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Synthesis, Antiprotozoal activity, Molecular Docking and ADME study of 2-(4-(5-cyano-2-(methylthio)-6-oxo-1,6-dihydropyrimidin-4-yl)phenoxy)-N-phenylacetamide derivatives

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Abstract

A series of chemotherapeutics comprising 2-(4-(5-cyano-2-(methylthio)-6-oxo-1,6-dihydropyrimidin-4-yl)phenoxy)-N-phenylacetamide derivatives **(4a -4i)** was synthesized, characterized and evaluated for antiprotozoal activity. These compounds were screened for the anti-amoebic potential for which HM1:IMSS strain of *Entamoeba histolytica* was taken and metronidazole (MTZ) was used as reference amoebicidal drug. Among the nine dihydropyrimidine-phenylacetamide derivatives, five were recorded with better anti-amoebic activity i.e. lower IC_{50} (0.12 µM to 0.82 µM) values than MTZ (IC_{50} = 1.8 µM). All the nine compounds displayed minimal antimalarial activity with more than 43% inhibition, with a low toxicity profile against three cell lines; human cell lines, Artemia and host red blood cell, but these compounds were more toxic than quinine. These compounds did not exhibit any significant inhibition against the viability of *Anopheles arabiensis* (KGB) larvae or ova. Docking study provided good binding affinity when interacted with the enzyme *O-acetyl-L-serine sulfohydrolase (EhOASS)* of *E. histolytica* (PDB id: 3BM5). These studies demonstrated the dihydropyrimidin-acetamide derivatives to be better anti-amoebic agents.

Keywords; Dihydropyrimidine; anti-amoebic, *Entamoeba histolytica,* antimalarial; cytotoxicity.

1. Introduction

Parasitic protozoan infections affect more than 500 million individuals in the world that is huge proportion of the world's population.[1] The widespread distribution of protozoan diseases poses significant challenges for public health. Factors such as poverty, lack of access to clean water, inadequate healthcare facilities, and climate change exacerbate the prevalence and impact of these infections. The two major examples of protozoan diseases are malaria and amoebiasis that have significant impact on global health.[2] Amoebiasis disease is caused by *Entamoeba histolytica* that continues to be a common but major infection of the human gastro-intestinal tract. The transmission mode of *E. histolytica* is carried out by the fecal-oral route, typically through the ingestion of cysts while consuming contaminated food or water.[3] The cysts are highly resistant and can survive harsh environmental conditions, allowing the parasite to spread easily in areas with poor sanitation. It primarily infects the large intestine, where it adheres to and invades the mucosal lining. The infection can lead to ulcer formation in the colon, resulting in ulcerative colitis. Symptoms may include abdominal pain, diarrhea, and the presence of blood and mucus in the stool. In severe cases, the infection can cause fulminant colitis, which is life-threatening and may require surgical intervention, but it can disseminate to other organs through the hematogenous (blood) route. The most common extraintestinal site of infection is the liver, where the parasite can cause an amoebic liver abscess.[4] The worldwide distribution of this infection is recorded about 50 million and deaths tolls about 110000 every year.[5] This infection is recorded high in Sub Saharan Africa (SSA), Sudan, Cote D'Ivoire, Nigeria, Egypt and South Africa and in some developing countries as India, Mexico, and Japan.[6] On the other hand, malaria, being the life-threatening disease, is also a parasitic infection. The causative agent *Plasmodium* species, especially *P. falciparum, P. vivax, P. ovale, P. malariae* and *P. knowlesi*, are causing malaria in human.[7] This infection was recorded with 249 million new clinical cases and approximately 6.08 lakhs deaths worldwide in 2022 according to WHO recent data.[8] Heterocyclic motifs such as nitroimidazoles and chloroquinolines are core moieties, having potential against amoebiasis and malaria, respectively.[9] It was a major setback in the effective control of these disease when parasites showed resistance against their first line drugs.[10] Metronidazole, being the main drug for the treatment of ameobiasis[11], found to be carcinogenic to human and having serious side effect such as neurological alterations and cardiac rhythm impairment.[12] According to literature survey, cross resistance also developed towards

metronidazole.[13] Chloroquine (CQ) also lost its efficiency as a first-line anti-malarial drug as a result of resistant parasites.[14] Thus, the lack of an effective drug is complicating the treatment and threatening the global health. Therefore, it is a requirement to investigate newer compounds for the development of drugs with improved biological activity and lesser toxicity. Considering the need for a novel therapeutic agent to treat protozoal disease, we had developed a series of pharmacophores containing pyrimidine as a core moiety.

Pyrimidines, being an essential constituent of all cells and thus of all living matter, are also pharmaceutically important heterocycles having nitrogen atoms at 1 and 3 positions in the ring.[15] Pyrimidine have played an important role in medicinal chemistry due to their variegated biological activities including anti-viral, antibacterial, antifungal, antioxidant, anticancer, antimalarial, analgesic and anti-inflammatory and antihypertensive activity.[16] Here are some pyrimidine-based scaffolds that are available in market and used in clinical treatment. 5-Fluorouracil is an oral drug to treat cancer disease.[17] While sulfadiazine is used to treat bacterial infections.[18] Flucytosine (5-FC) is an antifungal drug having pyrimidine analogue that interferes with DNA and RNA synthesis in fungal cells.[19] Particularly, the pyrimidine ring, as a core moiety can be found in pyrimethamine, a well-known anti-malarial and anti-toxoplasmosis drug.[20] Zidovudine (AZT) is pyrimidine nucleus analogue that is prescribed to prevent HIV/AIDS.[21] Epirizole[22], a pyrimidine-pyrazole hybrid, and Aronixil[23] are used to reduce inflammation and high lipid levels respectively. **(Fig-1)** Previously, some other pyrimidine-based derivatives were also reported by researchers for their anti-amoebic activity that possessed good potential. Parveen *et al* synthesized a new series of 2,4,6-trisubstituted bis-pyrimidines as anti-amoebic ranging their IC_{50} values from 0.10 to 9.57 μM.[24] Whilst, Hayat *et al* reported a series of 10 compounds, pyrazolo[3,4-d]pyrimidine-6-one derivatives.[25] Yadava *et al* synthesized pyrazolo[3,4-d]pyrimidine derivatives possessing good potential as anti-amoebic agent.[26] **(Fig-2)**

