, δ , ppm): 2.41 (s, 3H, CH₃), 7.38-8.05 (m, 8H, Ar-H), 7.73 (d, J = 15.2 Hz, 1H, HC=CH (H- α)), 8.04 (d, J = 15.2 Hz, 1H, HC=CH (H- β)), 9.69 (s, 1H, NH). ESI-MS (m/z): 386 [M+H]⁺. Molecular formula, C₁₉H₁₄Cl₂N₄O, Relative mol.mass- 385, M.P 143^oC, % Yield 66. *Anal.* Calcd : C, 59.24; H, 3.66; N, 14.54; Found: C, 59.25; H,3.61; N, 14.53%.

(*E*)-1-(3-(4,6-dichloro-1,3,5-triazin-2-ylamino)phenyl)-3-(4-methylphenyl)-2-propen-1-one (TCH-4): Light yellow crystals. FT-IR (KBr, ν_{\max} , cm⁻¹): 3122 (N-H), 3015 (C-H, aromatic), 2762 (C-H, aliphatic), 1705 (C=O), 1601 (C=C, aliphatic), 1440 (C=C, aromatic), 685 (C-Cl). ¹H NMR (400 MHz, DMSO-d₆, δ , ppm): 2.39 (s, 3H, CH₃), 7.31-7.66 (m, 8H, Ar-H), 7.73 (d, J = 15.2 Hz, 1H, HC=CH (H- α)), 8.02 (d, J = 15.2 Hz, 1H, HC=CH (H- β)), 9.62 (s, 1H, NH). ESI-MS (m/z): 386 [M+H]⁺. Molecular formula, C₁₉H₁₄Cl₂N₄O, Relative mol.mass-385, M.P 175^oC, % Yield 68. *Anal.* Calcd : C, 59.24; H, 3.66; N, 14.54; Found: C, 59.22; H,3.64; N, 14.51%.

(*E*)-1-(3-(4,6-dichloro-1,3,5-triazin-2-ylamino)phenyl)-3-(2-methoxyphenyl)-2-propen-1-one (TCH-5): Light yellow crystals. FT-IR (KBr, ν_{\max} , cm⁻¹): 3124 (N-H), 3027 (C-H, aromatic), 2975 (C-H, aliphatic), 1700 (C=O), 1603 (C=C, aliphatic), 1417 (C=C, aromatic), 713 (C-Cl), 1171 (C-O-C), 1054 (C-O). ¹H NMR (400 MHz, DMSO-d₆, δ , ppm): 3.86 (s, 3H, OCH₃), 7.20-8.05 (m, 8H, Ar-H), 7.48 (d, J = 15.2 Hz, 1H, HC=CH (H- α)), 8.05 (d, J = 15.2 Hz, 1H, HC=CH (H- β)), 9.66 (s, 1H, NH). ESI-MS (m/z): 402 [M+H]⁺. Molecular formula, C₁₉H₁₄Cl₂N₄O₂, Relative mol.mass-401, M.P 167^oC, % Yield 71. *Anal.* Calcd : C, 56.87; H, 3.52; N, 13.96; Found: C, 56.82; H,3.51; N, 13.95%.

(*E*)-1-(3-(4,6-dichloro-1,3,5-triazin-2-ylamino)phenyl)-3-(3-methoxyphenyl)-2-propen-1-one (TCH-6): Light yellow crystals. FT-IR (KBr, ν_{\max} , cm⁻¹): 3124 (N-H), 3027 (C-H, aromatic), 2977 (C-H, aliphatic), 1700 (C=O), 1605 (C=C, aliphatic), 1457 (C=C, aromatic), 687 (C-Cl), 1171 (C-O-C), 1054 (C-O). ¹H NMR (400 MHz, DMSO-d₆, δ , ppm): 3.88 (s, 3H, OCH₃), 7.12-8.21 (m, 8H, Ar-H), 7.71 (d, J = 15.2 Hz, 1H, HC=CH (H- α)), 8.06 (d, J = 15.2 Hz, 1H, HC=CH (H-

β)), 9.65 (s, 1H, NH). ESI-MS (m/z): 402 [M+H]⁺. Molecular formula, C₁₉H₁₄Cl₂N₄O₂, Relative mol.mass-401, M.P 129^oC, % Yield 58. *Anal.* Calcd : C, 56.87; H, 3.52; N, 13.96; Found: C, 56.83; H,3.51; N, 13.91%.

(*E*)-1-(3-(4,6-dichloro-1,3,5-triazin-2-ylamino)phenyl)-3-(4-methoxyphenyl)-2-propen-1-one (TCH-7): Light yellow crystals. FT-IR (KBr, ν_{\max} , cm⁻¹): 3122 (N-H), 3021 (C-H, aromatic), 2970 (C-H, aliphatic), 1690 (C=O), 1602 (C=C, aliphatic), 1455 (C=C, aromatic), 677 (C-Cl), 1170 (C-O-C), 1055 (C-O). ¹H NMR (400 MHz, DMSO-d₆, δ , ppm): 3.86 (s, 3H, OCH₃), 7.12-7.92 (m, 8H, Ar-H), 7.71 (d, J = 15.2 Hz, 1H, HC=CH (H- α)), 8.05 (d, J = 15.2 Hz, 1H, HC=CH (H- β)), 9.75 (s, 1H, NH). ESI-MS (m/z): 402 [M+H]⁺. Molecular formula, C₁₉H₁₄Cl₂N₄O₂, Relative mol.mass-401, M.P 145^oC, % Yield 61. *Anal.* Calcd : C, 56.87; H, 3.52; N, 13.96; Found: C, 56.84; H,3.56; N, 13.96%.

(*E*)-1-(3-(4,6-dichloro-1,3,5-triazin-2-ylamino)phenyl)-3-(3-hydroxyphenyl)-2-propen-1-one (TCH-8): Light yellow crystals. FT-IR (KBr, ν_{\max} , cm⁻¹): 3445 (O-H), 3124 (N-H), 3015 (C-H, aromatic), 2984 (C-H, aliphatic), 1689 (C=O), 1606 (C=C, aliphatic), 1415 (C=C, aromatic), 676 (C-Cl), 1054 (C-O). ¹H NMR (400 MHz, DMSO-d₆, δ , ppm): 7.36-8.01 (m, 8H, Ar-H), 7.67 (d, J = 15.6 Hz, 1H, HC=CH (H- α)), 8.18 (d, J = 15.6 Hz, 1H, HC=CH (H- β)), 9.85 (s, 1H, NH), 12.32 (s, 1H, OH). ESI-MS (m/z): 388 [M+H]⁺. Molecular formula, C₁₈H₁₂Cl₂N₄O₂, Relative mol.mass- 387, M.P 122^oC, % Yield 74. *Anal.* Calcd : C, 55.83; H, 3.12; N, 14.47; Found: C, 55.85; H,3.11; N, 14.42%.

