Research Article

Heterocylcic Based Curcumin: Design, Synthesis and Anticancer Efficacy against HeLa Cells

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ABSTRACT

Developing a new effective anticancer agent becomes an urgent need to overcome of current drug-resistance. In this study, we demonstrated that curcumin with heterocyclic moiety can function as an anticancer agent in a human. A new series of curcumin-based benzodiazepines, diazepines and diazoles were prepared using a simple one pot process. The process involved a condensation reaction of curcumin with various 1,2 diamino compounds and hydrazine. The structures of the prepared heterocycles were identified by the spectroscopic methods Fourier-Transform Infrared Spectroscopy (FT-IR), ^{1}H NMR (Proton Nuclear Magnetic Resonance), and ^{13}C NMR. The *in vitro* anticancer activities of the synthesized curcumin-based heterocycles against HeLa cancer cells were evaluated by the 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay. The viability of HeLa cells was reduced in the range of 4.48%-14.57% within the studied concentrations. Curcumin-based diazepine 6 showed the highest cytotoxic effect on the HeLa cells at all concentrations. It reduced the viability of the tested HeLa cells in range of 4.48% for the 400 µg/ml concentration to 4.95% for the 12.5 µg/ml concentration. Moreover, heterocyclic 6 showed the highest cytotoxic and cytostatic effect among the tested heterocyclics against HeLa cells. It exhibited IC_{50} (Half-maximal inhibitory concentration) and a cytostatic effect of 0.4572 and 0.08515 µg/ml, respectively at a nontoxic level, as the control L6 cells showed cytotoxic and cytostatic effect with IC_{50} values of 22.47 and 1.977 µg/ml, respectively. This study revealed that, the prepared curcumin-based compounds exhibit a promising anticancer activity against HeLa cancer cells at a nontoxic concentration.

Keywords: Curcumin, Benzodiazepine, Diazole, 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide (MTT), Anticancer, HeLa cells

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INTRODUCTION

Cancer is the second worldwide leading cause of death and one of the most common public health problems. World Health Organization (WHO) predicted a 70% increasing in cancer cases by the next few decades (Sawant S and Shegokar R, 2014). Despite the advances in the standard available therapies including chemotherapy, radiotherapy, surgery, immunotherapy and hormone therapy, Chemotherapy continues to be the most effective therapeutic option and widely used treatment for different types of malignancies. However, the side-effects associated with the cancer chemotherapy such as hair loss, fatigue, vomiting, nausea, and even death in severe cases which limits the scope of chemotherapeutic drugs. Therefore, continuous research for more efficient and less toxic cancer drug that can selectively target cancer cells with minimal or no side effects on normal cells is going on (Aslam MS, *et al.*, 2014).

Natural plants products have traditionally been a magnificent source of pharmaceutical agents for centuries. Curcumin (1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione; diferuloylmethane), is a phenolic, natural yellow to orange compound isolated from the plant *Curcuma longa L.* (Zingiberaceae) *(Figure 1)*. Curcumin has been used for centuries as a food colorant, and dietary spice in India, China, and Southeast Asia (Priyadarsini KI, 2013; Fang J, *et al.*, 2005; Borik RM, *et al.*, 2018; Ciochina R, *et al.*, 2014; Ahmed M, *et al.*, 2017; Sato T, *et al.*, 2018).

Figure 1: Curcumin based benzodiazepine

Curcumin and its derivatives possess numerous interesting pharmacological properties and wide variety of biological activities including anti-inflammatory, antioxidant, anti-microbial, antiviral, anti-fungal, antimalarial, anti-angiogenesis, anti-carcinogenic, antitumor, as well as medicinal applications, such as; their use against Alzheimer's disease and in HIV therapies (Fang J, *et al.*,

2005; Borik RM, *et al.*, 2018, Li Q, *et al.*, 2015; Ma'mani L, *et al.*, 2014; Ding L, *et al.*, 2015; Mishra S, *et al.*, 2019; Ramya PS, *et al.*, 2018). Further, curcumin is non-toxic and safe even at high dosages. The non-toxicity and the natural origin of curcumin and its wide range biological activities ensure that curcumin and its derivatives are promising lead compounds in medicinal chemistry. Curcumin contains three important functional groups that mainly contribute to its biological activities, α, β-unsaturated β-diketo group; a seven carbon olefinic linker, and an ortho-methoxy phenolic hydroxy group. Structure activity-based researches have investigated that α, β-unsaturated β-diketo group is an important functionality in anticancer activity, and the length of the olefinic linker is essential for the prevention of protein aggregation in Alzheimer's disease models. Moreover, the ortho-methoxy phenolic hydroxy group and methylenic hydrogen are responsible for the antioxidant activity. Curcumin is insoluble in both water and ether but it is soluble in ethanol, acetone, chloroform, methanol, dichloromethane, and dimethylsulfoxide. It exists in enolic and β-diketonic forms. Therefore, it can undergo keto-enol tautomerisms that are stabilized by intra-molecular hydrogen bonding between the enolic hydrogen atom and keto carbonyl oxygen. The enol is the predominant form of curcumin in solution due to the conjugation and intramolecular hydrogen bonding that contribute to its stability (Ciochina R, *et al.*, 2014; Ahmed M, *et al.*, 2017; Li Q, *et al.*, 2015; Mishra S, *et al.*, 2019; Ramya PS, *et al.*, 2018; Khor PY, *et al.*, 2019).

To date, a series of novel curcumin derivatives that exhibit potential synergistic anticancer activity were synthesized. The results indicated that the prepared derivatives can inhibit breast cancer stem cells growth by hindering the mediated efflux mechanism of P-glycoprotein (P-gp). Glucoside of curcumin derivatives showed higher affinity to bind with P-glycoprotein than other derivatives of curcumin, which have reduced the breast cancer stem cells growth (Mbese Z, *et al.*, 2019). The anticancer effect of the combination of 5-fluorouracil (5-FU) with curcumin against gastric cancer MKN45 and AGS cells (Human Gastric Adenocarcinoma Cells) was reported (Yang H, *et al.*, 2017). The results indicated that the combination of 5-FU and curcumin (2:1, mol/mol) enhanced cytotoxic effect compared to curcumin or 5-FU alone and showed synergistic effect. Moreover, the combination of 5-FU and curcumin also potentiated cytotoxicity in AGS cells compared to curcumin or 5-FU alone, but the effect was moderate. Further, synthesis of new heterocyclic derivatives based on curcumin was reported (Borik RM, *et al.*, 2018). The *in vitro* inhibition capacity of the synthesized compounds was screened in two human cancer cell lines (breast cancer (MCF-7) and Hepatocellular carcinoma (HEPG2) in addition to the normal cell line (human normal melanocyte, HFB4) in comparison to the known anticancer drugs: Doxorubicin and 5-flurouracil. The anticancer activity results investigated that the synthesized products showed growth inhibition activity against HEPG2 cell line and MCF-7 cell line, but with varying intensities compared to the known doxorubicin and 5-flurouracil anticancer drugs (Borik RM, *et al.*, 2018).

