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New 8-Heterocyclic Xanthine Derivatives as Antiproliferative Agents: Synthesis and Biological Evaluation

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Abstract: This research focuses on the synthesis, characterization, and evaluation of the anti-cancer activity of novel 8-aryl substituted 1,3-diethylxanthine derivatives. The anti-proliferative activities of all the compounds were assessed using an MTT assay on four human cancer cell lines: breast cancer MCF7, human lung cancer A549, human brain cancers LN229, and U87. One of the derivatives of 1,3-diethylxanthine with a thiazole structure displayed strong anti-proliferative activity. 1,3-Diethyl-8-(thiazol-4-yl)-3,7-dihydro-1*H*-purine-2,6-dione (5) exhibited the strongest activity against A549, MCF7, LN229, and U87 cell lines, with IC_{50} values of 16.70, 78.06, 22.07, and 25.07 μM, respectively. Furthermore, the scratch assay was conducted to evaluate the effect of compound 5 on the inhibition of cell migration in A549 cells. The consistent results demonstrate that compound 5 exhibits potent anti-cancer activity, which could be further investigated to enhance its biological potential.

Keywords: Xanthine, Heterocyclic compounds, Antiproliferative activity, Anti-cancer, MTT.

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1. INTRODUCTION

Cancer is a widespread public health problem that affects people of all ages, and it's caused by uncontrolled cell growth and spread, which can invade almost any tissue in the body (1). Despite significant advancements in the discovery and use of anticancer drugs, there are several challenges. These include the non-selectivity and high toxicity of the drugs (2). That's why studies aimed at developing new and effective cancer drugs are crucial. Many classes of organic compounds can be highly effective. Specifically, nitrogen-containing compounds are notable for their wide range of biological activities (3–6).

Xanthine (3,7-dihydropurine-2,6-dione) (Fig. 1) consists of a pyrimidine ring fused with an imidazole ring. They are purine alkaloids containing a nitrogen atom at the 1-, 3-, 7-, and 9 positions and a carbonyl group at the 2- and 6 positions. Different natural and synthetic xanthine derivatives are important therapeutic agents with a wide range of biological effects, such as antiasthmatic (7,8), antagonization of adenosine receptor (9), antibacterial (10), anti-inflammatory (11), anti-oxidant (12,13) and antitumor activity (14). The 8-(4-pyrazolyl)-xanthine derivative (Fig. 1) was found to be a selective and high-affinity A2B AdoR antagonist. It was determined to be safe and well tolerated in two phase 1 clinical studies (15). It has been reported as beneficial in various diseases, including asthma, chronic obstructive pulmonary disease, and pulmonary fibrosis (16,17). Theophylline, a xanthine derivative compound, is reported to have anti-asthma, anti-inflammatory, and immunomodulatory effects (18,19). Additionally, it has been reported to induce apoptosis in chronic lymphocytic leukemia cells and inhibit Bcl-2 expression in leukemic cells (20). According to reports, methylxanthines like theobromine and caffeine demonstrate strong anti-cancer activity (21–24). Studies have reported that xanthine derivatives induce apoptosis (25). Hence, it is crucial to explore the antiproliferative potential of derivatives of xanthine-containing compounds.

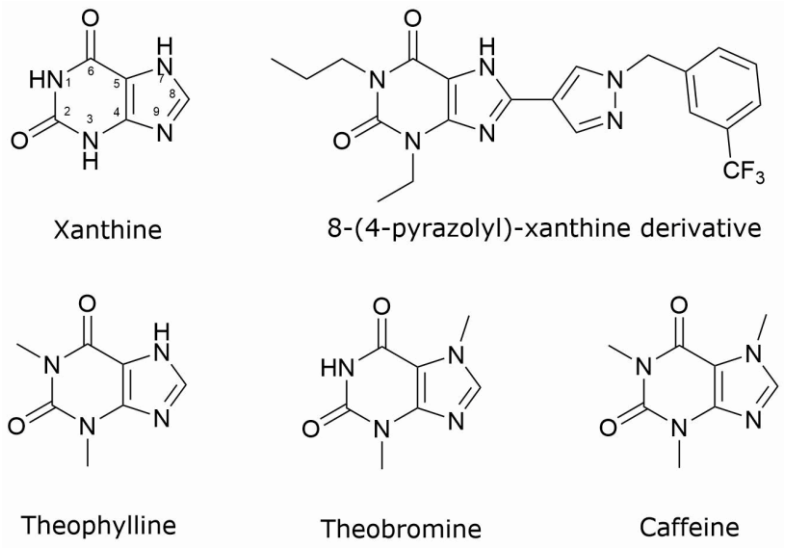


Figure 1: Structures of xanthine derivatives.