(*E*)-1-(3-(4,6-dichloro-1,3,5-triazin-2-ylamino)phenyl)-3-(4-hydroxyphenyl)-2-propen-1-one (TCH-9): Light yellow crystals. FT-IR (KBr, ν_{\max} , cm⁻¹): 3444 (O-H), 3124 (N-H), 3019 (C-H, aromatic), 2982 (C-H, aliphatic), 1684 (C=O), 1602 (C=C, aliphatic), 1412 (C=C, aromatic), 671 (C-Cl), 1055 (C-O). ¹H NMR (400 MHz, DMSO-d₆, δ , ppm): 7.16-7.62 (m, 8H, Ar-H), 7.68 (d, J = 15.6 Hz, 1H, HC=CH (H- α)), 8.14 (d, J = 15.6 Hz, 1H, HC=CH (H- β)), 9.82 (s, 1H, NH), 12.31 (s, 1H, OH). ESI-MS (m/z): 388 [M+H]⁺. Molecular formula, C₁₈H₁₂Cl₂N₄O₂, Relative mol.mass- 387, M.P 161^oC, % Yield 78. *Anal.* Calcd : C, 55.83; H, 3.12; N, 14.47; Found: C, 55.83; H,3.11; N, 14.45%.

(*E*)-1-(3-(4,6-dichloro-1,3,5-triazin-2-ylamino)phenyl)-3-(3,5-dihydroxyphenyl)-2-propen-1-one (TCH-10): Light yellow crystals. FT-IR (KBr, ν_{\max} , cm⁻¹): 3440 (O-H), 3122 (N-H), 3027 (C-H, aromatic), 2890 (C-H, aliphatic), 1700 (C=O), 1605 (C=C, aliphatic), 1511 (C=C, aromatic), 688 (C-Cl), 1054 (C-O). ¹H NMR (400 MHz, DMSO-d₆, δ , ppm): 7.21-8.02 (m, 7H, Ar-H), 7.79 (d, J = 15.3 Hz, 1H, HC=CH (H- α)), 8.03 (d, J = 15.3 Hz, 1H, HC=CH (H- β)), 9.89 (s, 1H, NH), 11.52 (s, 2H, OH). ESI-MS (m/z): 404 [M+H]⁺. Molecular formula, C₁₈H₁₂Cl₂N₄O₃, Relative mol.mass-

403, M.P 182^oC, % Yield 69. *Anal.* Calcd : C, 53.62; H, 3.00; N, 13.89; Found: C, 53.61; H,3.02; N, 13.81%.

(*E*)-1-(3-(4,6-dichloro-1,3,5-triazin-2-ylamino)phenyl)-3-(4,5-dihydroxy phenyl)-2-propen-1-one (TCH-11):Light yellow crystals.FT-IR (KBr, ν_{\max} , cm⁻¹): 3395 (O-H), 3127 (N-H), 3017 (C-H, aromatic), 2989 (C-H, aliphatic), 1686 (C=O), 1615 (C=C, aliphatic), 1545 (C=C, aromatic), 689 (C-Cl), 1054 (C-O). ¹H NMR (400 MHz, DMSO-d₆, δ , ppm): 7.55-8.03 (m, 7H, Ar-H), 7.83 (d, J = 15.3 Hz, 1H, HC=CH (H- α)), 8.08 (d, J = 15.3 Hz, 1H, HC=CH (H- β)), 9.58 (s, 1H, OH), 9.87 (s, 1H, NH), 10.57 (s, 1H, OH).ESI-MS (m/z): 404 [M+H]⁺. Molecular formula, C₁₈H₁₂Cl₂N₄O₃, Relative mol.mass- 403, M.P 154^oC, % Yield 51. *Anal.* Calcd : C, 53.62; H, 3.00; N, 13.89; Found: C, 53.61; H,3.04; N, 13.82%.

(*E*)-1-(3-(4,6-dichloro-1,3,5-triazin-2-ylamino)phenyl)-3-(2-methyl-5-hydroxyphenyl)-2-propen-1-one (TCH-12):Light yellow crystals.FT-IR (KBr, ν_{\max} , cm⁻¹): 3440 (O-H), 3122 (N-H), 3021 (C-H, aromatic), 2975 (C-H, aliphatic), 1690 (C=O), 1641 (C=C, aliphatic), 1486 (C=C, aromatic), 678 (C-Cl), 1054 (C-O). ¹H NMR (400 MHz, DMSO-d₆, δ , ppm): 2.47 (s, 3H, CH₃), 7.62-8.01 (m, 7H, Ar-H), 7.81 (d, J = 15.3 Hz, 1H, HC=CH (H- α)), 8.08 (d, J = 15.3 Hz, 1H, HC=CH (H- β)), 9.01 (s, 1H, NH), 10.52 (s, 1H, OH).ESI-MS (m/z): 402 [M+H]⁺. Molecular formula, C₁₉H₁₄Cl₂N₄O₂, Relative mol.mass- 401, M.P 169^oC, % Yield 55. *Anal.* Calcd : C, 56.87; H, 3.52; N, 13.96; Found: C, 56.86; H,3.51; N, 13.93%.

(*E*)-1-(3-(4,6-dichloro-1,3,5-triazin-2-ylamino)phenyl)-3-(2-aminophenyl)-2-propen-1-one (TCH-13):Light yellow crystals.FT-IR (KBr, ν_{\max} , cm⁻¹): 3367 (NH₂), 3117 (N-H), 2978 (C-H, aromatic), 2763 (C-H, aliphatic), 1693 (C=O), 1597 (C=C, aliphatic), 1413 (C=C, aromatic), 688 (C-Cl), 1296 (C-N). ¹H NMR (400 MHz, DMSO-d₆, δ , ppm): 7.74-8.11 (m, 8H, Ar-H), 7.58 (d, J = 15.2 Hz, 1H, HC=CH (H- α)), 8.06 (d, J = 15.2 Hz, 1H, HC=CH (H- β)), 9.65 (s, 1H, NH), 10.51 (s, 2H, Ar-NH₂).ESI-MS (m/z): 387 [M+H]⁺. Molecular formula, C₁₈H₁₃Cl₂N₅O, Relative mol.mass- 386, M.P 154^oC, % Yield 67. *Anal.* Calcd : C, 55.97; H, 3.39; N, 18.13; Found: C, 55.95; H,3.31; N, 18.11%.

(*E*)-1-(3-(4,6-dichloro-1,3,5-triazin-2-ylamino)phenyl)-3-(3-aminophenyl)-2-propen-1-one (TCH-14):Light yellow crystals.FT-IR (KBr, ν_{\max} , cm⁻¹): 3367 (NH₂), 3117 (N-H), 2978 (C-H, aromatic), 2763 (C-H, aliphatic), 1693 (C=O), 1597 (C=C, aliphatic), 1413 (C=C, aromatic), 688 (C-Cl), 1290 (C-N). ¹H NMR (400 MHz, DMSO-d₆, δ , ppm): 7.72 (d, J = 15.2 Hz, 1H, HC=CH (H- α)), 7.74-8.11 (m, 8H, Ar-H), 8.01 (d, J = 15.2 Hz, 1H, HC=CH (H- β)), 9.67 (s, 1H, NH), 10.54 (s, 2H, Ar-NH₂).ESI-MS (m/z): 387 [M+H]⁺. Molecular formula, C₁₈H₁₃Cl₂N₅O, Relative mol.mass- 386, M.P 133^oC, % Yield 71. *Anal.* Calcd : C, 55.97; H, 3.39; N, 18.13; Found: C, 55.94; H,3.32; N, 18.12%.