In the present work, we report synthesis of new curcumin-based benzodiazepines, diazepines and diazoles *via* an acid catalyzed condensation cyclization reaction of curcumin with different 1,2 diamines, pyridine, pyrazine, or pyrimidine. Moreover, synthesis of hydrogenated curcumin (H-curcumin) based amine using a two-step process was also reported. The *in vitro* anticancer activities of the synthesized heterocycles against HeLa cancer cells were screened using the MTT colorimetric assay.

MATERIALS AND METHODS

All chemicals used in this study were purchased from Aldrich Chemical Company and used as they were received. All prepared compounds were characterized by ^{1}H NMR, ^{13}C NMR, MS/MS (Tandem Mass Spectrometry) and Infra-Red (IR) spectroscopy. Nuclear Magnetic Resonance spectra were recorded on Varian Gemini 2000, 300 MHz instruments. Infrared spectra were recorded on a Shimadzu 820 PC (Polycarbonate) FT-IR spectrometer. The solvent used in the NMR was DMSO-d$_6$ (Dimethyl sulfoxide-d$_6$), ^{1}H NMR experiments were reported in δ units, parts per million (ppm) downfield from Tetramethyl Silane (TMS). All ^{13}C NMR spectra were reported in ppm relative to DMSO-d$_6$ (39.52 ppm). The reactions progress was monitored using Thin Layer Chromatography (TLC) analysis performed on silica gel plates, pre-coated with Merck Kieselgel 60 F254, and visualization was done using an Ultraviolet (UV) lamp. The mobile phase used was hexane: Ethyl acetate (6:4) mixture. All melting points were uncorrected and were determined in an open capillary tube. Each melting point was carried out at least in duplicate. The sample purifications were carried out by either crystallization or flash chromatography with silica gel (100-200) mesh.

Preparation of curcumin-based heterocyclic compounds

In a round-bottomed flask equipped with a condenser and a magnetic stirring bar, curcumin (1.357 mmoles, 0.5 g) was dissolved in (30.0 mL) ethanol. The desired amine, pyridine, pyrazine, or pyrimidine (1.357 mmoles) was added to the curcumin solution followed by adding a few drops of the catalyst concentrated sulfuric acid. The reaction mixture was then refluxed in a paraffin oil bath until completion of the reaction (12 to 24 hr), which was monitored by TLC. The excess solvent was removed under reduced pressure using a rotary evaporator; the residue was washed with sodium bicarbonate solution (5%), filtered, washed again with water and dried. Residual starting materials were washed out from the product by suspending the product in either diethyl ether or ethyl acetate. The products were further purified by flash chromatography or crystallization. In the preparation of compound 2 glacial acetic acid was used as a solvent (Hamed O, *et al.*, 2020).

5,7-bis [(E)-2-(4-hydroxy-3-methoxyphenyl) ethenyl]-1H-1,4-diazepine-2,3-dicarbonitrile (2): A mixture of curcumin (1.357 mmole, 0.5 g) and diaminomaleonitrile (0.146 g, 1.357 mmol) were refluxed in glacial acetic acid (30.0 mL) for about 60 h. The product was recrystallized from hexane/EtOAc (2:1 by volume) to give 0.126 g (yield 21.1%), mp 250°C-254°C, IR: v$_{max}$ cm^{-1} 3417.74 (O-H), 3366.66 (N-H), 2362.39 (C≡N), 1650.55 (C=N), 1558.32 (C=C). ^{1}H-NMR (400 MHz, DMSO-d$_6$) δ: 3.82 (s, 6H, OCH$_3$), 4.05 (bs, 1H, NH), 5.10 (1H, s), 5.41 (bs, 2H, OH), 5.71 (d, 1H, J=15.1 Hz), 6.82 (m, 4H), 7.03 m, 3H; 7.25 (d, 2H, J=7.5 Hz).^{13}C-NMR (400 MHz, DMSO-d$_6$) δ: 56.1, 103.6, 105.0, 11.3, 138, 1147.9, 115.2, 116.8, 120.2, 122.9, 124.3, 127.6, 135, 149.1, 149.4, 164.6. LC/MS [M+1] for C$_{25}$H$_{20}$N$_4$O$_4$: Calculated 441.15, found: 441.82.

4-[(E)-2-{2-[(E)-2-(4-hydroxy-3-methoxyphenyl) ethenyl]-5H-pyrido [2,3-b][1,4] diazepin-4-yl} ethenyl]-2-methoxyphenol (3): A 2,3-diaminopyridine (0.148 g, 1.357 mmol) was added to a solution of curcumin (0.5 g, 1.357 mmol) in ethanol (30.0 mL), followed by adding a catalytic amount of H$_2$SO$_4$ (3 drops). The produced mixture was stirred under reflux until reaction completion. The product was recrystallized from hexane/EtOAc (2:1 by volume) to give 0.35 g (yield 58.5%), m.p is 118-120. IR: v$_{max}$ cm^{-1} 3344 (O-H, and N-H stretching), 3022 (=C-H), 2974 (C-H, aliphatic), 1605 (C=N), 1584 (C=C, conjugated), 1389 (C-N), 1085 (C-O ether and alcohol). ^{1}H-NMR (400 MHz, DMSO-d$_6$) δ: 3.83 (s, 6H, OCH$_3$), 4.0 (bs, 1H, NH), 5.06 (1H, d), 5.35 (bs, 2H, OH), 5.67 (d, 1H, J=15.1 Hz), 6.80-6.98 (m, 7H); 7.13 (m, 1H); 7.26 (m, 2H); 7.36 (d, 1H, J=7.5 Hz); 8.12 (d, 1H).^{13}C-NMR (400 MHz, DMSO-d$_6$) δ: 56.1, 88.69, 111.9, 113.0, 116.8, 122.9, 124.0, 127.6, 132.6, 135.0, 138.1, 146.6, 147.9, 149.1, 149.5, 160.0, 164.6. LC/MS [M+1] for C$_{26}$H$_{23}$N$_3$O$_4$: Calculated 442.17, found: 442.43