Xanthine provides many substitution possibilities, so it is possible to synthesize a wide variety of derivatives. Research indicates that the incorporation of substituents at positions 1, 3, and 8 can lead to the synthesis of more potent compounds (26). Some of the synthesized 8-(substituted)aryloxycaffeine derivatives showed strong inhibitory activity against the tested gram-negative (-) bacteria Salmonella enteritidis, while others also showed strong inhibitory activity against topoisomerase II (27). 8-(3-phenylpropyl)-1,3,7-triethylxanthine compound is reported to be a potent potential adenosine A1 receptor antagonist as a result of in vitro and in vivo activity studies (28). According to studies, incorporating specific groups at the 8th position can significantly enhance the stability and pharmaceutical properties of xanthines (29).

In line with the literature studies mentioned above, a new 1,3-diethyl xanthine derivative with heterocyclic structures substituted at C8 was synthesized and characterized to search for new anti-cancer agents. The anti-cancer activities of these compounds on the proliferation of lung cancer cell line (A-549), breast cancer cell line (MCF-7), and glioblastoma cancer cell lines (LN229 and U87) were determined by MTT assay. In addition, the pharmacokinetic properties of the derivatives were analyzed to evaluate their potential as drug candidates, which showed that all compounds conformed to Lipinski's rule of five.

2. EXPERIMENTAL SECTION

2.1. Materials and Methods

All chemicals and reagents were obtained from Merck, Sigma-Aldrich, and TCI. Reactions were monitored by thin-layer chromatography (TLC). The purity of the compounds was checked on TLC. Column chromatography purifications were performed on Merck Silica gel 60. Melting points were taken in open capillary tubes using a SRS OptiMelt apparatus. High-resolution mass Spectra (HRMS) were measured using the Thermo ORBITRAP Q-EXACTIVE instrument. The ^{1}H and ^{13}C (APT) nuclear magnetic resonance (NMR) spectra were measured in $CDCl_3$ on Agilent 500 MHz NMR spectrophotometer. MCF7 (ATCC, HTB-22), A549 (ATCC, CCL-185), LN229 (ATCC, CRL- 2611), and U87 (ATCC, HTB-14) cells were available in our laboratory. *In vitro* experiments were conducted using fetal bovine serum (FBS), high and low glucose Dulbecco's Modified Eagle Medium (DMEM), Penicillin-Streptomycin, L-Glutamine, and Trypsin/EDTA 0.25%. Antiproliferative activities were performed using the 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) Sigma-Aldrich.

2.2. Chemistry

*2.2.1. General procedure for the synthesis of xanthine analogues (**5-7**):* 5,6-diamino-1,3-diethyl-2,4(*1H,3H*)-pyrimidinedione hydrochloride (1 mmol) was dissolved in a minimum amount of dioxane/H_2O (1:1). EDC.HCl (1.3 mmol) and the corresponding carboxylic acid (1 mmol) were added. The reaction was stirred for 2 h at room temperature. The reaction was neutralized with the addition of 1 N NaOH. The reaction was then heated at reflux for 2 h at room temperature. The precipitate formed after cooling was filtered and washed with water (30). The obtained crude material was purified by SiO_2 column using Ethyl Acetate/*n*-Hexane as eluent.