(*E*)-1-(3-(4,6-dichloro-1,3,5-triazin-2-ylamino)phenyl)-3-(4-aminophenyl)-2-propen-1-one (TCH-15):Light yellow crystals.FT-IR (KBr, ν_{\max} , cm⁻¹): 3362 (NH₂), 3115 (N-H), 2979 (C-H, aromatic), 2761 (C-H, aliphatic), 1690 (C=O), 1590 (C=C, aliphatic), 1410 (C=C, aromatic), 684 (C-Cl), 1290 (C-N). ¹H NMR (400 MHz, DMSO-d₆, δ , ppm): 7.71 (d, J = 15.2 Hz, 1H, HC=CH (H- α)), 7.77-8.14 (m, 8H, Ar-H), 8.12 (d, J = 15.2 Hz, 1H, HC=CH (H- β)), 9.65 (s, 1H, NH), 10.52 (s, 2H, Ar-NH₂). ESI-MS (m/z): 387 [M+H]⁺. Molecular formula, C₁₈H₁₃Cl₂N₅O, Relative mol.mass- 386, M.P 139^oC, % Yield 68. *Anal.* Calcd : C, 55.97; H, 3.39; N, 18.13; Found: C, 55.93; H,3.35; N, 18.14%.

(*E*)-1-(3-(4,6-dichloro-1,3,5-triazin-2-ylamino)phenyl)-3-(2-nitrophenyl)-2-propen-1-one (TCH-16):Light yellow crystals.FT-IR (KBr, ν_{\max} , cm⁻¹): 3122 (N-H), 3024 (C-H, aromatic), 2776 (C-H, aliphatic), 1700 (C=O), 1604 (C=C, aliphatic), 1414 (C=C, aromatic), 688 (C-Cl), 1529 (N=O), 1291 (C-N). ¹H NMR (400 MHz, DMSO-d₆, δ , ppm): 6.86-8.18 (m, 8H, Ar-H), 8.05 (d, J = 15.2 Hz, 1H, HC=CH (H- α)), 8.35 (d, J = 15.2 Hz, 1H, HC=CH (H- β)), 9.72 (s, 1H, NH). ESI-MS (m/z): 417 [M+H]⁺. Molecular formula, C₁₈H₁₁Cl₂N₅O₃, Relative mol.mass- 416, M.P 120^oC, % Yield 52. *Anal.* Calcd : C, 51.94; H, 2.66; N, 16.83; Found: C, 51.95; H,2.62; N, 16.82%.

(*E*)-1-(3-(4,6-dichloro-1,3,5-triazin-2-ylamino)phenyl)-3-(3-nitrophenyl)-2-propen-1-one (TCH-17):Light yellow crystals.FT-IR (KBr, ν_{\max} , cm⁻¹): 3115 (N-H), 3026 (C-H, aromatic), 2775 (C-H, aliphatic), 1700 (C=O), 1599 (C=C, aliphatic), 1412 (C=C, aromatic), 688 (C-Cl), 1522 (N=O), 1290 (C-N). ¹H NMR (400 MHz, DMSO-d₆, δ , ppm): 7.55-8.39 (m, 8H, Ar-H), 7.86 (d, J = 15.2 Hz, 1H, HC=CH (H- α)), 8.06 (d, J = 15.2 Hz, 1H, HC=CH (H- β)), 9.73 (s, 1H, NH). ESI-MS (m/z): 417 [M+H]⁺. Molecular formula, C₁₈H₁₁Cl₂N₅O₃, Relative mol.mass- 416, M.P 140^oC, % Yield 77. *Anal.* Calcd : C, 51.94; H, 2.66; N, 16.83; Found: C, 51.92; H,2.65; N, 16.85%.

(*E*)-1-(3-(4,6-dichloro-1,3,5-triazin-2-ylamino)phenyl)-3-(4-nitrophenyl)-2-propen-1-one (TCH-18):Light yellow crystals.FT-IR (KBr, ν_{\max} , cm⁻¹): 3205 (N-H), 3016 (C-H, aromatic), 2895 (C-H, aliphatic), 1710 (C=O), 1589 (C=C, aliphatic), 1442 (C=C, aromatic), 680 (C-Cl), 1520 (N=O), 1287 (C-N). ¹H NMR (400 MHz, DMSO-d₆, δ , ppm): 7.54-8.29 (m, 8H, Ar-H), 7.83 (d, J = 15.2 Hz, 1H, HC=CH (H- α)), 8.07 (d, J = 15.2 Hz, 1H, HC=CH (H- β)), 9.23 (s, 1H, NH). ESI-MS (m/z): 417 [M+H]⁺. Molecular formula, C₁₈H₁₁Cl₂N₅O₃, Relative mol.mass- 416, M.P 124^oC, % Yield 84. *Anal.* Calcd : C, 51.94; H, 2.66; N, 16.83; Found: C, 51.93; H,2.62; N, 16.81%.

(*E*)-1-(3-(4,6-dichloro-1,3,5-triazin-2-ylamino)phenyl)-3-(2-chlorophenyl)-2-propen-1-one (TCH-19):Light yellow crystals.FT-IR (KBr, ν_{\max} , cm⁻¹): 3127 (N-H),

3027 (C–H, aromatic), 2893 (C–H, aliphatic), 1689 (C=O), 1597 (C=C, aliphatic), 1450 (C=C, aromatic), 688 (C–Cl), 786 (C–Cl). ¹H NMR (400 MHz, DMSO-d₆, δ, ppm): 7.60 (d, J = 15.2 Hz, 1H, HC=CH (H-α)), 7.62-8.24 (m, 8H, Ar-H), 7.78 (d, J = 15.2 Hz, 1H, HC=CH (H-β)), 9.65 (s, 1H, NH). ESI-MS (m/z): 406 [M+H]⁺. Molecular formula, C₁₈H₁₁Cl₃N₄O, Relative mol.mass- 405, M.P 138^oC, % Yield 81. Anal. Calcd : C, 53.29; H, 2.73; N, 13.81; Found: C, 53.21; H,2.71; N, 13.82%.

(E)-1-(3-(4,6-dichloro-1,3,5-triazin-2-ylamino)phenyl)-3-(3-chlorophenyl)-2-propen-1-one (TCH-20):Light yellow crystals.FT-IR (KBr, v_{max}, cm-1): 3121 (N–H), 3025 (C–H, aromatic), 2891 (C–H, aliphatic), 1686 (C=O), 1594 (C=C, aliphatic), 1451 (C=C, aromatic), 786 (C–Cl). ¹H NMR (400 MHz, DMSO-d₆, δ, ppm): 7.45 (d, J = 15.2 Hz, 1H, HC=CH (H-α)), 7.62-7.74 (m, 8H, Ar-H), 7.79 (d, J = 15.2 Hz, 1H, HC=CH (H-β)), 9.65 (s, 1H, NH). ESI-MS (m/z): 406 [M+H]⁺. Molecular formula, C₁₈H₁₁Cl₃N₄O, Relative mol.mass-405, M.P 181^oC, % Yield 71. Anal. Calcd : C, 53.29; H, 2.73; N, 13.81; Found: C, 53.22; H,2.74; N, 13.81%.

(E)-1-(3-(4,6-dichloro-1,3,5-triazin-2-ylamino)phenyl)-3-(4-chlorophenyl)-2-propen-1-one (TCH-21):Light yellow crystals.FT-IR (KBr, v_{max}, cm-1): 3126 (N–H), 3023 (C–H, aromatic), 2883 (C–H, aliphatic), 1690 (C=O), 1588 (C=C, aliphatic), 1442 (C=C, aromatic), 681 (C–Cl), 785 (C–Cl). ¹H NMR (400 MHz, DMSO-d₆, δ, ppm): 7.61 (d, J = 15.2 Hz, 1H, HC=CH (H-α)), 7.67-7.82 (m, 8H, Ar-H), 7.87 (d, J = 15.2 Hz, 1H, HC=CH (H-β)), 9.63 (s, 1H, NH). ESI-MS (m/z): 406 [M+H]⁺. Molecular formula, C₁₈H₁₁Cl₃N₄O, Relative mol.mass- 405, M.P 149^oC, % Yield 73. Anal. Calcd : C, 53.29; H, 2.73; N, 13.81; Found: C, 53.23; H,2.71; N, 13.84%.