6,8-bis [(E)-2-(4-hydroxy-3-methoxyphenyl) ethenyl]-5H-pyrazino [2,3-b] [1,4] diazepine-2,3-dicarbonitrile (4): A sample of 5,6-diamino-2,3-pyrazindicarbonitrile (0.217g, 1.357 mmol) was added to a solution of 1,7- curcumin (1.357 mmole, 0.5 g) in ethanol (30.0 mL) followed by adding catalytic amount of H$_2$SO$_4$ (3 drops). Then the solution was refluxed until reaction completion. The product was recrystallized

from hexane/EtOAc (2:1 by volume) to give 0.67 g (Yield 99.55%), m.p. 206°C-208°C, IR: v_{max} cm^{-1} 3338.69 (N-H), 3157.25 (=C-H), 2961.48 (C-H), 2231.89 (C≡N stretch), 1671 (C=N), 1628.62 (C=C). ^{1}H-NMR (400 MHz, DMSO-d$_6$) δ: 3.81 (s, 6H, OCH$_3$), 4.1 (bs, 1H, NH), 5.09 (1H, s), 5.41 (bs, 2H, OH), 5.67 (d, 1H, J=15.1 Hz), 6.81 (m, 3H), 6.83 (d, 1H); 6.88 (d, 1H); 6.99 (d, 2H); 7.21 (d, 2H, J=7.5 Hz).^{13}C-NMR (400 MHz, DMSO-d$_6$) δ: 56.2, 112.1, 117.3, 122.9, 127.9, 131.3, 135.1, 124.4, 137.9, 148.1, 149.3, 149.7, 154.5, 147.5, 155.2, 160.2, 164.5. LC/MS [M+1] for C$_{27}$H$_{20}$N$_6$O$_4$: Calculated 493.15, found: 493.73.

4-[(E)-2-{3-bromo-8-[(E)-2-(4-hydroxy-3-methoxyphenyl)ethenyl]-5H-pyrazino[2,3-b][1,4]diazepin-6-yl} ethenyl]-2-methoxyphenol (5): A sample of 2, 3-diamino-5-bromopyrizine (0.1276 g, 0.678 mmole) was added to a solution of curcumin (0.678 mmole, 0.25 g) in ethanol (30.0 mL), followed by adding catalytic amount of H$_2$SO$_4$ (1 drop). Then the solution was refluxed until reaction completion. The product was recrystallized from hexane/EtOAc (2:1 by volume) to give 0.31g (Yield 87.85%), mp 88°C-90°C, IR (KBr): v_{max} Cm^{-1} 1621.33 (-C=N), 542.15 (C-Br), and 3384.14 (-C-N-H). ^{1}H-NMR (400 MHz, DMSO-d$_6$) δ: 3.82 (s, 6H, OCH$_3$), 3.98 (bs, 1H, NH), 5.06 (1H, s), 5.35 (bs, 2H, OH), 5.67 (d, 1H, J=15.1 Hz), 6.79 (m, 2H), 6.85 (d, 1H, J=15.1 Hz), 6.86 (d, 2H); 6.99 (d, 2H); 7.16 (d, 2H, J=7.5 Hz), 7.96 (s, 1H).^{13}C-NMR (400 MHz, DMSO-d$_6$) δ: 56.2, 103.1, 111.5, 116.4, 121.2, 122.5, 124.2, 135.6, 139.3, 147.2, 149.3, 150.6, 159.3, 164.8. LC/MS [M+1] for C$_{25}$H$_{21}$BrN$_4$O$_4$: Calculated 521.08, found: 521.38 and 533.43 (bromine isotope).

4-[(E)-2-{8-bromo-4-[(E)-2-(4-hydroxy-3-methoxyphenyl)ethenyl]-1H-pyrido[2,3-b][1,4]diazepin-2-yl]ethenyl]-2-methoxyphenol (6): A 2, 3-diamino-5-bromopyridine (0.2538 g, 1.357 mmol) was added to a solution of curcumin (1.357 mmole, 0.5 g) in ethanol (30.0 mL), followed by the addition of concentrated H$_2$SO$_4$ (2 drops). The produced solution was then refluxed until reaction completion. The product was recrystallized from hexane/EtOAc (2:1 by volume) to give 0.34 g (Yield 48.14%), mp 108°C-110°C, IR (KBr): v_{max} cm^{-1} 1623.19 (-C=N), 3374.27 (-C-NH), 568.88 (C-Br), and 1030.66 (C-O ether) of (-O-CH$_3$). ^{1}H-NMR (400 MHz, DMSO-d$_6$) δ: 3.83 (s, 6H, OCH$_3$), 4.02 (bs, 1H, NH), 5.05 (1H, s), 5.33 (bs, 2H, OH), 5.68 (d, 1H, J=15.1 Hz), 6.81 (m, 4H), 6.87 (d, 1H, J=15.1 Hz); 6.97 (d, 2H, J=7.5 Hz); 7.16 (s, 2H), 7.67 (s, 1H), 8.14 (s, 1H). ^{13}C-NMR (400 MHz, DMSO-d$_6$) δ: 56.2, 103.1, 111.5, 116.4, 121.2, 122.5, 123.2, 124.2, 135.6, 139.3, 147.2, 149.3, 150.6, 159.3, 164.8. LC/MS [M+1] for C$_{26}$H$_{22}$BrN$_3$O$_4$: Calculated 521.08, found: 521.38 and 533.43 (bromine isotope).

4-[(E)-2-{5-[(E)-2-(4-hydroxy-3-methoxyphenyl)ethenyl]-1-(pyrimidin-2-yl)-1H-pyrazol-3-yl} ethenyl]-2-methoxyphenol (7): A 2-hydrazinopyrimidine hydrate (0.15 g, 1.357 mmol) was added to a solution of 1,7-Bis(4-hydroxy-3-methoxyphenyl)hepta-1,6-diene-3,5-dione (curcumin) (1.357 mmole, 0.5 g) in ethanol (30.0 mL), followed by the addition of concentrated H$_2$SO$_4$ (2 drops). The produced solution was refluxed for about 12 h. The product was recrystallized from hexane/EtOAc (2:1 by volume) to give 0.4 g (Yield 66.66%), mp 88°C-90°C, IR (KBr): v_{max} cm^{-1} 1639.9 (-C=N), 1061 (C-O ether) of (-O-CH$_3$), and 1214.80 (N-N). ^{1}H-NMR (400 MHz, DMSO-d$_6$) δ: 3.81 (s, 6H, OCH$_3$), 5.38 (sb, 2H, OH), 6.76 (s, 1H), 6.97 (m, 6H); 7.12 (d, 2H, J=7.7 Hz), 7.18 (m, 2H, J=7.7 Hz); 7.68 (m, 1H), 8.83 (d, 2H, J=7.9 Hz). ^{13}C-NMR (400 MHz, DMSO-d$_6$) δ: 56.2, 107.5, 109.3, 116.4, 116.6, 118.5, 122.7, 123.8, 130.3, 131.4, 147.3, 147.5. 148.1, 149.3, 155.7, 156.6. LC/MS [M+1] for C$_{25}$H$_{22}$N$_4$O$_4$: Calculated 444.17, Found: 444.67.