*2.2.2. 1,3-diethyl-8-(thiazol-4-yl)-3,7-dihydro-1H-purine-2,6-dione (**5**):*
1,3-thiazole-4-carboxylic acid **2** (0.129 g, 1 mmol) was treated with 5,6-diamino-1,3-diethyl-2,4(*1H,3H*)-pyrimidinedione hydrochloride **1** (0.235 g, 1 mmol) and processed as described in the general procedure section. White solid; yield 62%; m.p. 258-260 °C; R_f: 0.3 (4:1 EtOAc:*n*-hexane); ^{1}H NMR (500 MHz, $CDCl_3$) δ 12.03 (s,

1H), 8.92 (d, *J* = 2.1 Hz, 1H), 8.22 (d, *J* = 2.0 Hz, 1H), 4.26 (q, *J* = 7.1 Hz, 2H), 4.19 (q, *J* = 7.0 Hz, 2H), 1.40 (t, *J* = 7.1 Hz, 3H), 1.32 (t, *J* = 7.1 Hz, 3H). ^{13}C NMR (126 MHz, $CDCl_3$) *δ* 155.29, 154.37, 150.92, 149.11, 146.12, 146.01, 119.48, 107.72, 39.05, 37.06, 13.61. HRMS-ESI (m/z) calc. for $C_{12}H_{13}N_5O_2S$ $[M+H]^+$: 292.08682, found: 292.08588.

*2.2.3. 1,3-diethyl-8-(3-methylisoxazol-5-yl)-3,7-dihydro-1H-purine-2,6-dione (**6**):*
3-methylisoxazole-5-carboxylic acid **3** (0.127 g, 1 mmol) was treated with 5,6-diamino-1,3-diethyl-2,4(*1H,3H*)-pyrimidinedione hydrochloride **1** (0.235 g, 1 mmol) and processed as described in the general procedure section. White solid; yield 56%; m.p. 274 °C (decomposed); R_f: 0.48 (4:1 EtOAc:*n*-hexane); ^{1}H NMR (500 MHz, $CDCl_3$) *δ* 6.95 (s, 1H), 4.27 (q, *J* = 7.1 Hz, 4H), 2.42 (s, 3H), 1.38 (dt, *J* = 16.6, 7.1 Hz, 6H). ^{13}C NMR (126 MHz, $CDCl_3$) *δ* 160.39, 159.54, 155.68, 150.25, 148.96, 139.95, 108.49, 105.12, 39.07, 37.29, 13.27, 13.18, 11.32. HRMS-ESI (m/z) calc. for $C_{13}H_{15}N_5O_3$ $[M+H]^+$: 290.12531, found: 290.12433.

*2.2.4. 1,3-diethyl-8-(oxazol-5-yl)-3,7-dihydro-1H-purine-2,6-dione (**7**):*
Oxazole-5-carboxylic acid **4** (0.113 g, 1 mmol) was treated with 5,6-diamino-1,3-diethyl-2,4(*1H,3H*)-pyrimidinedione hydrochloride **1** (0.235 g, 1 mmol) and processed as described in the general procedure section. White solid; yield 26%; m.p. 262 °C; R_f: 0.3 (4:1 EtOAc:*n*-hexane); ^{1}H NMR (500 MHz, $CDCl_3$) *δ* 13.42 (s, 1H), 8.05 (s, 1H), 8.00 (s, 1H), 4.26 (dq, *J* = 21.5, 7.1 Hz, 4H), 1.41 (t, *J* = 7.1 Hz, 3H), 1.37 (t, *J* = 7.1 Hz, 3H). ^{13}C NMR (126 MHz, $CDCl_3$) *δ* 155.97, 151.91, 150.45, 149.54, 142.62, 141.10, 127.83, 108.10, 39.37, 37.49, 13.57, 13.39. HRMS-ESI (m/z) calc. for $C_{12}H_{13}N_5O_3$ $[M-H]^-$: 274.09402, found: 274.09467; $[M+Na]^+$: 298.09161, found: 298.09076.

2.3. Biological evaluation

2.3.1. Cell Culture
A549, MCF7, LN229, and U87 were used for cell viability analysis. Cell lines were cultured with high glucose Dulbecco's Modified Eagle (DMEM) containing 10% FBS, 1% Penicillin- Streptomycin, and 1% L-Glutamine. Cells were grown on a 10 cm petri dish at 37 °C in a 5% CO_2 incubator. It was removed from the flask with 0.25% trypsin/EDTA to perform cell viability experiments. A549, MCF7, LN229, and U87 were seeded in 96-well black plates at a cell density of 9×10^3, 6.5×10^3, 7×10^3 and 7.5×10^3 cells per well, respectively.