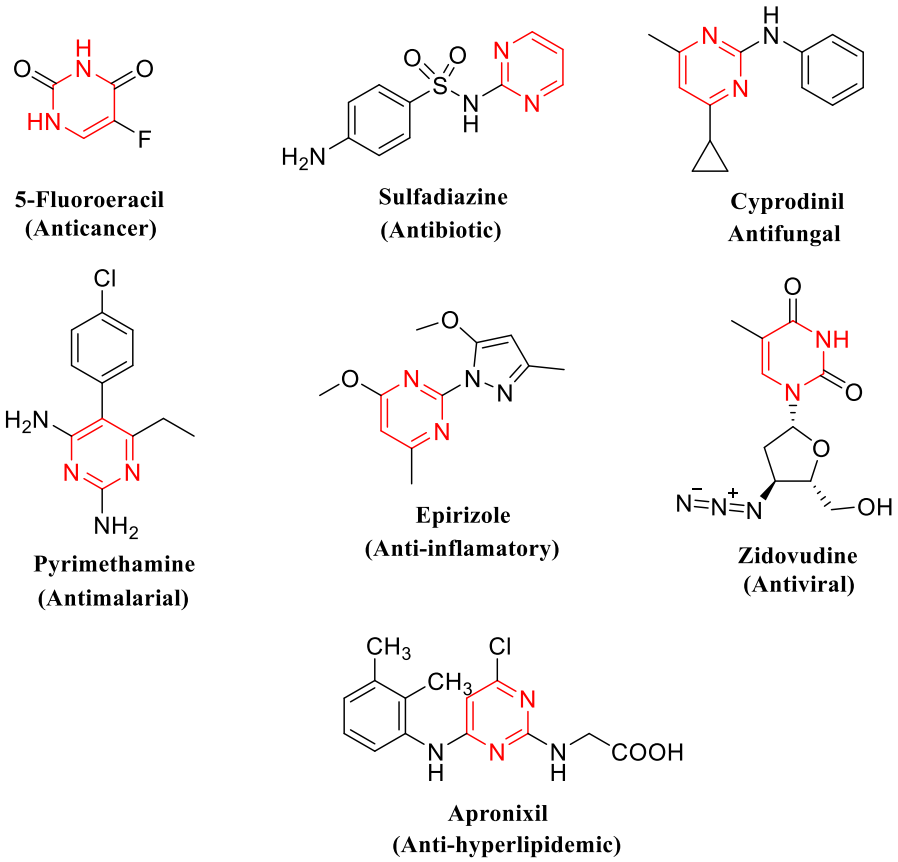


Fig-1: Clinically available drugs containing pyrimidine motif

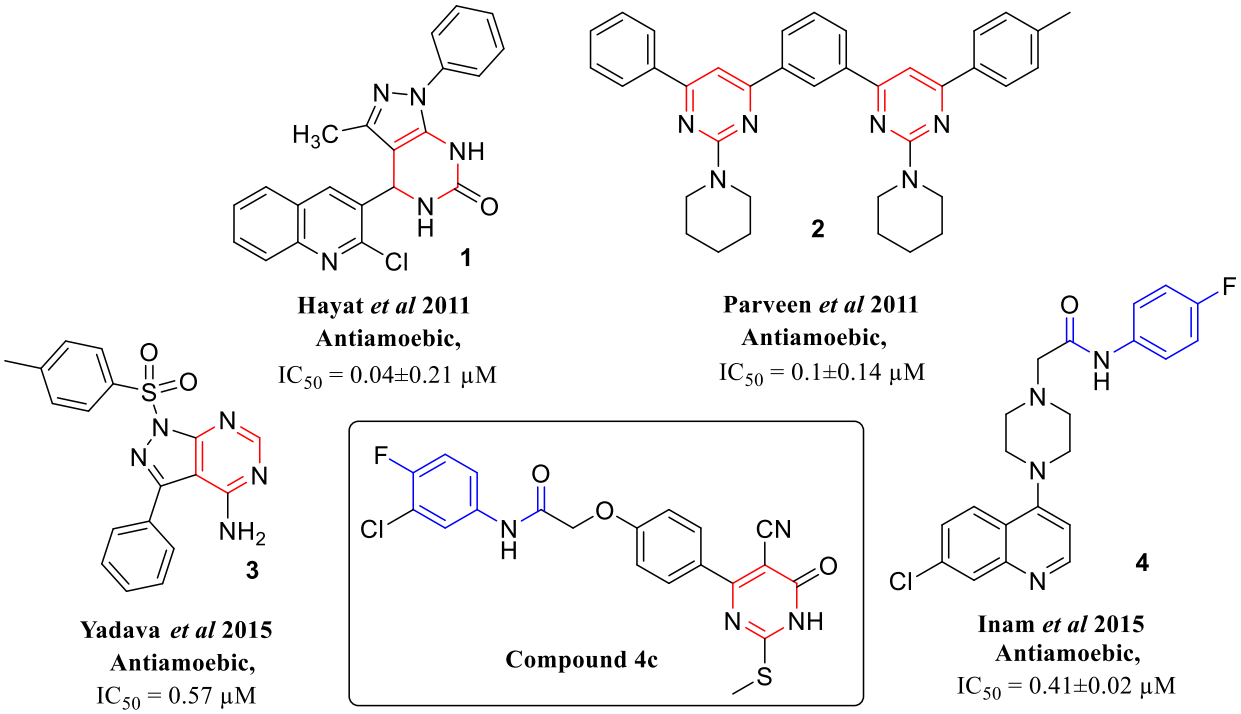


Fig-2: Structural similarity of dihydropyrimidine-phenylacetamide derivative **4c**

2. Materials and methods

2.1. Experimental protocol

All necessary chemicals were obtained from Merck and Aldrich Chemical Company (USA). Thin-layer chromatography (TLC) was performed using precoated aluminum sheets (Silica gel 60 F254, Merck, Germany), and spots were visualized under UV light. Melting points were determined using a Veego REC-22038 A2 instrument and were uncorrected. Elemental analyses were conducted on an Elementar Vario analyzer, with results within ±0.4% of theoretical values. IR spectra were recorded on a Bruker FT-IR spectrophotometer. ^{1}H NMR spectra was obtained on Bruker Spectrospin DPX spectrometers at 300 MHz using DMSO-d_6 as a solvent and TMS as the internal standard. Chemical shifts are reported in ppm with splitting patterns noted as s (singlet), d (doublet), t (triplet), and m (multiplet). Mass spectra were acquired using ESI-MS (AB-Sciex 2000, Applied Biosystems).

***General procedure for the synthesis of chloroacetamide derivatives* (2a-2i)**

Chloroacetamide derivatives were synthesized by a method reported in the literature.[27]

***Synthesis of aldehyde intermediates* (3a-3i)**

The aldehyde intermediates which contained 2-(4-formyl-phenoxy)-*N*-substituted-phenyl acetamides were synthesized by a reported method.[28]