(E)-1-(3-(4,6-dichloro-1,3,5-triazin-2-ylamino)phenyl)-3-(2,4-dichlorophenyl)-2-propen-1-one (TCH-22):Light yellow crystals.FT-IR (KBr, v_{max}, cm-1): 3124 (N–H), 3018 (C–H, aromatic), 2891 (C–H, aliphatic), 1689 (C=O), 1641 (C=C, aliphatic), 1485 (C=C, aromatic), 691 (C–Cl), 786 (C–Cl). ¹H NMR (400 MHz, DMSO-d₆, δ, ppm): 7.65-8.23 (m, 7H, Ar-H), 7.78 (d, J = 15.2 Hz, 1H, HC=CH (H-α)), 8.06 (d, J = 15.2 Hz, 1H, HC=CH (H-β)), 9.69 (s, 1H, NH). ESI-MS (m/z): 441 [M+H]⁺. Molecular formula, C₁₈H₁₀Cl₄N₄O, Relative mol.mass- 440, M.P 192^oC, % Yield 59. Anal. Calcd : C, 49.12; H, 2.29; N, 12.73; Found: C, 49.11; H,2.25; N, 12.71%.

(E)-1-(3-(4,6-dichloro-1,3,5-triazin-2-ylamino)phenyl)-3-(2,4-dichlorophenyl)-2-propen-1-one (TCH-23):Light yellow crystals.FT-IR (KBr, v_{max}, cm-1): 3117 (N–H), 3017 (C–H, aromatic), 2977 (C–H, aliphatic), 1693 (C=O), 1605 (C=C, aliphatic), 1415 (C=C, aromatic), 688 (C–Cl), 1116 (C–F). ¹H NMR (400 MHz, DMSO-d₆, δ, ppm): 7.36-8.03 (m, 8H, Ar-

H), 7.55 (d, J = 15.2 Hz, 1H, HC=CH (H-α)), 7.82 (d, J = 15.2 Hz, 1H, HC=CH (H-β)), 9.68 (s, 1H, NH). ESI-MS (m/z): 390 [M+H]⁺. Molecular formula, C₁₈H₁₁Cl₂FN₄O, Relative mol.mass- 389, M.P 152^oC, % Yield 67. Anal. Calcd : C, 55.55; H, 2.85; N, 14.39; Found: C, 55.53; H,2.82; N, 14.35%.

(E)-1-(3-(4,6-dichloro-1,3,5-triazin-2-ylamino)phenyl)-3-(3-fluorophenyl)-2-propen-1-one (TCH-24):Light yellow crystals.FT-IR (KBr, v_{max}, cm-1): 3112 (N–H), 3011 (C–H, aromatic), 2974 (C–H, aliphatic), 1690 (C=O), 1602 (C=C, aliphatic), 1412 (C=C, aromatic), 680 (C–Cl), 1011 (C–F). ¹H NMR (400 MHz, DMSO-d₆, δ, ppm): 7.16-7.73 (m, 8H, Ar-H), 7.75 (d, J = 15.2 Hz, 1H, HC=CH (H-α)), 7.81 (d, J = 15.2 Hz, 1H, HC=CH (H-β)), 9.78 (s, 1H, NH). ESI-MS (m/z): 390 [M+H]⁺. Molecular formula, C₁₈H₁₁Cl₂FN₄O, Relative mol.mass- 389, M.P 132^oC, % Yield 55. Anal. Calcd : C, 55.55; H, 2.85; N, 14.39; Found: C, 55.52; H,2.84; N, 14.35%.

(E)-1-(3-(4,6-dichloro-1,3,5-triazin-2-ylamino)phenyl)-3-(4-fluorophenyl)-2-propen-1-one (TCH-25):Light yellow crystals.FT-IR (KBr, v_{max}, cm-1): 3114 (N–H), 3212 (C–H, aromatic), 2975 (C–H, aliphatic), 1694 (C=O), 1602 (C=C, aliphatic), 1412 (C=C, aromatic), 1106 (C–F), 685 (C–Cl). ¹H NMR (400 MHz, DMSO-d₆, δ, ppm): 7.22-7.63 (m, 8H, Ar-H), 7.65 (d, J = 15.2 Hz, 1H, HC=CH (H-α)), 7.82 (d, J = 15.2 Hz, 1H, HC=CH (H-β)), 9.77 (s, 1H, NH). ESI-MS (m/z): 390 [M+H]⁺. Molecular formula, C₁₈H₁₁Cl₂FN₄O, Relative mol.mass- 389, M.P 145^oC, % Yield 51. Anal. Calcd : C, 55.55; H, 2.85; N, 14.39; Found: C, 55.51; H,2.81; N, 14.32%.

(E)-1-(3-(4,6-dichloro-1,3,5-triazin-2-ylamino)phenyl)-3(2,4difluorophenyl)-2-propen-1-one (TCH-26):Light yellow crystals.FT-IR (KBr, v_{max}, cm-1): 3122 (N–H), 3021 (C–H, aromatic), 2884 (C–H, aliphatic), 1693 (C=O), 1605 (C=C, aliphatic), 1415 (C=C, aromatic), 688 (C–Cl), 1114 (C–F). ¹H NMR (400 MHz, DMSO-d₆, δ, ppm): 7.39-8.31 (m, 7H, Ar-H), 7.76 (d, J = 15.2 Hz, 1H, HC=CH (H-α)), 8.08 (d, J = 15.2 Hz, 1H, HC=CH (H-β)), 9.69 (s, 1H, NH). ESI-MS (m/z): 408 [M+H]⁺. Molecular formula, C₁₈H₁₀Cl₂F₂N₄O, Relative mol.mass- 407, M.P 160^oC, % Yield 67. Anal. Calcd : C, 53.09; H, 2.48; N, 13.76; Found: C, 53.01; H,2.42; N, 13.72%.

(E)-1-(3-(4,6-dichloro-1,3,5-triazin-2-ylamino)phenyl)-3-(furan-2-yl)-2-propen-1-one (TCH-27):Light yellow crystals.FT-IR (KBr, v_{max}, cm-1): 3420 (N–H), 3062 (C–H, aromatic), 3030 (C–H, aliphatic), 1671(C=O), 1591 (C=C, aliphatic), 1453 (C=C, aromatic), 696 (C–Cl), 1155 (C–O–C), 1053 (C–O). ¹H NMR (400 MHz, DMSO-d₆, δ, ppm): 6.74 (s, 1H, Ar-H), 6.21 (m, 1H, Ar-H), 7.16-7.50 (m, 5H, Ar-H), 7.62 (d, J = 16 Hz, 1H, HC=CH (H-α)), 8.06 (d, J = 16 Hz, 1H, HC=CH (H-β)), 9.73 (s, 1H, NH). ESI-MS (m/z): 362 [M+H]⁺.

Molecular formula, $C_{16}H_{10}Cl_2N_4O_2$, Relative mol.mass-361, M.P 188^oC, % Yield 71. *Anal.* Calcd : C, 53.21; H, 2.79; N, 15.51; Found: C, 53.22; H,2.75; N, 15.50%.