Preparation of H-Curcumin Based Amine

1,7-bis (4-hydroxy-3-methoxyphenyl) heptane-3,5-dione (8): A low-pressure reaction bottle was charged with a solution of curcuminoids 10.0 g in absolute ethanol (100 ml) and in the presence of Pd/C (0.35 g) which was used as a heterogeneous catalyst. The bottle was attached to the low-pressure hydrogenation apparatus and evacuated, and then hydrogen

was admitted to a pressure slightly above 5 atm. The contents of the flask were shaken until absorption of hydrogen stopped (about 4 hrs). The catalyst was removed by filtration and ethanol was removed under vacuum to afford 4.6 g (91.8%) of pale-yellow gummy material. The gummy material was purified by flash chromatography using ethyl acetate as eluent. The prepared compound 8, was analyzed by ^{1}H NMR and ^{13}C NMR. ^{1}H NMR (300 MHz) (CDCl$_3$): δ=6.8 (d, J=8.24, 2H), 6.62 (d, J=8.42=2H), 6.6 (s, 2H), 5.6 (br, 2H, OH), 5.4 (s, 0.75 H, vinylic), 3.90 (s, 0.5H, diketone), 3.85 (s, 6H, OCH$_3$), 2.9 (t, J=7.97, 3H), 2.6 (t, J=7.14, 3H). ^{13}C NMR (CDCl$_3$ d$_6$) δ: 193.2 (Cc and Ce), 144.5 (C4'), 143.9 (C3'), 132.5 (C1'), 120.7 (C6'), 114.3 (C2'), 110.9 (C5'), 99.8 (Cd), 55.8 (OMe), 40.4 (Cb, Cf), 31.1 (Ca, Cg).

4-[(4Z)-5-amino-7-(4-hydroxy-3-methoxyphenyl)-3-iminohept-4-en-1-yl]-2-methoxyphenol (9): A sample of 1,7-Bis(4-hydroxy-3-methoxyphenyl) hepta-1,6-diene-3,5-dione (H-curcumin, 8) (0.5 g, 12.5 mL, 1.357 mmol) was placed in a single-neck round bottom flask (50 mL) and dissolved in 2.0 mL ethanol. To the solution was added ammonium acetate (0.313 g, 1.357 mmol). The produced mixture was stirred at 90°C until complete solvent evaporation. Then it was heated at 140°C until reaction completion (an hour), which was monitored by TLC. After cooling down to room temperature, it was purified by washing with water, isopropyl alcohol and diethyl ether. The purification was further completed by crystallization from ethanol/diethyl ether to give 0.3 g (yield 60%) of yellow solid. Melting point is 160°C-163°C, IR: v_{max} cm^{-1} 3216.78 (C-NH), (C=N), 2939.29 (C-N), 1605.51 (C=C), 1515.17 (C=N). ^{1}H NMR (400 MHz, DMSO-d$_6$) δ: 1.86 (t, 2H, CH$_2$), 2.29 (t, 2H, CH$_2$), 2.56 (t, 4H, CH$_2$), 3.83 (s, 6H, OCH$_3$, methyl), 3.88 (s, 1H), 5.35 (bs, 2H, OH), 6.68 (d, 2H, CH benzene), 6.71 (s, 2H, CH benzene), 6.79 (d, 2H, CH benzene) 8.56 (bs, 2H, NH$_2$).

^{13}C NMR (400 MHz, DMSO-d$_6$) δ: 29.7, 34.8, 35.7, 36.7, 51.7, 56.1, 113.2, 115.5, 122.5, 133.0, 145.9, 147.1, 147.4, 164.6.

Biological assays

Preparation of stock solutions: Solutions of curcumin-based heterocyclics were prepared at a concentration of 400 μg per 1 mL of Dimethyl Sulfoxide (DMSO) solvent and then incubated at 4°C.

Various concentrations of (200, 100, 50, 25, and 12.5 μg/ml) were then prepared using serial dilution method.

Cell lines: The human cervical cancer cell lines (HeLa cells) were obtained from the American Type Culture Collection [ATCC], Manassas, VA, USA. HeLa cells were grown in RPMI medium supplemented with 10% fetal calf serum, 1% non-essential amino acid, 1% l-glutamine, 1% penicillin streptomycin and 1% amphotericin B. All cells were grown in a humidified atmosphere of 95% air, 5% CO$_2$ at 37°C, the culture medium was changed at least twice a week as needed. All chemicals used were purchased from Biological Industries except for the amphotericin B and MTT reagent from SIGMA Aldrich.

For screening experiment, the cells were grown into 12-well plates in 950 μl of RPMI medium (Roswell Park Memorial Institute Medium) (Biological Industries, USA) containing 5% FBS, 2×10^4 cells/well plating density. After that 50 μl of diverse concentrations (400, 200, 100, 50, 25, and 12.5 μg/ml) of curcumin-based heterocyclics was added in duplicates to the prepared 12-well plates and incubated for 24 hr at 37°C, 5% CO$_2$, 95% air and 100% relative humidity. An inverted microscope (Labomed, USA) was used to observe the morphological changes of the cells.

Cytotoxicity assay

Cells at 70%-80% confluence were detached from culture flask by removing the culture medium then adding 0.05% trypsin-EDTA and a suspension of 100 μl (2.0×10^4 cells/well) of viable cells were seeded in a 96-well plate and incubated for 24 hr at 37°C. After the removal of media cells

were treated with 50 μl stock solution (400 μg/ml) serially diluted to reach concentrations of (200, 100, 50, 25, and 12.5 μg/ml) of curcumin-based heterocyclics and then incubated for 24 hr at 37°C to perform the MTT assay.

MTT assay

The anticancer effect of curcumin-based heterocyclics against cell lines (both normal and cancer cell lines) was estimated by the 3-[4, 5-dimethylthiazole-2-yl]-2, 5-diphenyltetrazolium bromide (MTT) assay using (cell growth determination kit MTT based, Sigma). Cells (2×10^4 cells/well) for cytotoxic assay and (1.0×10^4 cells/well) for cytostatic Assay were incubated with various concentrations of the compounds (400, 200, 100, 50, 25, and 12.5 μg/ml) in 5% CO_2, 95% air and 100% relative humidity at 37°C for 24 h in an Fetal bovine serum (FBS)-free medium. Aseptically MTT solution was added in an amount equal to 10% of the culture volume. Then cultures were returned to incubator and incubated for 4 hours. After the incubation period, the resulting MTT formazan crystals were dissolved by the addition of MTT solvent in an amount equal to the original culture volume. The addition of MTT solvent was performed after the removal and disposal of the culture fluid as HeLa cells and L6 were still attached to the culture surface. The absorbance at 570 nm was measured using microplate reader (Labtech, UK). The relative cell viability was determined by the amount of MTT converted to the insoluble formazan salt. The data are expressed as *v* the mean percentage of viable cells as compared to the respective control.