***Synthesis of 2-(4-(5-cyano-2-(methylthio)-6-oxo-1,6-dihydropyrimidin-4-yl)phenoxy)-N-phenylacetamide derivatives derivatives* (4a-4i)**

2-(4-(5-cyano-2-(methylthio)-6-oxo-1,6-dihydropyrimidin-4-yl)phenoxy)-N-phenylacetamide derivatives were synthesized by a reported method.[29]

2-(4-(5-cyano-2-(methylthio)-6-oxo-1,6-dihydropyrimidin-4-yl)phenoxy)-N-phenylacetamide (4a)

Yield 81.3 %; mp: 290°C; FT-IR ν_{max} (cm^{-1}): 3330 (NH), 2212 (C=N), 1666(C=O); ^{1}H NMR (300 MHz, DMSO-d_6) δ (ppm): 10.30(s, 1H, NH), 7.84 (d, 1H, *J*=7.8 Hz, Ar-H), 7.80(d,2H, *J*=8.4 Hz, Ar-H), 7.65(d, 2H, *J*=7.8 Hz, Ar-H), 7.39-7.23(m, 2H, Ar-H), 7.08(d, 2H, *J*=7.5 Hz, Ar-H), 6.97(s, 1H, NH), 4.86 (s,2H,CH_2), 2.34(s,3H,CH_3); Anal. Calc. (%) for $C_{20}H_{16}N_4O_3S$: C, 61.21; H, 4.11; N, 14.28; O, 12.23; S, 8.17; found: C, 61.20; H, 4.10; N, 14.29; O, 12.24; S, 8.16; ESI-MS: m/z = 393 (M+1).

N-(3-chlorophenyl)-2-(4-(5-cyano-2-(methylthio)-6-oxo-1,6-dihydropyrimidin-4-yl)phenoxy)acetamide (4b):

Yield 79.3 %; mp: 275°C; FT-IR ν_{max} (cm^{-1}): 3335 (NH), 2214 (C=N), 1660(C=O); ^{1}H NMR (300 MHz, DMSO-d_6) δ (ppm): 10.33(s, 1H, NH), 7.85 (d, 3H, *J*=9.0 Hz, Ar-H), 7.58(d,1H, *J*=7.8 Hz, Ar-H), 7.38-7.31(m, 2H, Ar-H), 7.24(d, 2H, *J*=8.4 Hz, Ar-H), 6.95(s, 1H, NH), 4.78 (s,2H,CH_2), 2.35(s,3H,CH_3); Anal. Calc. (%) for $C_{20}H_{15}ClN_4O_3S$: C, 56.27; H, 3.54; Cl, 8.31; N, 13.12; O, 11.24; S, 7.51; found: C, 56.26; H, 3.53; Cl, 8.32; N, 13.11; O, 11.25; S, 7.52; ESI-MS: m/z = 428 (M+1).