(*E*)-1-(3-(4,6-dichloro-1,3,5-triazin-2-ylamino)phenyl)-3-(thiophen-3-yl)-2-propen-1-one (TCH-28):Light yellow crystals.FT-IR (KBr, ν_{max} , cm^{-1}): 3430 (N-H), 3019 (C-H, aromatic), 2973 (C-H, aliphatic), 1689 (C=O), 1599 (C=C, aliphatic), 1414 (C=C, aromatic), 1531(C-S), 688 (C-Cl). ¹H NMR (400 MHz, DMSO- d_6 , δ , ppm): 6.68 (s, 1H, Ar-H), 6.91 (s, 1H, Ar-H), 7.12 (s, 1H, Ar-H), 7.33-7.58 (m, 4H, Ar-H), 7.76 (d, J = 15.2 Hz, 1H, HC=CH (H- α)), 8.02 (d, J = 15.2 Hz, 1H, HC=CH (H- β)), 9.68 (s, 1H, NH). ESI-MS (m/z): 378 [M+H]⁺. Molecular formula, $C_{16}H_{10}Cl_2N_4OS$, Relative mol.mass- 377, M.P 177^oC, % Yield 78. *Anal.* Calcd : C, 50.94; H, 2.67; N, 14.85; Found: C, 50.97; H,2.65; N, 14.82%.

(*E*)-1-(3-(4,6-dichloro-1,3,5-triazin-2-ylamino)phenyl)-3-(pyrrol-2-yl)-2-propen-1-one (TCH-29):Light yellow crystals.FT-IR (KBr, ν_{max} , cm^{-1}): 3144 (N-H), 3052 (N-H), 3017 (C-H, aromatic), 2973 (C-H, aliphatic), 1695 (C=O), 1615 (C=C, aliphatic), 1414 (C=C, aromatic), 678 (C-Cl), 1308 (C-N). ¹H NMR (400 MHz, DMSO- d_6 , δ , ppm): 6.46 (s, 1H, Ar-H), 7.44 (m, 1H, Ar-H), 7.55-7.61 (m, 5H, Ar-H), 7.76 (d, J = 15.2 Hz, 1H, HC=CH (H- α)), 8.03 (d, J = 15.2 Hz, 1H, HC=CH (H- β)), 9.64 (s, 1H, NH), 10.55 (s, 1H, NH).ESI-MS (m/z): 361 [M+H]⁺. Molecular formula, $C_{16}H_{11}Cl_2N_5O$, Relative mol.mass- 360, M.P 121^oC, % Yield 66. *Anal.* Calcd : C, 66.25; H, 3.42; N, 11.89; Found: C, 66.22; H,3.41; N, 11.86%.

(*E*)-1-(3-(4,6-dichloro-1,3,5-triazin-2-ylamino)phenyl)-3-(pyridin-2-yl)-2-propen-1-one (TCH-30):Light yellow crystals.FT-IR (KBr, ν_{max} , cm^{-1}): 3127 (N-H), 3019 (C-H, aromatic), 2931 (C-H, aliphatic), 1689 (C=O), 1604 (C=C, aliphatic), 1417 (C=C, aromatic), 688 (C-Cl), 1308 (C-N). ¹H NMR (400 MHz, DMSO- d_6 , δ , ppm): 6.98 (d, J = 16 Hz, 1H, HC=CH (H- α)), 7.13-7.69 (m, 8H, Ar-H),7.78 (d, J = 16 Hz, 1H, HC=CH (H- β)), 9.60 (s, 1H, NH). ESI-MS (m/z): 373 [M+H]⁺. Molecular formula, $C_{17}H_{11}Cl_2N_5O$, Relative mol.mass-372, M.P 124^oC, % Yield 72. *Anal.* Calcd : C, 54.86; H, 2.98; N, 18.82; Found: C, 54.82; H,2.96; N, 18.88%

(*E*)-1-(3-(4,6-dichloro-1,3,5-triazin-2-ylamino)phenyl)-3-(pyridin-3-yl)-2-propen-1-one (TCH-31):Light yellow crystals.FT-IR (KBr, ν_{max} , cm^{-1}): 3122 (N-H), 3011 (C-H, aromatic), 2922 (C-H, aliphatic), 1679 (C=O), 1609 (C=C, aliphatic), 1422 (C=C, aromatic), 1308 (C-N), 681 (C-Cl). ¹H NMR (400 MHz, DMSO- d_6 , δ , ppm): 7.22 (d, J = 16 Hz, 1H, HC=CH (H- α)), 7.23-7.59 (m, 8H, Ar-H), 7.68 (d, J = 16 Hz, 1H, HC=CH (H- β)), 9.58 (s, 1H, NH). ESI-MS (m/z): 373 [M+H]⁺. Molecular formula, $C_{17}H_{11}Cl_2N_5O$, Relative mol.mass-372, M.P 151^oC, % Yield 79. *Anal.* Calcd : C, 54.86; H, 2.98; N, 18.82; Found: C, 54.81; H,2.95; N, 18.89%

(*E*)-1-(3-(4,6-dichloro-1,3,5-triazin-2-ylamino)phenyl)-3-(pyridin-4-yl)-2-propen-1-one (TCH-32):Light yellow crystals.FT-IR (KBr, ν_{max} , cm^{-1}): 3127 (N-H), 3019 (C-H, aromatic), 2931 (C-H, aliphatic), 1689 (C=O), 1604 (C=C, aliphatic), 1417 (C=C, aromatic), 688 (C-Cl), 1308 (C-N). ¹H NMR (400 MHz, DMSO- d_6 , δ , ppm): 6.98 (d, J = 16 Hz, 1H, HC=CH (H- α)), 7.13-7.69 (m, 8H, Ar-H), 7.78 (d, J = 16 Hz, 1H, HC=CH (H- β)), 9.60 (s, 1H, NH). ESI-MS (m/z): 373 [M+H]⁺. Molecular formula, $C_{17}H_{11}Cl_2N_5O$, Relative mol.mass-372, M.P 197^oC, % Yield 77. *Anal.* Calcd : C, 54.86; H, 2.98; N, 18.82; Found: C, 54.85; H,2.92; N, 18.81%

(*E*)-1-(3-(4,6-dichloro-1,3,5-triazin-2-ylamino)phenyl)-3-(naphthalen-2-yl)-2-propen-1-one (TCH-33) Light yellow crystals.FT-IR (KBr, ν_{max} , cm^{-1}): 3102 (N-H), 3015 (C-H, aromatic), 2926 (C-H, aliphatic), 1684 (C=O), 1602 (C=C, aliphatic), 1416 (C=C, aromatic), 682 (C-Cl). ¹H NMR (400 MHz, DMSO- d_6 , δ , ppm): 7.62-7.83 (m, 11H, Ar-H), 7.87 (d, J = 15.2 Hz, 1H, HC=CH (H- α)), 8.16 (d, J = 15.2 Hz, 1H, HC=CH (H- β)), 9.70 (s, 1H, NH). ESI-MS (m/z): 422 [M+H]⁺. Molecular formula, $C_{22}H_{14}Cl_2N_4O$, Relative mol.mass-421, M.P 105^oC, % Yield 81. *Anal.* Calcd : C, 62.72; H, 3.35; N, 13.30; Found: C, 62.71; H,3.32; N, 13.32%