RESULTS AND DISCUSSION

In preceding studies, a non-toxic and a naturally occurring curcumin was used as a skeleton for synthesizing various heterocyclic compounds including pyrazole, diazepine, benzodiazepine, diazoles, and isoxazole (Hamed O, *et al.*, 2020; Hamed OA, *et al.*, 2013; Qneibi M, *et al.*, 2021). The antibacterial activity and cytotoxicity of the prepared curcumin-based heterocycles were tested against gram-positive and gram-negative bacteria. Some showed excellent antibacterial activities against the tested strains. Moreover, some of the prepared diazepines showed a synergistic effect with ampicillin antibiotic. The results indicated that the curcumin-based heterocycles are promising for designing a potentially active anticancer agent. The compound selected for this purpose are curcumin with heterocyclic moiety, since as shown in (*Figure 2*), they have the functionalities that make ideal for this purpose.
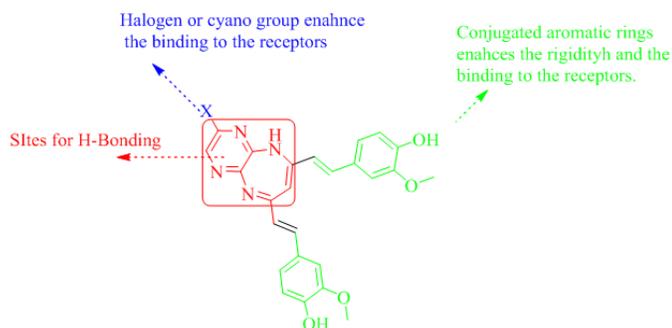

Figure 2: Functional groups present in curcumin bases heterocycles

Based on these results, we intend to extend the work for development of curcumin-based reagents with better bioactivity. The anticancer activities of the prepared heterocycles against HeLa cancer cells were evaluated. Unsophisticated and convenient synthetic methods were employed in this work in preparation of benzodiazepines, diazepines, diazoles, and amines *via* condensation cyclization of curcumin with various 1, 2 diamino and

hydrazine compounds. Schemes I, II and III show a summary of the prepared compounds structures and reaction conditions (*Figures 3 and 4*).
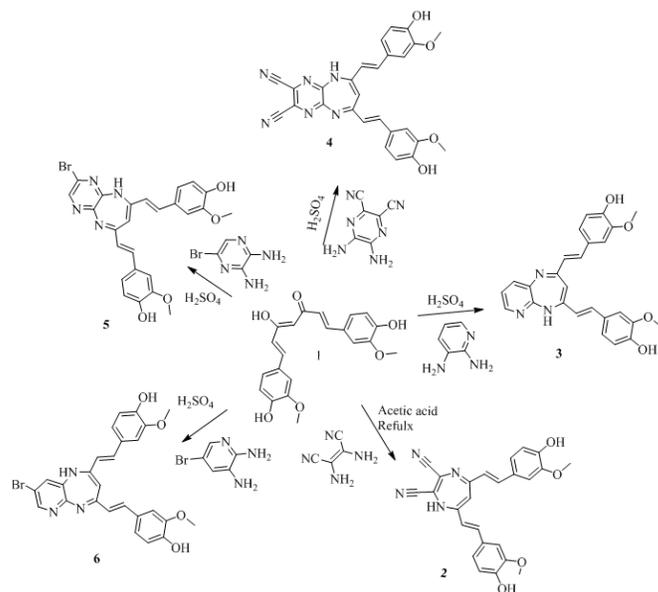

Figure 3: Chemical structures and reaction condensations for making curcumin-based benzodiazepines and diazepines

In the preparation of curcumin-based benzodiazepines, diazepines and diazole, ethanol was used as a solvent and H_2SO_4 as a catalyst. However, in the condensation reaction for making diazepine 2, curcumin was refluxed with diaminomaleonitrile in a glacial acetic acid, which was performed as a catalyst and a solvent. The reaction progress was monitored by TLC. Curcumin-based benzodiazepines, diazepines 2, 3, 4, 5, and 6 required more reflux time than diazole 7. Two methods were employed in purification of the prepared heterocycles, either column chromatography or crystallization. The purified products were analyzed by FT-IR, ^{1}H NMR, and ^{13}C NMR; the detailed descriptions of all the procedures and of the final analytical characterization are summarized in the experimental part. The yield was quantitative for derivatives; it ranged between 21% and 99%.

Hydrogenated curcumin (H-Curcumin-based amine, 9) was prepared in a two-step process, as shown in *Figure 5*. The first step involved the hydrogenation of curcumin 1 using a hydrogenation apparatus charged, absolute ethanol was used as a solvent and Pd/C was the catalyst. The pale-yellow gummy product 8 was purified by flash chromatography using ethyl acetate as eluent and analyzed by FT-IR, ^{1}H NMR, and ^{13}C NMR as described in the experimental part.
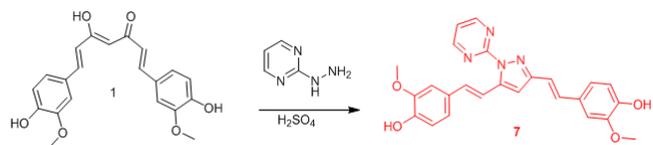

Figure 4: Chemical structures and reaction conditions for making curcumin-based diazole 7

The second step involves condensation reaction of amination of H-Curcumin 8 with Ammonium acetate as shown in *Figure 5*. The progress of reaction was monitored using TLC. The synthesized product was purified as previously described in the experimental part. The structure of compound 9 was identified by FT-IR, ^{1}H NMR, and ^{13}C NMR.
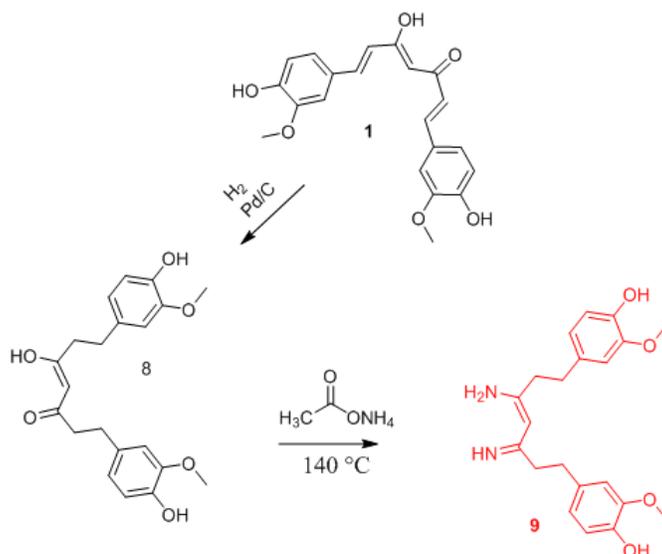
Figure 5: Chemical structures and reaction conditions for making H-curcumin-based amine 9