N-(3-chloro-4-fluorophenyl)-2-(4-(5-cyano-2-(methylthio-6-oxo-1,6-dihydropyrimidin-4-yl)phenoxy)acetamide **(4c)**

Yield 82.5 %; mp: 287°C; FT-IR ν_{max} (cm^{-1}): 3310 (NH), 2215(C=N), 1655(C=O); ^{1}H NMR (300 MHz, DMSO-d_6) δ (ppm): 10.37(s, 1H, NH), 7.77 (d, 1H, *J*=8.7 Hz, Ar-H), 7.47 (d, 1H, *J*=8.1 Hz, Ar-H), 7.35 (t, 1H, *J*=7.8 Hz, Ar-H), 7.30-7.22 (m, 2H, Ar-H), 7.07 (d, 2H, *J*=7.8 Hz, Ar-H), 6.97 (s, 1H, NH), 4.81 (s,2H,CH_2), 2.34 (s,3H,CH_3); Anal. Calc. (%) for $C_{20}H_{14}ClFN_4O_3S$: C, 54.00; H, 3.17; Cl, 7.97; F, 4.27; N, 12.59; O, 10.79; S, 7.21; found: C, 53.91; H, 3.16; Cl, 7.98; F, 4.25; N, 12.58; O, 10.77; S, 7.22; ESI-MS: m/z = 446 (M+1).

2-(4-(5-cyano-2-(methylthio)-6-oxo-1,6-dihydropyrimidin-4-yl)phenoxy)-N-(2,6-dimethyl phenyl)acetamide (4d): Yield 79%; mp: 278°C; FT-IR ν_{max} (cm^{-1}): 3330 (NH), 2212 (C=N), 1668(C=O); ^{1}H NMR (300 MHz, 300 MHz, DMSO-d_6) δ (ppm): 10.14(s, 1H, NH), 7.84 (d, 1H, *J*=9.0 Hz, Ar-H), 7.64 (d, 1H, *J*=7.8 Hz, Ar-H), 7.41-7.28 (m, 1H, Ar-H), 7.20 (d, 2H, *J*=7.8 Hz, Ar-H), 7.08 (d, 2H, *J*=8.1 Hz, Ar-H), 6.95 (s, 1H, NH), 4.80 (s, 2H, CH_2), 2.35 (s, 3H, CH_3), 2.06 (s, 6H, CH_3); Anal. Calc. (%) for $C_{22}H_{20}N_4O_3S$: C, 62.84; H, 4.79; N, 13.32; O, 11.41; S, 7.63; found: C, 62.85; H, 4.78; N, 13.31; O, 11.42; S, 7.64; ESI-MS: m/z = 421 (M+1).

2-(4-(5-cyano-2-(methylthio)-6-oxo-1,6-dihydropyrimidin-4-yl)phenoxy)-N-4-fluorophenyl)acetamide (4e):

Yield 86.1 %; mp: 265°C; FT-IR ν_{max} (cm^{-1}): 3347 (NH), 2216 (C=N), 1664(C=O); ^{1}H NMR (300 MHz, DMSO-d_6) δ (ppm): 10.39 (s, 1H, NH), 7.88 (d, 2H, *J*=8.7 Hz, Ar-H), 7.74 (d, 2H, *J*=8.7 Hz, Ar-H), 7.52 (d, 2H, *J*=9.0 Hz, Ar-H), 7.07 (d, 2H, *J*=8.7 Hz, Ar-H), 6.88 (s, 1H, NH),4.77 (s, 2H, CH_2) , 2.50 (s, 3H, CH_3); Anal. Calc. (%) for $C_{20}H_{15}FN_4O_3S$: C, 58.53; H, 3.68; F, 4.63; N, 13.65; O, 11.69; S, 7.81; found: C, 58.52; H, 3.69; F, 4.62; N, 13.64; O, 11.68; S, 7.82; ESI-MS: m/z = 411 (M+1).

2-(4-(5-cyano-2-(methylthio)-6-oxo-1,6-dihydropyrimidin-4-yl)phenoxy)-N-(naphthalen-1-yl)acetamide (4f):

Yield 86.3 %; mp: 295°C; FT-IR ν_{max} (cm^{-1}): 3320 (NH), 2212 (C=N), 1668(C=O); ^{1}H NMR (300 MHz, DMSO-d_6) δ (ppm): 10.19 (s, 1H, NH), 7.94 (d, 2H, *J*=7.8 Hz, Ar-H), 7.48 (t, 2H, *J*=8.1 Hz, Ar-H), 7.74 (d, 2H, *J*=7.2 Hz, Ar-H), 7.64 (d, 2H, *J*=7.2 Hz, Ar-H), 7.54-7.49 (m,1H,Ar-H), 7.49 (d, 2H, *J*=8.4 Hz, Ar-H), 6.53 (s, 1H, NH),4.91(s, 2H, CH_2) , 2.50 (s, 3H, CH_3); Anal. Calc. (%) for $C_{24}H_{18}N_4O_3S$: C, 65.14; H, 4.10; N, 12.66; O, 10.85; S, 7.25; found: C, 65.15; H, 4.11; N, 12.65; O, 10.84; S, 7.26; ESI-MS: m/z = 443 (M+1)

N-(4-acetylphenyl)-2-(4-(5-cyano-2-(methylthio)-6-oxo-1,6-dihydropyrimidin-4-yl)phenoxy)acetamide (4g): Yield 84.5 %; mp: 298°C; FT-IR ν_{max} (cm^{-1}): 3343 (NH), 2212 (C=N), 1660(C=O); ^{1}H NMR (300 MHz, DMSO-d_6) δ (ppm): 10.36 (s, 1H, NH), 8.28 (m, 1H, Ar-H), 7.93-7.83 (m, 2H, Ar-H), 7.71 (d, 1H, *J*=6.9 Hz, Ar-H), 7.25 (d, 2H, *J*=7.5 Hz, Ar-H), 7.16-7.09 (m,2H,Ar-H), 6.55 (s, 1H, NH), 4.80 (s, 2H, CH_2), 2.56 (s,3H,CH_3), 2.50 (s, 3H, CH_3); Anal. Calc. (%) for $C_{22}H_{18}N_4O_4S$: C, 60.82; H, 4.18; N, 12.90; O, 14.73; S, 7.38; found: C, 60.81; H, 4.19; N, 12.91; O, 14.72; S, 7.39; ESI-MS: m/z = 435 (M+1).