(*E*)-1-(3-(4,6-dichloro-1,3,5-triazin-2-ylamino)phenyl)-3-(naphthalen-3-yl)-2-propen-1-one (TCH-34):Light yellow crystals.FT-IR (KBr, ν_{max} , cm^{-1}): 3115 (N-H), 3019 (C-H, aromatic), 2931 (C-H, aliphatic), 1689 (C=O), 1604 (C=C, aliphatic), 1417 (C=C, aromatic), 688 (C-Cl). ¹H NMR (400 MHz, DMSO- d_6 , δ , ppm): 7.62-8.33 (m, 11H, Ar-H), 7.89 (d, J = 15.2 Hz, 1H, HC=CH (H- α)), 8.26 (d, J = 15.2 Hz, 1H, HC=CH (H- β)), 9.71 (s, 1H, NH). ESI-MS (m/z): 422 [M+H]⁺. Molecular formula, $C_{22}H_{14}Cl_2N_4O$, Relative mol.mass-421, M.P 117^oC, % Yield 87. *Anal.* Calcd : C, 62.72; H, 3.35; N, 13.30; Found: C, 62.72; H,3.31; N, 13.33%

(*E*)-1-(3-(4,6-dichloro-1,3,5-triazin-2-ylamino)phenyl)-3-(anthracen-9-yl)-2-propen-1-one (TCH-35):Light yellow crystals.FT-IR (KBr, ν_{max} , cm^{-1}): 3127 (N-H), 3019 (C-H, aromatic), 2931 (C-H, aliphatic), 1689 (C=O), 1604 (C=C, aliphatic), 1417 (C=C, aromatic), 688 (C-Cl). ¹H NMR (400 MHz, DMSO- d_6 , δ , ppm): 6.98-7.41 (m, 13H, Ar-H), 7.59 (d, J = 15.6 Hz, 1H, HC=CH (H- α)), 8.06 (d, J = 15.6 Hz, 1H, HC=CH (H- β)), 9.75 (s, 1H, NH). ESI-MS (m/z): 472 [M+H]⁺. Molecular formula, $C_{26}H_{16}Cl_2N_4O$, Relative mol.mass-471, M.P 220^oC, % Yield 68. *Anal.* Calcd : C, 66.25; H, 3.42; N, 11.89; Found: C, 66.22; H,3.40; N, 11.85%

Brine Shrimp Lethality Assay (BSLT)

A general bioassay that appears capable of detecting a board spectrum of bioactivity is the brine shrimp lethality bioassay (BSLT). The technique is easily mastered, costs little, and utilizes small amount of test material. The aim of this method is to provide a front-line screen that can be backed up by more specific

and more expensive bioassays. It appears that brine shrimp lethality bioassay is predictive of cytotoxicity and pesticidal activity. Since its introduction in 1982 by Meyer *et al.*, [15] this *in vivo* lethality test has been successively employed for bioassay-guide fractionation of active Cytotoxic and antitumor agents.

Test Materials Used for the Study

Artemia is a genus of aquatic crustaceans known as brine shrimp. *Artemia*, the only genus in the family Artemiidae, has changed little externally since the Triassic period. The historical record of the existence of *Artemia* dates back to 982 from Urmia Lake, Iran, although the first unambiguous record is the report and drawings made by Schlosser in 1756 of animals from

Lymington, England. *Artemia* populations are found worldwide in inland saltwater lakes, but not in oceans. *Artemia* are able to avoid cohabiting with most types of predators, such as fish, by their ability to live in waters of very high salinity (up to 25%). The ability of the *Artemia* to produce dormant eggs, known as cysts, has led to extensive use of *Artemia* in aquaculture. The cysts may be stored for long periods and hatched on demand to provide a convenient form of live feed for larval fish and crustaceans. Nauplii of the brine shrimp *Artemia* constitute the most widely used food item, and over 2000 tonnes of dry *Artemia* cysts are marketed worldwide annually. In addition, the resilience of *Artemia* makes them ideal animals for running biological toxicity assays and it is now one of the standard organisms for testing the toxicity of chemicals.

Hatching the brine shrimp

The cytotoxicity potential of the synthesized compounds was determined by Brine Shrimp Lethality assay as described by Meyer *et al.* 70 mg of brine shrimp eggs (*Artemia salina*, Sanders TM Great Salt Lake, Brine Shrimp Company L.C., U.S.A.) were purchased from e-bay and then hatched in artificial sea water prepared from commercial sea salt (Aqua Marine, Thailand) 40 g/L and supplemented with 60 mg/L dried yeast. The two unequal compartments plastic chamber with several holes on the divider was used for hatching. The eggs were sprinkled into the larger compartment

which was darkened, while the smaller compartment was illuminated. Container was placed beside a light ray precisely the window blind for rays of light and proper ventilation. After 48 hours incubation at room temperature (25-29°C), phototropic nauplii were collected using Pasteur pipettes from the illuminated compartment, whereas their shells were left in another side.

Sample preparation

0.5 ml of salty sea water was poured into in wells of micro-titer plate. A control was prepared for each synthesized compound being assayed by adding 0.5 ml of sea water and 0.5 ml of dimethyl sulphoxide (DMSO). Samples were prepared by dissolving 30 mg of each compound in 3 ml of DMSO to give stock solutions 10000µg/mL, from this 1mL of sample withdrawn and made to volume 10mL to get 1000µg/mL. Five different concentrations namely 100 µg/ml, 50 µg/ml, 25 µg/ml, 12.5µg/ml, 6.25 µg/ml and 3.125 µg/ml were prepared in triplicate by serial dilutions from the stock solution.

Bioassay

Serial dilutions were made in the wells of 96-well microplates (Nunc, Denmark) in triplicate in 120 µL sea water. Control wells with DMSO were included in each experiment. A suspension of nauplii containing 25-30 organisms (100 µL) was added to each well. The plates were covered and incubated at room temperature (25-29°C) for 24 hours. Plates were then examined under the 3X magnifying glass (3572, Konus Optima) and the numbers of number of survived napulii in each in each well were counted. Larvae were considered dead only if they did not move their appendages for 10 s during observation.

The results for the test compounds were compared with the positive control podophyllotoxin, and the data of cytotoxicity study was given in Table-1. Each time the experiment was conducted along with control (vehicle treated) at various concentrations of the test substances. Analysis of the data was performed by probit analysis on a Finney computer program to determine the lethal concentration to half of the test organisms (LC₅₀) [16].

$$\text{percentage of mortality (PM)} = \frac{\text{live count in control} - \text{live count in test}}{\text{live count in control}} \times 100$$

% Mortality rate of Brine shrimp nauplii determined as follows: A suspension of nauplii containing 30 organisms (100 µL) was added to each well. The wells were prepared in triplicates for each time point and were incubated at (25-29°C) for 24 hours. The numbers of survived nauplii in each well were counted. The % Mortality rate of Brine shrimp nauplii were plotted against the LC₅₀ values of

synthesized compounds using standard reference drug podophyllotoxin.

Insilico molecular docking analysis

METHODOLOGY

Insilico molecular docking analysis [17] was performed using Schrodinger Maestro (9.5) GLIDE commercial version is used as graphical user interface.