2.3.2. Assay for antiproliferative effect
To explore the anti-proliferative potential of compounds, an MTT assay was performed using different cell lines. Cells were seeded in 96-well black plates and cultured for 24 hours at 37 °C in 5 % CO_2 incubator. The growth medium was withdrawn, and the cells were treated with varied concentrations of novel compounds (6.25, 12.5, 25, 50, 100, and 200 µM). After 48 hours of treatment, MTT solution (5 µl, 5 mg/mL in PBS) was added to each well and incubated for 3 h. MTT solution was aspirated, and 200 µl DMSO was added to dissolve the formazan crystals. UV–Vis spectrophotometer was used to measure the optical density at 570 nm. The relative cell viability was expressed as a percentage relative to Tween 20 treated cells as 0% viable and untreated control cells as 100% viable (31). GraphPad Prism software was used to calculate the IC_{50} values.

2.3.3. Scratch Assay
A549 cells were seeded on a 35 mm plate at 6×10^5 per well for the scratch assay. The seeded cells were incubated. Then, the cells were scraped off with a p100 pipette tip. The culture medium was withdrawn, and the cells were treated with 10 µM compound **5** and equal concentrations of DMSO solvent for 48 hours. Markings were made close to the drawn area to obtain the same area during image acquisition. Images of the determined reference points were taken at 0, 24, and 48 hours under the microscope. Images were analyzed with ImageJ software.

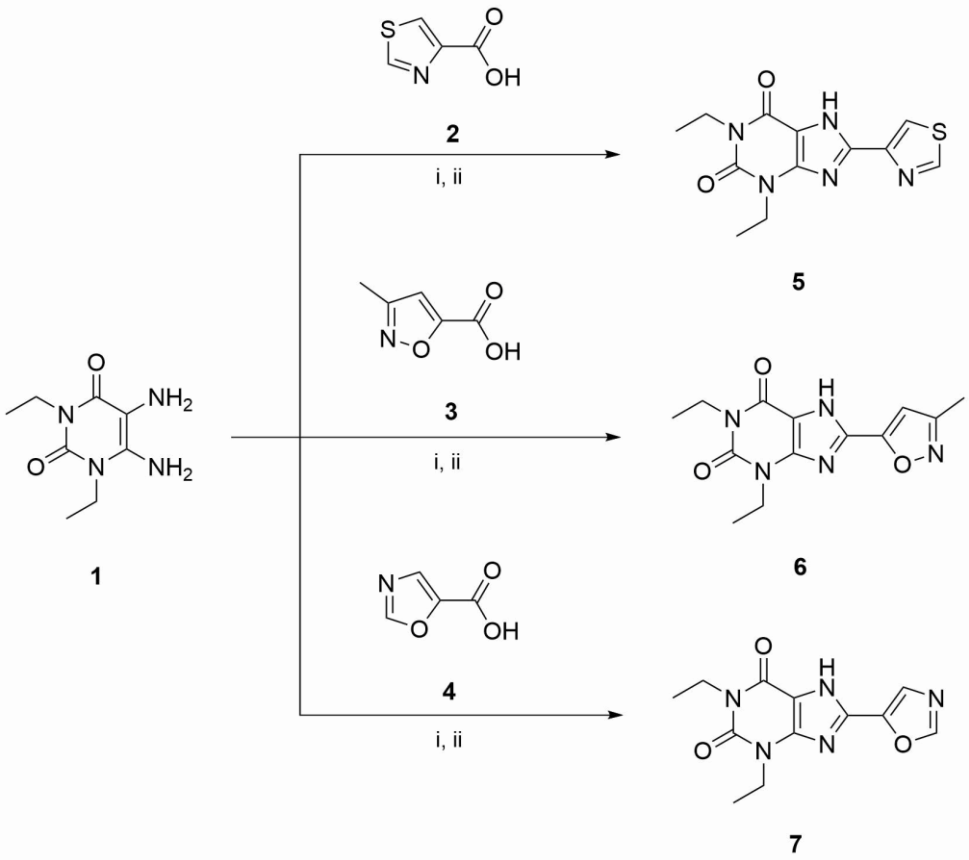
2.3.4. ADME Properties
The ADME properties of the synthesized compounds were predicted using the SwissADME online property calculator (32). Molecular weight, number of rotatable bonds, topological polar surface area (TPSA), the logarithm of the partition coefficient (XLOGP3), and Lipinski's rule of five criteria were calculated.