N-(4-bromophenyl)-2-(4-(5-cyano-2-(methylthio)-6-oxo-1,6-dihydropyrimidin-4-yl)phenoxy)acetamide (4h):

Yield 82.6 %; mp: 274°C; FT-IR ν_{max} (cm^{-1}): 3310 (NH), 2212 (C=N), 1658(C=O); ^{1}H NMR (300 MHz, DMSO-d_6) δ (ppm): 10.40 (s, 1H, NH), 7.79 (d, 2H, *J*=8.7 Hz, Ar-H), 7.65 (d, 2H, *J*=8.7 Hz, Ar-H), 7.52 (d, 2H, *J*=9.0 Hz, Ar-H), 7.07 (d, 2H, *J*=8.7 Hz, Ar-H), 6.88 (s, 1H, NH), 4.77 (s, 2H, CH_2) , 2.50 (s, 3H, CH_3); Anal. Calc. (%) for $C_{20}H_{15}BrN_4O_3S$: C, 50.97; H, 3.21; Br, 16.95; N, 11.89; O, 10.18; S, 6.80 ; found: C, 50.98; H, 3.20; Br, 16.96; N, 11.88; O, 10.17; S, 6.81; ESI-MS: m/z = 472 (M+1).

2-(4-(5-cyano-2-(methylthio)-6-oxo-1,6-dihydropyrimidin-4-yl)phenoxy)-N-p-tolylacetamide (4i):

Yield 88.1 %; mp: 292°C; FT-IR ν_{max} (cm^{-1}): 3350 (NH), 2218 (C=N), 1655(C=O); ^{1}H NMR (300 MHz, DMSO-d_6) δ (ppm): 10.05 (s, 1H, NH), 7.80 (d, 2H, *J*=8.4 Hz, Ar-H), 7.48-7.41 (m, 2H, Ar-H), 7.20 (d, 2H, *J*=7.8 Hz, Ar-H), 7.08 (d, 2H, *J*=8.7 Hz, Ar-H), 6.96 (s, 1H, NH), 4.74 (s, 2H, CH_2) , 2.76 (s, 3H, CH_3), 2.28 (s, 3H, CH_3); Anal. Calc. (%) for $C_{21}H_{18}N_4O_3S$: C, 62.05; H, 4.46; N, 13.78; O, 11.81; S, 7.89 ; found: C, 62.04; H, 4.45; N, 13.79; O, 11.82; S, 7.88; ESI-MS: m/z = 407 (M+1).

2.2. Biological Evaluation

2.2.1. Anti-amoebic activity

Examination of *in vitro* anti-amoebic activity of all dihydropyrimidine-phenylacetamide derivatives **(4a -4i)** against HM1:IMSS strain of *E. histolytica* was performed using the microdilution method, a standard procedure for assessing the efficacy of potential antimicrobial agents.[30] For per ml in Dimethyl sulfoxide (DMSO) 1 mg of each compounds was used to prepared the fresh stock solutions to ensure stability and effectiveness during the testing process.[31] For each experiment, a 96-well microtiter plate was used, which included several controls: Metronidazole (positive control), a well-established anti-amoebic drug, was used as the standard reference to compare the efficacy of the test compounds, negative control Included the parasite (*E. histolytica*) in culture medium without any treatment to monitor the natural growth of the amoeba, and a blank contained only the culture medium without amoeba to serve as a baseline for absorbance readings. The parasite suspension was prepared by adjusting the amoeba concentration to 10^5 cells per mL for with fresh culture medium was taken. This suspension was then distributed evenly into the wells of the 96-well microtiter plate (Corning). To test the compounds' ability to suppress the amoeba growth, various concentrations of compounds under research added to the assigned wells to assess their inhibitory effects. The plate was sealed to keep out contamination after compounds were added, then nitrogen gas was flushed through in for 10 minutes that created an anaerobic environment, an optimal condition for *E. histolytica* growth. After 72 hours incubation period of plate, the amoeba growth was assessed under a microscope to ensure the presence of viable cells. Once the incubation was completed, the culture medium was removed carefully and gently shaking it to discard the supernatant so that unadhered cells or residual compounds get removed. Then, the plate was cleaned with 0.9% sodium chloride solution at 37°C to remove debris. After washing, the plate was dried and methanol was used to maintain cell integrity and adhere them to the plate surface followed by straining with 0.5% (w/v) aqueous eosin for 15 minutes. Using a microplate reader, the optical density (OD) of each well was determined at 490 nm (wavelength allowed for the detection of stained cells) upon straining. The anti-amoebic activity of each compound was determined by calculating the percentage inhibition of parasite growth relative to the negative control. The point was fitted to a curve using a non-linear regression technique, which enabled the determination of the half-maximal inhibitory concentration (IC_{50}) values. At least two independent experiments were conducted for each drug to ensure reproducibility of the results.