Anticancer activity

Curcumin and its derivatives have been known to have a wide variety of therapeutic effects, ranging from anti-inflammatory, chemo-preventive, anti-proliferative, and anti-metastatic. In this work a novel set curcumin-based heterocyclics were screened for their anti-tumor effect against HeLa cells. The *in vitro* cytotoxic and cytostatic effect of the prepared hetero-cyclics was evaluated using MTT test.

Cytotoxic effect of the curcumin-based heterocyclics

Cells (2×10^4 cells/well) with 40%-50% confluence were seeded on 96-well plate, the cells were then treated with different concentrations of the prepared curcumin-based heterocyclics (400, 200, 100, 50, 25, and 12.5 μg/ml) and incubated for 24 hours.

MTT assay was used to determine the cell viability by adding MTT solution to the plate and incubated for four hours, after that the isopropyl alcohol was added and incubated in dark for 15 minutes. The microplate reader (Labtech, UK) was used to measure the absorbance at 570 nm.

Cytotoxic effect of the curcumin-based heterocyclics on HeLa cells

The *in vitro* anticancer activities of a novel set of curcumin-based benzo-diazepine, diazepines, diazoles, and H-curcumin based amine against HeLa cancer cell were evaluated. The results indicate that the prepared curcumin-based heterocyclics have varying cytotoxic effect on the HeLa cells at different concentrations. The viability of HeLa cells has reduced in the range of 4.48%-14.57% within the studied concentration range as shown in *(Table 1)*. In general, cells growth was decreased as the concentration of the prepared curcumin-based heterocyclics increased *(Figure 6)*.

Curcumin-based benzodiazepine and diazepines 2, 3, 4, 5, and 6 showed higher potency on HeLa cancer cells than the other tested compounds 7 and 9. The viability of HeLa cells that were treated with benzodiazepine and diazepines 2, 3, 4, 5, and 6 was reduced in the range of 4.48%-6.73%. Curcumin-based diazepines 6 showed the highest cytotoxic effect on the HeLa cells at all concentrations. It reduced the viability of the tested HeLa cells in range of 4.48% for the 400 μg/ml concentration to 4.95% for the 12.5 μg/ml concentration. Among the prepared curcumin-based benzodi-azepine 3 and 5 are more effective against HeLa cancer cells than 4. As they reduced the viability of the tested HeLa cells in range of 4.78% and 4.63% for the 400 μg/ml concentration to 5.95% and 5.9% for the 12.5 μg/ml concentration for 3 and 5, respectively. IC_{50} of the prepared heterocycles tested on HeLa cancer cells were; compound 2:0.2490, compound 3:0.6811, compound 4:4.454, compound 5:2.120, compound 6:0.4572, and compound 7: 1.245. *(Figures 7 and 8, Table 2)*. The lines represent the mean of three independent experiments carried out in triplicates.

Table 1: *In vitro* effects of curcumin-based heterocyclics on the viability of HeLa cells at different concentrations (μg/ml)

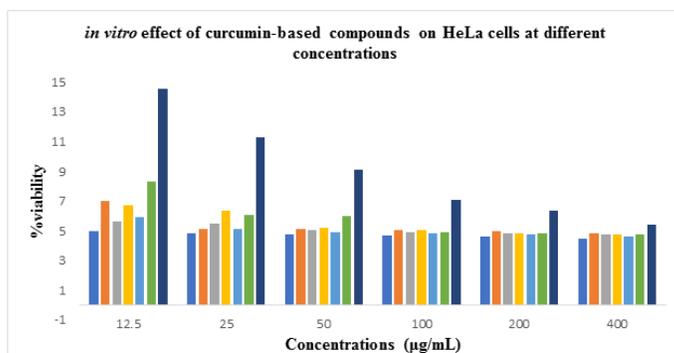| Curcumin derivatives	Concentration (μg/ml)					
	400	**200**	**100**	**50**	**25**	**12.5**
2	4.84%	4.95%	5.07%	5.08%	5.10%	6.96%
3	4.78%	4.84%	4.88%	5.07%	5.44%	5.59%
4	4.72%	4.84%	5.07%	5.19%	6.32%	6.73%
5	4.63%	4.75%	4.82%	4.88%	5.12%	5.90%
6	4.48%	4.60%	4.70%	4.72%	4.84%	4.95%
7	4.71%	4.84%	4.90%	5.95%	6.02%	8.31%
9	5.43%	6.37%	7.08%	9.07%	11.28%	14.57%

Figure 6: *In vitro* effects of curcumin-based heterocyclics on HeLa cells at different concentrations (µg/ml). Note: Series 1(■): Compound 2, series 2 (■): Compound 3, series 3 (■): Compound 4, series 4 (■): Compound 5, series 5 (■): Compound 6, series 6 (■): Compound 7, series 7 (■): Compound 9
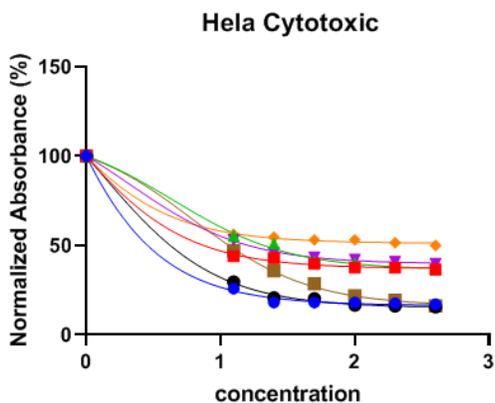

Figure 7: Cytotoxic effect of the prepared heterocycles on HeLa cancer cells. Note: Series 1 (●): Compound 2, series 2 (■): Compound 3, series 3 (▲): Compound 4, series 4 (▼): Compound 5, series 5 (◆): Compound 6, series 6 (●): Compound 7, series 7 (■): Compound 9
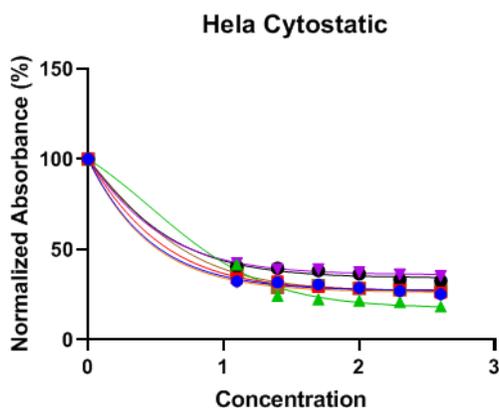