2.3.5. Statistical Analysis
The experiments were carried out in three sets. All statistical comparisons were made using the Student's t-test. The differences were declared statistically significant at $*p < 0.0001$. The data was expressed as a standard error of the mean (SEM).

3. RESULTS AND DISCUSSION

3.1. Chemistry
The target xanthine derivatives, compounds **5-7** were synthesized as described in Scheme 1. 5,6-diamino-1,3-diethyl-2,4(*1H,3H*)-pyrimidinedione hydrochloride **1** was reacted with a carboxylic acid (1,3-thiazole-4-carboxylic acid **2**, 3-methylisoxazole-5-carboxylic acid **3** and oxazole-5-carboxylic acid **4**) in the presence of the dehydrating agent *N*-(3-Dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (EDC.HCl) to yield the corresponding intermediary amides. Ring closure was achieved by heating the intermediate amides with aqueous NaOH (1 N), yielding the corresponding 1,3-dialkyl-7*H*-xanthine analogs (**5-7**). All compounds were characterized using ^{1}H NMR, ^{13}C (APT) NMR, and HRMS analysis as cited in the Supplementary material. The methyl peaks of the ethyl groups at the 1- and 3-positions of the xanthine nucleus are observed as triplets between 1.32 - 1.41 ppm in the ^{1}H NMR spectra, and

between 13.18 - 13.81 ppm in the ^{13}C (APT) NMR spectra. The methylene peaks are between 4.19 - 4.27 ppm in the ^{1}H NMR spectra, and between 39.37 - 37.06 ppm in the ^{13}C (APT) NMR spectra. The methyl group at the 3-position of the isoxazole ring in compound **6** was observed as a singlet at 2.42 ppm in the ^{1}H NMR and 11.32 ppm in the ^{13}C (APT) NMR spectra. Aromatic protons showed between 6.25–8.92 ppm. In compounds **5** and **7**, a characteristic peak for the -NH group was also observed at ~ 12-13.5 ppm in ^{1}H-NMR.



Scheme 1: Synthetic pathway to xanthine derivatives **5-7**. *Reagents and conditions: (i) EDC.HCl, dioxane:H_2O (1:1); (ii) NaOH (aq), reflux.*

3.2. Biological evaluation

Compounds **5-7** were evaluated for their antiproliferative activity against four cancer cell lines, lung carcinoma (A549), breast cancer cell line (MCF-7), and glioblastoma (GB) cell lines (LN229 and U87) using MTT assay, and tamoxifen citrate was used as the reference compound. Graph Pad Prism software was used to calculate the median inhibition concentration (IC_{50}) for all compounds. Results are illustrated in Table 1. Compounds **6** and **7**, containing isoxazole and oxazole moieties, respectively, displayed weak antiproliferative activity against all cancer cell lines. Compound **5**, which has a thiazole moiety at the 8-substitution in the xanthine nucleus, exhibited significant antiproliferative activity in all cell lines.

Table 1: IC_{50} values (μM) for compounds against the selected cancer cell lines.

Compound	Antiproliferative activity IC_{50} (μM)			
	A549	**MCF7**	**U87**	**LN229**
5	16.70	78.06	22.07	25.07
6	123.0	148.3	>200	159.8
7	190.6	182.8	>200	171.2
Tamoxifen citrate	15.0	15.78	12.68	13.44