2.2.2. Antimalarial activity

The *P. faliparum* (NF54) strain that was susceptible to chloroquine, was continuously cultured *in vitro*. The parasites were cultured in supplement RPMI-1640 culture media for providing the necessary nutrients and conditions for parasite growth. The culture was kept at 37°C in a controlled atmosphere consisting of 5% CO_2, 3% O_2 and 92% N_2. To synchronize the parasite culture to the ring stage, a critical developmental stage of *P. falciparum*, 5% D-sorbitol was used. D-sorbitol selectively lyses mature parasites, leaving only the ring stage intact.[32] After synchronization, titration of ring stage parasites to a final parasitemia (the percentage of infected red blood cells) and hematocrit (the percentage of red blood cells in the culture) of 2%. These parameters were chosen to standardize the starting conditions across all experimental wells. The test compounds, along with the positive control drug quinine, were added to the cultures after 24 hours. After the addition of the compounds, additional culture incubation period was 48 hours at 37°C that allowed the compounds to exert their potential antimalarial effects on the synchronized parasites. Next, the plates were frozen for 1 hour at -70°C. After thawing for 2 hours at room temperature, 25 µL of the lysate was transferred from each well to a non-sterile plate. 100 µL of Malstat™ reagent (a commercially available reagent that reacts with pLDH to produce a colored product) was added to each well followed by addition of 20 µL of a nitroblue tetrazolium (NBT) and phenazine ethosulfate (PES) mixture (1:1). Then, plate was again incubated for 40 minutes at 37°C, allowing the reaction to proceed. After the incubation, addition of 5% acetic acid to each well ended the reaction. The amount of pLDH activity which correlates with parasite viability, is directly proportional to the presence of formazan, a purple-colored product.[33] The 620 nm wavelength was preferred for absorbance of the formazan product using a microplate reader. Percentage inhibition was computed by comparing the absorbance values of the wells treated with the test compounds to the appropriate controls (untreated and quinine-treated wells).

2.2.3. Larvicidal activity

To evaluate the potential larvicidal effects of the synthesized compounds, Anopheles arabiensis (KGB) mosquito larvae were obtained from a permanent colony housed at the Botha de Meillon Insectary at the National Institute for Communicable Diseases/WITS Research Institute for Malaria, Johannesburg. The larvae were reared and fed according to established WHO protocols for mosquito maintenance.[34] Fourth instar larvae, the last larval stage before pupation, were chosen for the experiment. 25 larvae were placed in each experimental group and incubated with

the test compounds. The tested concentration of compounds (50 µM), dissolved in single distilled water. The larvae were incubated for 24 hours at a temperature between 25-28°C, a condition suitable for Anopheles mosquito larvae. A well-established positive control, DDT (dichlorodiphenyltrichloroethane), was used. After 24 hours, percentage mortality was recorded for each cohort. The mortality rate was determined by counting the number of dead larvae in each group and comparing it to the total number of larvae exposed to the compounds. The morphological appearance of both the dead and surviving larvae was recorded at 3x magnification using a photographic setup. The morphological traits of the treated larvae were contrasted with those of the untreated controls and the DDT-treated group to identify any unique characteristics caused by the compounds.[35]

2.2.4. Anopheles ovicidal assay

Anopheles arabiensis (KGB) eggs were incubated with dihydropyrimidine-phenylacetamide derivatives and controls (DDT and 1% formalin) to determine the interferance with the ability of the larvae to hatch. The morphological appearance of the eggs and developing larvae were noted for abnormalities.[36] Each derivative was incubated at 25-28°C with 20-30 eggs at 50 µM concentration. At 48- and 72-hours' time duration, the number of hatched larvae were recorded. At 3x magnification, the % of hatched *Anopheles* eggs were counted and then expressed as a percentage of the untreated water control. The experiment was performed in triplicate for the data accuracy.

2.2.5. Cytotoxicity assays

Two cancer cell lines: myelogenous leukemia (K562) and human neuroblastoma (SH-SY5Y) were used to assess the anticancer property of targeted compounds, both cultured under conditions similar to the HEK-293 cell line. To evaluate the cytotoxicity of these compounds, these cells were cultured as a monolayer in Dulbecco's Modified Eagle's Medium (DMEM), (a nutrient-rich medium commonly used for the development of variety of mammalian cells). The medium was supplemented with 10% fetal bovine serum (FBS) for essential growth factors and nutrients followed by addition of penicillin (100 IU/mL) and streptomycin (100 µg/mL) to prevent bacterial contamination. The cells maintenance was done at 37°C in a humidified atmosphere containing 5% CO2 to promote optimal cell growth. To start the assay, a suspension of 15,000 for K562 cells and 20,000 for HEK-293 and SH-SY5Y cells per well was prepared

followed by addition of serial dilutions of the test compounds or positive control to each well. DMSO was used at a final concentration of less than 1%, as it had no impact on cell viability. After incubating the cells at 37°C for 48 hours, each well was treated 40 µL of MTT reagent (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide; 5 mg/mL in phosphate-buffered saline, pH 7.3). After the additional 2 hours of incubation period, the culture medium containing the unreacted MTT was carefully removed. The formazan crystals were dissolved by adding DMSO to each well. The absorbance was measured at 540 nm, with a reference wavelength of 690 nm, using a microplate reader (Labsystems Multiskan RC).[36] Then calculation of percent cellular viability by comparing the absorbance readings from treated wells to the control wells, was done and IC_{50} values were determined for each compound and compared with the positive control, camptothecin, a known anticancer agent. The entire experiment was conducted in triplicate for reproducible results.