Figure 8: Cytostatic effect of the prepared heterocycles on HeLa cancer cells. Note: Series 1 (●): Compound 2, series 2 (■): Compound 3, series 3 (▲): Compound 4, series 4 (▼): Compound 5, series 5 (◆): Compound 6, series 6 (●): Compound 7, series 7 (■): Compound 9

Table 2: Cytotoxic and cytostatic effect of the prepared heterocycles on HeLa cancer cells and L6 Cells (normal cells)

Cancer cells	Curcumin	2	3	4	5	6	7	9
HeLa cytotoxic	12.46	0.249	0.6811	4.454	2.12	0.4572	1.245	6.164
HeLa cytostatic	10.21	~0.1036	0.3536	2.947	0.3425	~0.08515	0.523	0.8659
L6 cytotoxic	12.7	12.48	13.76	9.307	10.75	22.47	25	15.61
L6 cytostatic	10.5	3.947	1.12	4.088	0.9463	1.977	1.202	1.09

Cytotoxic effect of the prepared heterocycles on L6 Cells (normal cells)

Results show that most of the prepared heterocycles have a cytotoxic effect on L6 cells at concentrations higher than 12.5 µg/ml. IC_{50} of the prepared heterocycles were; compound 2:12.43, compound 3:13.76, compound 4: 9.307, compound 5: 10.75, compound 6: 22.47, compound 7: 25.00 and compound 9: 15.61 *(Figure 9, Table 2)*. The lines represent the mean of three independent experiments carried out in triplicates.

Results of L6 cells showed that all the tested curcumin-based heterocyclics exhibited cytotoxic and cytostatic effects in a dose-dependent manner as presented in *Figures 9 and 10*, and *Table 2*. Most of curcumin-based benzodiazepine and diazepines 2, 3, and 6 induced higher cytotoxic and cytostatic effects compared to curcumin-based diazole 7 and H-curcumin-based amine 9 at the same concentrations. Heterocyclic 2 exhibited cytotoxic and cytostatic effect against HeLa cells with IC_{50} values of 0.2490 and ~0.1036 µg/ml respectively at nontoxic levels as the control L6 cells showed cytotoxic and cytostatic effect with IC_{50} values of 12.48 and 3.947 µg/ml respectively. Heterocyclic 3 exhibited cytotoxic and cytostatic effect against HeLa cells with IC_{50} values of 0.6811 and 0.3536 µg/ml respectively at nontoxic levels as the control L6 cells showed cytotoxic and cytostatic effect with IC_{50} values of 13.76 and 1.120 µg/ml respectively. Heterocyclic 4 exhibited cytotoxic and cytostatic effect against HeLa cells with IC_{50} values of 4.454 and 2.947 µg/ml respectively at nontoxic levels as the control L6 cells showed cytotoxic and cytostatic effect with IC_{50} values of 2.947 and 4.088 µg/ml respectively. Heterocyclic 5 exhibited cytotoxic and cytostatic effect against HeLa cells with IC_{50} values of 2.120 and 0.3425 µg/ml respectively at nontoxic levels as the control L6 cells showed cytotoxic and cytostatic effect with IC_{50} values of 10.75 and 0.9463 µg/ml respectively. Heterocyclic 6 exhibited cytotoxic and cytostatic effect against HeLa cells with IC_{50} values of 0.4572 and ~0.08515 µg/ml respectively at nontoxic levels as the control L6 cells showed cytotoxic and cytostatic effect with IC_{50} values of 22.47 and 1.977 µg/ml respectively.

In general, heterocyclic 6 showed the highest cytotoxic and cytostatic effect among the tested heterocyclics against HeLa cells at same concentration.

It exhibited IC_{50} values of 0.4572 and ~0.08515 µg/ml respectively at non-toxic levels as the control L6 cells showed cytotoxic and cytostatic effect with IC_{50} values of 22.47 and 1.977 µg/ml respectively.

Curcumin-based diazole 7 showed the lowest effect on the HeLa cells. It showed lower effect than curcumin-based benzodiazepine and diazepines 2, 3, 4, 5, and 6. Compound 7 reduced the viability of the tested HeLa cells in range of 4.71% for the 400 µg/ml concentration to 8.31% for the 12.5 µg/ml concentration. Curcumin-based diazole 7 exhibited cytotoxic and cytostatic effect against HeLa cells with IC_{50} values of 1.245 and 0.5230 µg/ml respectively at nontoxic levels as the control L6 cells showed cytotoxic and cytostatic effect with IC_{50} values of 25.00 and 1.202 µg/ml respectively. The anticancer activities shown above could explain by structure activity relationship. Curcumin with benzodiazepine moiety 6 showed the highest activity. The presence of the hetero atom and nitrogen in addition to bromine gives this molecule the ability to accommodate well into the binding site and interact with the receptor site of the cancer cells and interact through H-bonding with functional groups present in the receptor site. As more substituents are introduced in the benzodiazepine ring the activity decreases as the case with compound 4, in this case the steric factor effect becomes the predominant factor that tend to reduce the interaction with the receptor sites and thus the potency drops.

On the other hand, H-curcumin amine 9 showed the lowest cytotoxic effect on the HeLa cells at all concentrations among the prepared heterocyclics. It reduced the viability of the tested HeLa cells in range of 5.43% for the 400 µg/ml concentration to 14.57% for the 12.5 µg/ml concentration. H-curcumin-based amine 9 exhibited cytotoxic and cytostatic effect against HeLa cells with IC_{50} values of 6.164 and 0.8659 µg/ml respectively at nontoxic levels as the control L6 cells showed cytotoxic and cytostatic effect with IC_{50} values of 15.61 and 1.090 µg/ml respectively.