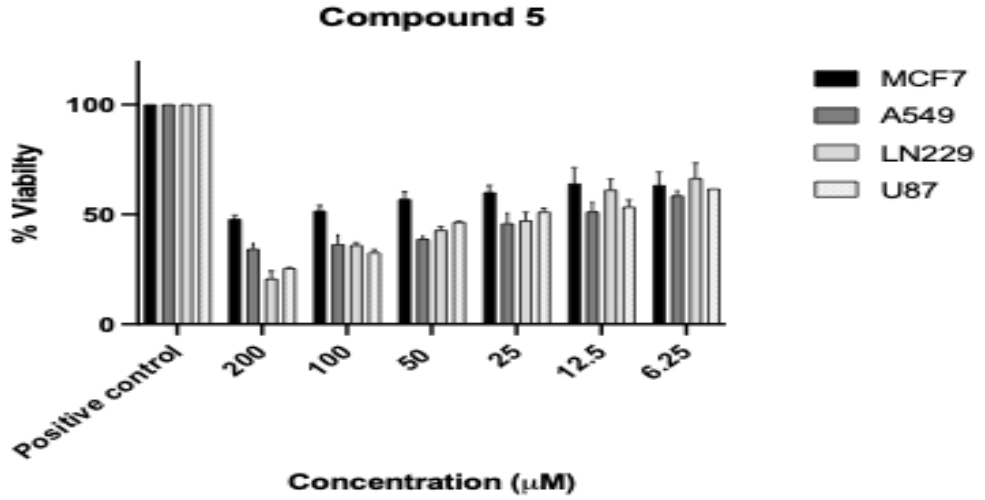


Figure 2: Cell viability results of compound 5 on cancer cells. The error bars show SEM.

Scratch assay was performed to evaluate the inhibition of cell migration in A549 cells treated with compound **5**. Images at various time points, along with the wound closure rate of cells treated with 10 μM compound **5** compared to the control, are presented in Figure 2. The wound closure rate in A549 control cells and cells treated with compound **5** for 48 hours was 85 % and 28 %, respectively. The data demonstrate that cell migration was considerably inhibited in A549 cells treated with compound **5** for 48 hours.