2.2.6. Haemolysis assay

Fresh human red blood cells (RBCs) were collected and prepared to a 1% hematocrit in RPMI-1640 culture medium supplemented with necessary nutrients. The RBC suspension was then incubated with 25 µL of each test compound or control solution. The concentration of the test compounds was 50 µM, chosen to evaluate the potential hemolytic effects at a standardized concentration. The incubation period lasted for 48 hours to allow sufficient time for any potential hemolytic effects to occur.[25] The supernatant was then analyzed for its absorbance at 412 nm. The % haemolysis was achieved using 0.2% (v/v) Triton X-100, a detergent known to completely lyse red blood cells. Triton X-100 serves as a positive control for maximum hemolysis.

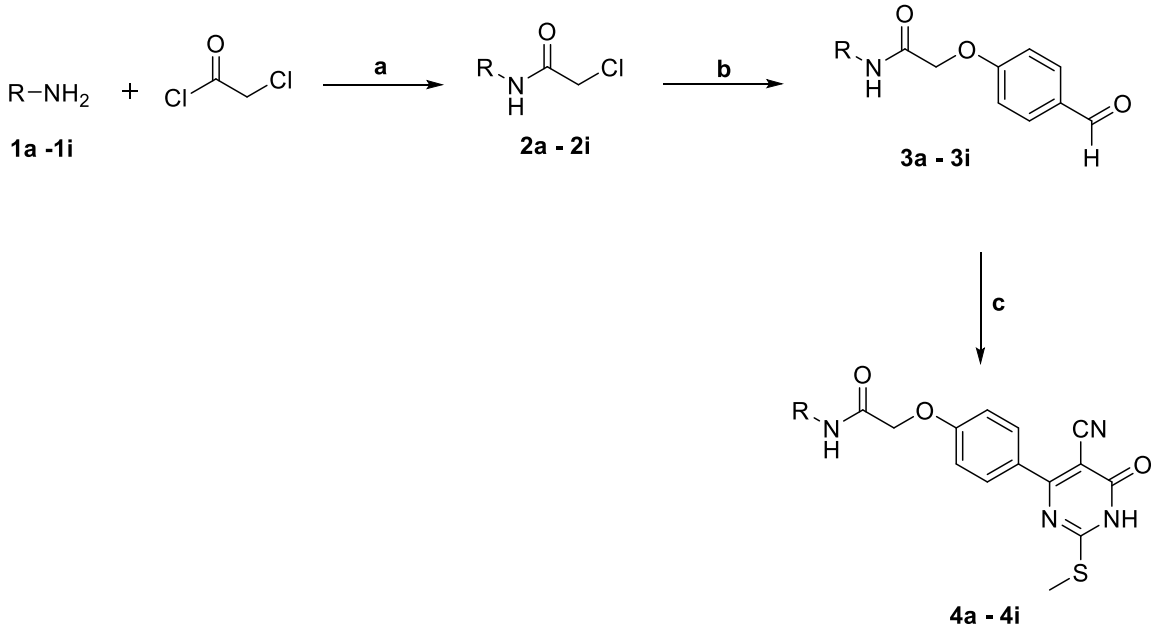
2.2.7. Artemia lethality assay

Artemia franciscana eggs placed in sea salt (38g/L) were hatched in an aerated environment at an optimal temperature for 18 hrs.[37] To each well, 40-60 live *Artemia* in 400 µL of water were added with the test/control drugs and the plates left over night at room temperature. Dead *Artemia* nauplii were then counted at 0, 24 and 48 hr. All *Artemia* were killed following the addition of 25% acetic acid and total number of *Artemia* per well counted. Percentage mortality was then calculated taking the controls into account, where potassium dichromate was used as the positive control. The experiment was repeated in triplicate.

3. Results and Discussion

3.1. Chemistry

The synthetic route leading to the titled compounds, dihydropyrimidine-phenylacetamide derivatives **(4a -4i)**, is given in **Scheme 1**. In the first step, different chloro-acetamides **(2a -2i)** were synthesized by substitution of the amine with chloro acetyl chloride by the proposed method. In the second step, the formylphenoxy acetamide intermediates **(3a -3i)** were formed by the reaction of chloro-acetamides and 4-hydroxybenzaldehyde using K_2CO_3 in acetone by a reported method. Then, in the final step, heating a mixture of ethyl cyanoacetate with the formylphenoxy acetamide intermediate and S-methylisothiourea hemisulfate gave the corresponding dihydropyrimidin-phenylacetamide derivatives **(4a -4i)** as the final product with good yield.



Scheme 1; Synthesis of dihydropyrimidine-phenylacetamide derivatives. **Reagents and conditions: (a)** TEA, CH_2Cl_2 **(b)** KI, K_2CO_3, 4-hydroxybenzaldehyde, ethanol, reflux, 24 h; **(c)** $CNCH_2COOC_2H_5$, S-methylisothiourea hemisulfate, ethanol, reflux.