Protein is prepared using protein preparation wizard and following functions are performed:

- Automatically imported full PDB files or any chain within a PDB file from local databases or the PDB website.
- Automatically missing hydrogen atoms are added.
- Metal ionization states are corrected to ensure proper formal charge and force field treatment
- Bond orders are enumerated to HET groups
- Co-crystallized water molecules are removed at the user's discretion.
- Residues with missing atoms or multiple occupancies are highlighted.
- Quickly and easily determine the most likely ligand protonation state as well as the energy penalties associated with alternate protonation states
- Optimal protonation states for histidine residues are determined.
- Potentially transposed heavy atoms in arginine, glutamine, and histidine side chains are corrected.
- The protein's hydrogen bond network is optimized by means of a systematic, cluster-based approach, which greatly decreases preparation times.
- A restrained minimization is performed that allows hydrogen atoms to be freely minimized, while allowing for sufficient heavy-atom movement to relax strained bonds, angles, and clashes.

Phase-1 ligand preparation

The chemical structures of the title compounds synthesized in the present study 1,3,5-triazine-chalcone hybrid molecules TCH 1-35 were drawn using Chemdraw ultra v10.0 (Chemical Structure Drawing Standard; Cambridge Soft corporation, USA), all the structures were converted into compatible mol2 format copied to Schrodinger Glide software (Maestro 9.5) version to create a 3D model and, finally subjected to energy minimization using molecular mechanics (MM₂). The minimization was executed until the root mean square gradient value reached a value smaller

than 0.001kcal/mol. Such energy minimized structures are considered for molecular docking studies.

Ligand preparation was performed using LigPrep for the corrections on the ligands such as the addition of hydrogens, 2D to 3D conversion, bond lengths and bond angles, low energy structure, and ring conformation followed by minimization and optimization in optimized potential for liquid simulations force field. Glide is a ligand docking program for predicting protein–ligand binding modes and ranking ligands via high-throughput virtual screening. Glide molecular docking needs an X-ray crystal structure of protein binding with ligands for determining active site receptor grid.

Phase-2 protein selection and preparation

The protein was first prepared using the Protein Preparation wizard and the docking studies were performed using the Schrodinger Glide software within Maestro 9.5, which executes the correction of raw PDB structure, where amendments such as the addition of hydrogen atoms, assigning bond orders, creating disulfide bonds, fixing of the charges and orientation of groups were incorporated. Finally the resultant protein target was prepared for molecular docking simulation in such a way that all hetero atoms (i.e., non receptor atoms such as water, ions, etc.) were removed. Kollmann charges were assigned [18].

RESULTS

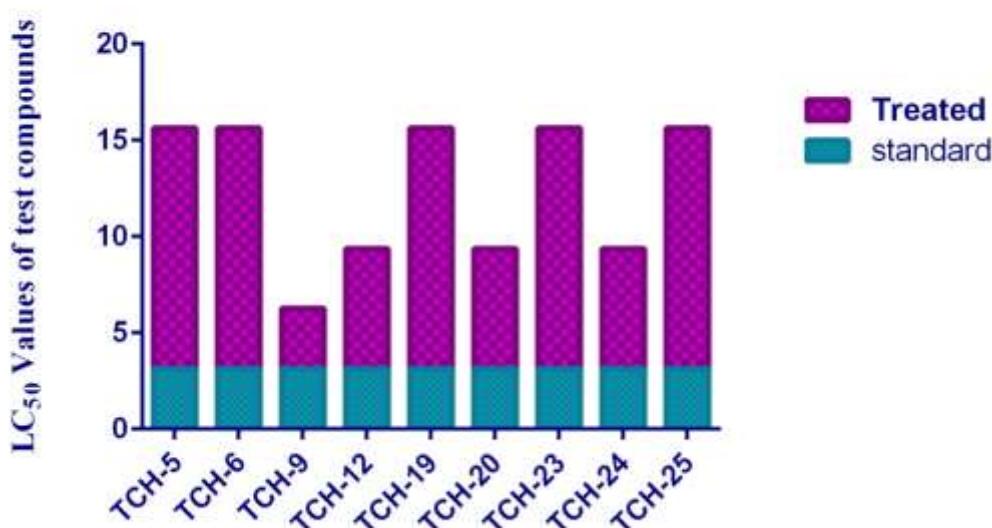
The procedure determines LC₅₀ value in µg/ml of active compounds in the brine medium. The activities of compounds are manifested as toxicity to shrimps. The advantages of this method are being rapid results; reliability, inexpensive and convenient assay. This bioassay has good correlation with cytotoxic activity determined using molecular docking studies in some cancer targets. The best of synthesized compounds with least LC₅₀ Values were selected and Tabulated in comparison with Control podophyllotoxin.

Table-1: Brine shrimp Lethality assay results of Synthesized Triazine-chalcone hybrids. Lethality Dose Concentration expressed in µg/mL.

Compound code	R-Substituent	Lethality Concentration(LC ₅₀)
TCH-5	2-OMeC ₆ H ₄	12.5±0.005
TCH-6	3-OMeC ₆ H ₄	12.5±0.003
TCH-9	4-OHC ₆ H ₄	3.125±0.003
TCH-12	2-Me,5-OHC ₆ H ₃	6.25±0.001
TCH-19	2-ClC ₆ H ₄	12.5±0.003
TCH-20	3-ClC ₆ H ₄	6.25±0.001
TCH-23	2-FC ₆ H ₄	12.5±0.003
TCH-24	3-FC ₆ H ₄	6.25±0.001
TCH-25	4-FC ₆ H ₄	12.5±0.003
Podophyllotoxin	--	3.125±0.001

Table-2: The number of shrimp nauplii that survived after 24 hrs of treating on an average with the test compounds and the percentage mortality in the cytotoxicity assay

Dose($\mu\text{g}/\text{ml}$)	Total No. shrimps	No. of survivors shrimps	No. of dead shrimps	%Mortality	Probit
100	30	6	24	80.13	6.28
50	30	9	21	70.64	5.31
25	30	12	18	60.72	4.95
12.2	30	14	16	53.91	3.16
6.25	30	14	16	53.91	3.16
3.125	30	14	16	53.91	3.16
Control	30	11	19	63.39	5.92

% Mortality rate of Nauplii treated with Test & standard**Fig-1: Percent Mortality rate of Brine shrimp nauplii Vs LC₅₀ values of selected 9 synthesized Triazine-chalcone hybrids and Podophyllotoxin against Artemia Salina nauplii by the Brine shrimp Lethality assay.**

Molecular docking results for selected five anticancer targets using schrodinger glide software (maestro 9.5) version.

Table-3: Software validation data for selected anticancer drug targets.