The reason for the low bioactivity of compound 9 could be due to the loss of the olefinic part of curcumin due to hydrogenation. As mentioned in the introduction this functionality is important functionality in anticancer activity. For comparison purpose we tried converting curcumin to curcumin amine as shown in *(Figure 11)* but the effort was unsuccessful.
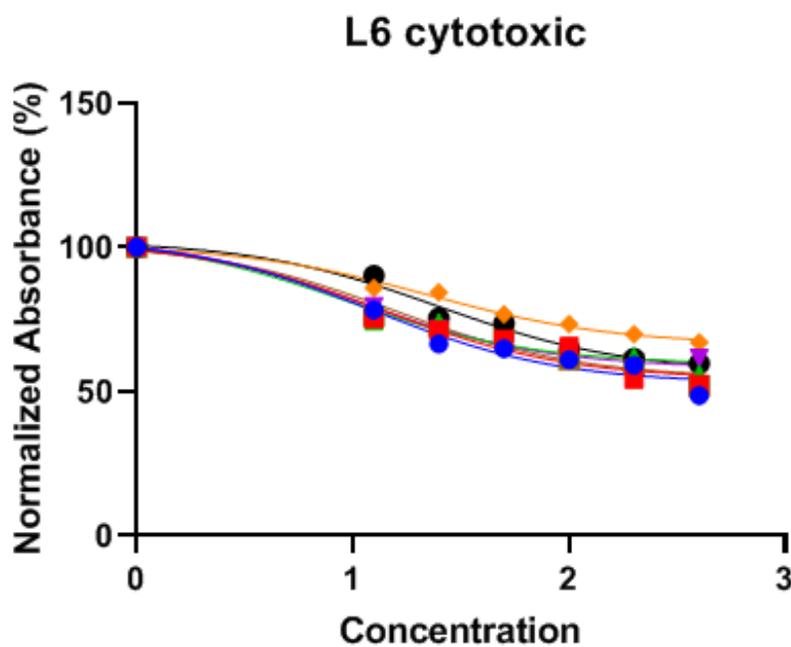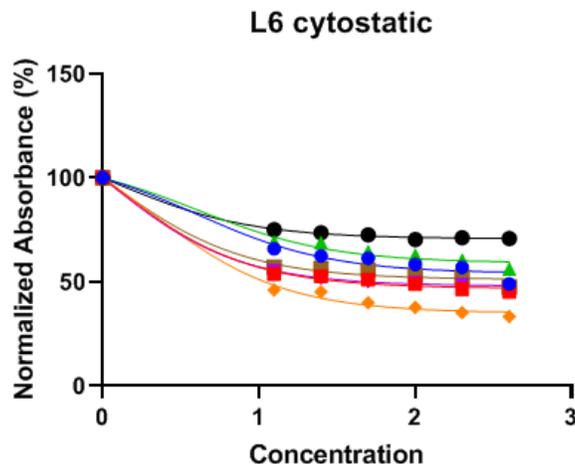

Figure 9: Cytotoxic effect of the prepared heterocycles on L6 Cells (normal cells). Note: Series 1 (—●—): Compound 2, series 2 (—■—): Compound 3, series 3 (—▲—): Compound 4, series 4 (—▼—): Compound 5, series 5 (—◆—): Compound 6, series 6 (—●—): Compound 7, series 7 (—■—): Compound 9

Figure 10: Cytostatic effect of the prepared heterocycles on L6 Cells (normal cells). Note: Series 1 (⬤): Compound 2, series 2 (◼): Compound 3, series 3 (▲): Compound 4, series 4 (▼): Compound 5, series 5 (◆): Compound 6, series 6 (⬤): Compound 7, series 7 (◼): Compound 9
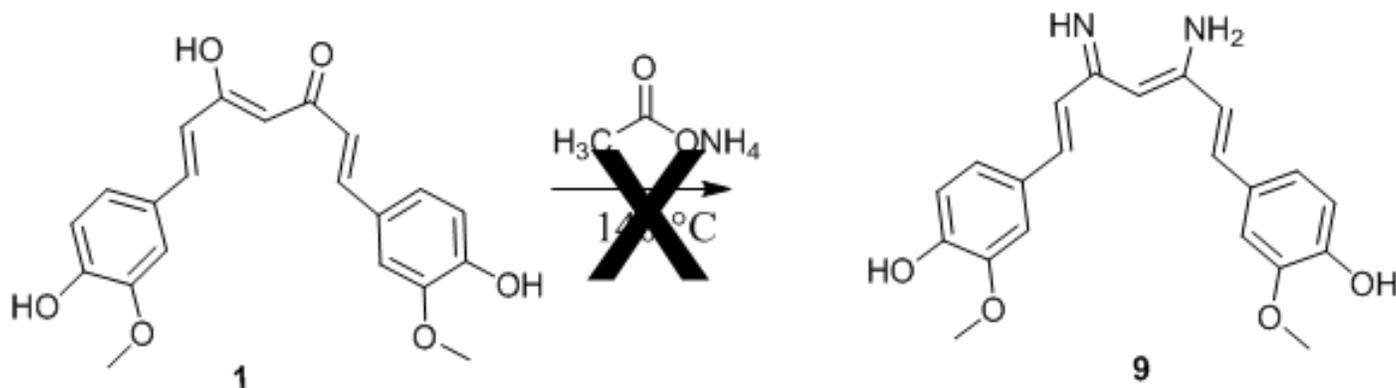

Figure 11: Reaction of curcumin with ammonium acetate

CONCLUSION

The *in vitro* anticancer activities of a novel synthesized set of heterocycles against HeLa cancer cells and normal muscle cells L6 as a control were evaluated. The results indicated that all the tested curcumin-based heterocyclics have varying cytotoxic effect on the HeLa cells at different concentrations. The viability of HeLa cells was reduced in the range of 4.48%-14.57% within the studied concentration. Cell growth was decreased as the concentration of the prepared curcumin-based heterocyclics increased. Curcumin-based benzodiazepine and diazepines showed higher potency on HeLa cancer cells than the other tested heterocyclics. The viability of HeLa cells that were treated with benzodiazepine and diazepines was reduced in the range of 4.48%-6.73%. On the other hand, curcumin-based diazepine 6 showed the highest cytotoxic effect on the HeLa cells at all concentrations. It reduced the viability of the tested HeLa cells in range of 4.48% for the 400 µg/ml concentration to 4.95% for the 12.5 µg/ml concentration. Heterocyclic 6 showed the highest cytotoxic and cytostatic effect among the tested heterocyclics against HeLa cells at same concentration. It exhibited IC_{50} values of 0.4572 and ~0.08515 µg/ml respectively at nontoxic levels as the control L6 cells showed cytotoxic and cytostatic effect with IC_{50} values of 22.47 and 1.977 µg/ml respectively. H-curcumin-based amine 9 showed the lowest cytotoxic effect on the HeLa cells at all concentrations among the prepared heterocyclics. It reduced the viability of the tested HeLa cells in range of 5.43% for the 400 µg/ml concentration to 14.57% for the 12.5 µg/ml concentration. Strictly speaking all the synthe-sized curcumin-based compounds exhibited promising anticancer activity against HeLa cancer cells, which indicates that these compounds have anti-cancer effect at nontoxic concentrations.

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