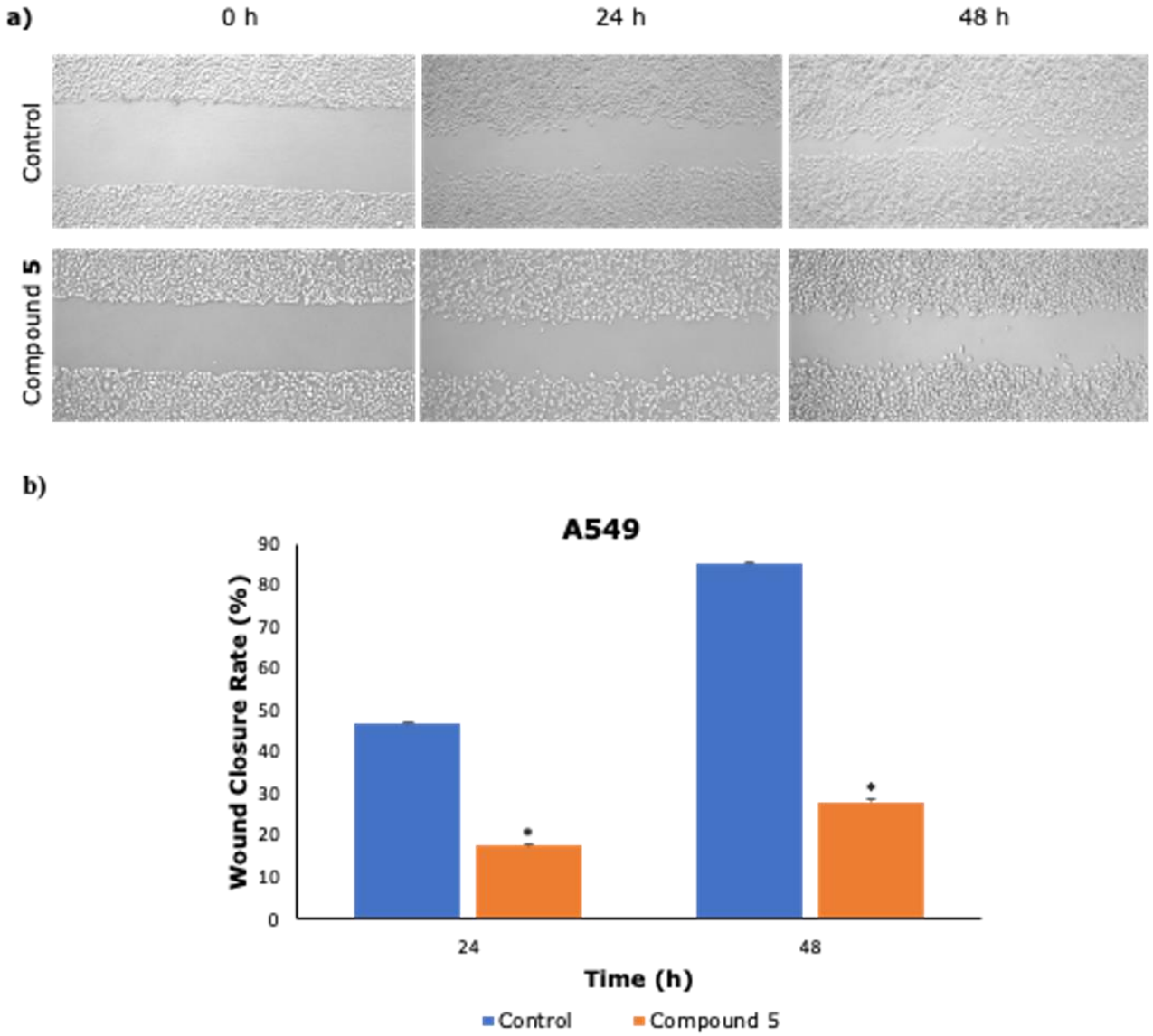


Figure 3: Results of Scratch Assay. Image of A549 cells treated with 10 μM compound **5** for 24 h and 48 h (a). Quantification of the scratch area by using ImageJ analysis (b). Control cells were treated with equal concentrations of DMSO solvent. Differences were considered statistically significant at *p < 0.0001, and the error bars were calculated by SEM.

Evaluating pharmacokinetic properties such as solubility, permeability, and bioavailability is crucial for conducting clinical testing during the drug development process. Lipinski's "rule of five" defines physicochemical parameters that are important for the understanding of molecular properties in the design of new drug candidates (33). The bioavailability and potency of the novel compounds were calculated using the SwissADME calculator, and the results are shown in Table 2. All of the novel compounds adhered to the Lipinski rules, including the number of hydrogen bond donors (n-OHNH< 5), number of hydrogen bond acceptors (n-ON<10), molecular weight of the compounds (Mw< 500 Dalton), octanol-water partition coefficient (logP<5), and the topological polar surface area (TPSA) of the compounds. These data showed that each of the molecules has good cell permeability and drug-like properties. In addition, most of the synthesized compounds were classified as soluble.

Table 2: *In silico* prediction of some pharmacokinetic properties for compounds 5-7.

Compound	Mw g/mol	#rotatable bonds	n-OHNH	n-ON	Log P	Tpsa Å^2	Lipinski's Violation
5	291.33	3	1	4	2.00	13.81	0
6	289.29	3	1	5	1	98.71	0
7	275.26	3	1	5	1.38	98.71	0

MW: Molecular weight. #Rotatable bonds: number of rotatable bonds, determines flexibility, must be less than 9. N-OHNH: The number of H-bond donors must be less than 5. n-ON: the number of H-bond acceptors must be less than 10. LogP: The partition coefficient between n-octanol and water calculated by XLOGP3 method, determines lipophilicity, must be between -0.7 and +5.0. TPSA: Topological Polar Surface Area, determines polarity, must be between 20 and 130 Å^2.

4. CONCLUSION

In the current work, synthesized new xanthine derivatives and characterized utilizing diverse spectroscopic methods like HRMS, ^{1}H NMR, and ^{13}C (APT) NMR. The cytotoxic activity of the target compounds against several cancer cell lines (A549, MCF7, LN229, and U87) was investigated using the MTT test. Compound **5**, containing thiazole, exhibited more antiproliferative activity compared to the tested aryl groups (oxazole, isoxazole, and thiazole). The predicted ADME properties of the new compounds followed Lipinski's rule, indicating that they have sufficient bioavailability and drug-likeness for further biological investigations.

5. CONFLICT OF INTEREST

The author declares no competing interests.

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