S.No	Name of the target protein	PDB ID	Co-Crystallized Ligand Code	RMSD (Å ^o)
1	PI ₃ K γ	4FHK	OUO	1.44
2	VEGF	1Y6A	AAZ	1.21
3	CDK-2	4BGH	3I6	1.25
4	BRAF	5CSX	54J	1.23
5	FAK	4Q9S	30G	1.11

Following figures represents 2D diagram Hydrogen-bond interactions of the best of all synthesized compounds. Active molecules with high

glide score when docked with Targets are represented (Color figure online)

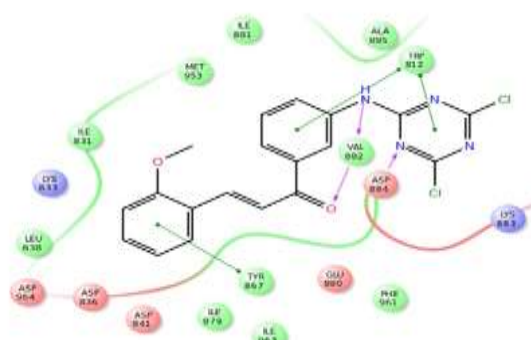


Fig-2: TCH-5

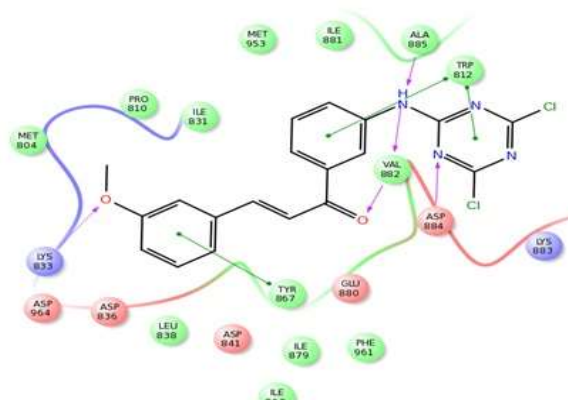


Fig-3: TCH-6

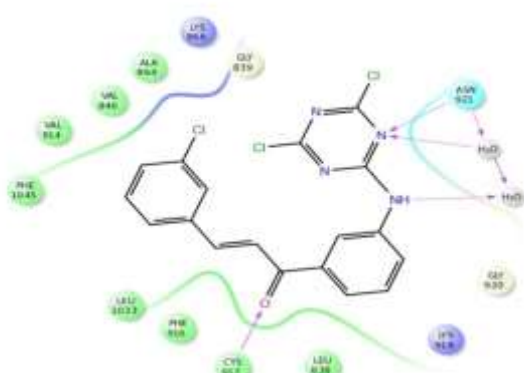


Fig-4: TCH-20

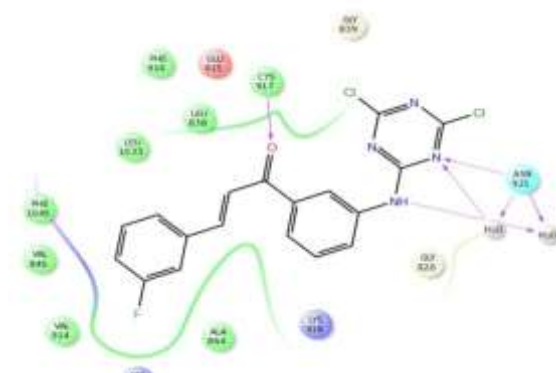


Fig-5: TCH-24

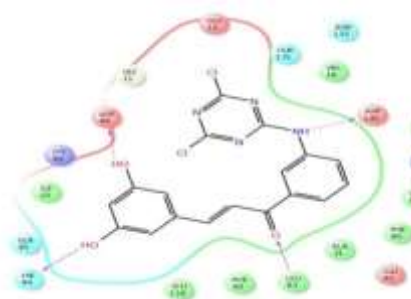


Fig-6: TCH-10

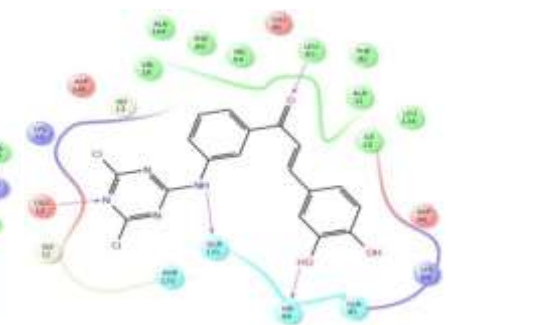


Fig-7: TCH-11

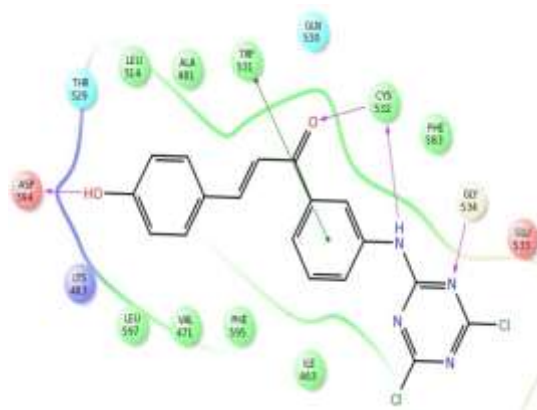


Fig-8: TCH-9

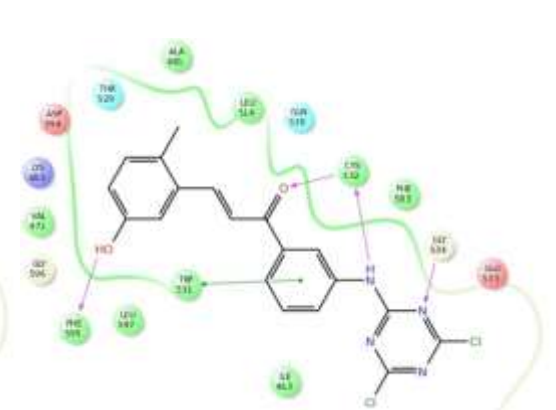


Fig-9: TCH-12

DISCUSSION

The brine shrimp lethality assay was carried out to determine the lethal concentration of the synthesized compounds. The concentrations were prepared in micro gram. It was observed that the compound TCH-9 and 12 were found to be significant, killed the brine shrimp with percentage lethality/mortality of 80.13, 70.64, 60.72, 53.91, 53.91, 53.91 and 63.39% at a concentration of 100, 50, and 25, 12.5, 6.25, 3.125 $\mu\text{g/mL}$ respectively. While for the other compounds the lethality concentration was mentioned in Table-1. The lethality test was compared with the control group. Podophyllotoxin was used as test standard in determining the LC_{50} of the synthesized compounds.

From the results, TCH-9, 12 was accomplished best binding efficiency against BRAF with, similarly, compound TCH-20, 24 against VEGFR, Compounds TCH-5, 6 against PI3K γ Correspondingly compound TCH-10 and TCH-11 showed good binding efficiency against CDK-2 and FAK respectively. The corresponding Schrodinger Glide scores were reported in Table-4 and 5.

A direct revision into the Structure-Activity Relationship (SAR) of these compounds clearly exhibited the intrinsic property of Lethality activity associated with the basic skeleton consisting of 1,3,5-triazine and α, β -unsaturated ketone moieties with LC_{50} values range 50-3.125 $\mu\text{g/mL}$. It is noteworthy that the observed inhibitory activity of 1,3,5-triazine-chalcone hybrid molecules **TCH 1-35** against *Artemia salina* (Brine shrimp) revealed the importance of the type of substituted aromatic/heteroaromatic aldehyde from which the corresponding 1,3,5-triazine-chalcone hybrid molecules **TCH 1-35** were obtained, which in some cases was enhanced by the influence of some substituents and decreased by some other substituents

CONCLUSION

The brine shrimp lethality bioassay is considered as a useful tool for the preliminary assessment of toxicity. It can be deduced that the synthesized triazine chalcone hybrids were useful and potent compounds that can be further modified to complex structures into useful therapeutic drugs. Molecular docking of the compounds provides some basic information about the hydrogen bonds and the interacting amino acid residues. Although, the brine shrimp lethality cytotoxicity bioassay is rather inadequate as to the elucidation of the mechanism of action but it offers a front line screen for the establishment of the LC_{50} of test compounds which can be backed up by a more specific and expensive bioassays.

Conflict of interest

We declare that we have no conflict of interest.

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