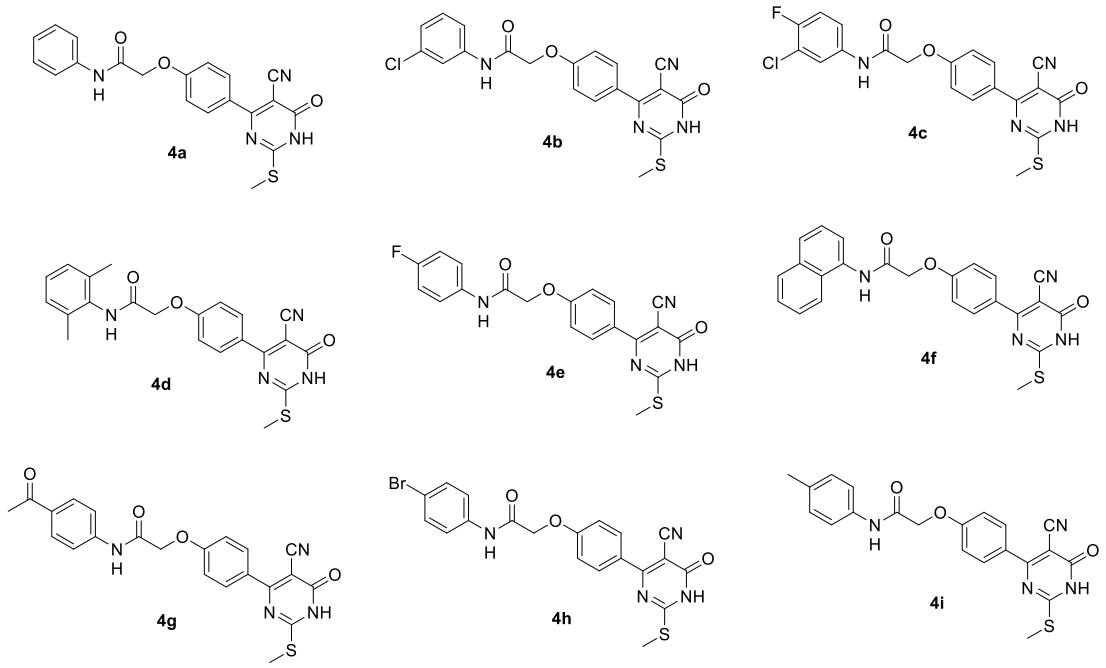


Fig-3: Structure of dihydropyrimidine-phenylacetamide derivatives

All the dihydropyrimidine-phenylacetamide derivatives **(4a -4i) (Fig-3)** were stable in solid state at room temperature. A satisfactory spectral data was observed for the proposed structures of these derivatives. Characteristic IR spectra showed CN absorption band around 2212 -2218 cm^{-1} in dihydropyrimidine-phenylacetamide derivatives **(4a -4i)**. The ^{1}H NMR indicated that the disappearance of the peak of aldehyde proton (a singlet in the range δ 9.94-9.96 ppm) and the appearance of the peak of NH proton of pyrimidine ring as a broad singlet in the range δ 6.53-6.97 ppm along with the three protons of S-CH_3 shows a sharp singlet in range δ 2.34-2.76 ppm confirms the formation of dihydropyrimidine. The signals due to the aromatic protons appeared in the expected region. The CN carbon showed peak around δ 115 ppm and S-CH_3 around 13.7 ppm in the ^{13}C NMR of these compounds. The structure of the synthesized compounds was further elucidated by mass spectral studies with M+1 peak. The purity of the compounds was confirmed by elemental analysis.

3.2. Biological evaluation

3.2.1 Anti-amoebic activity

In order to examine the effectiveness of dihydropyrimidine-phenylacetamide derivatives **(4a -4i)** as the anti-amoebic potential against *E. histolytica*, HM1: IMSS strain was taken and

metronidazole (MTZ) (IC_{50} =1.80 μM) was utilized as reference drug. The experiments were done in triplicate at each concentration level with three repetitions. The results of anti-amoebic activity are summarized in **Table 1**. All the dihydropyrimidine-phenylacetamide derivatives had IC_{50} values in the range 0.12-7.16 μM. Among these derivatives, five compounds: **4b** (IC_{50} = 0.82 μM), **4c** (IC_{50} = 0.12 μM), **4e** (IC_{50} = 0.34 μM), **4g** (IC_{50} = 0.51 μM), and **4h** (IC_{50} = 1.66 μM) were found to exhibit better anti-amoebic activity than MTZ (IC_{50} =1.80 μM). Compound **4c**, having weak deactivating chloro group at meta position and fluoro group at the para position of phenyl ring, exhibited most promising activity (IC_{50} = 0.12 μM) in the series. Compound **4e** (IC_{50} = 0.34 μM) having strong electronegative fluoro group at para position exhibited better activity. Introduction of strong electron withdrawing *para* substituted acetyl group in compound **4g** (IC_{50} = 0.51 μM), exerts significant inhibitory activity. Compound **4b** (IC_{50} = 0.82 μM) having chloro group at meta position and compound **4h** (IC_{50} = 1.66 μM) with bromo group at the para position also had better activity. Whereas the presence of weak activating methyl group at both *ortho* positions in compound **4d** (IC_{50}= 5.91 μM) exhibited less activity. Compound **4a** (IC_{50} = 3.25 μM) without any substitution had better activity than compound **4d**. The substitution of the phenyl ring with bulky naphthyl group in compound **4f** (IC_{50} = 7.16 μM) reduced the activity Thus, from the above discussion, all the dihydropyrimidine-phenylacetamide derivatives **(4a -4i)** were good to potent anti-amoebic, with compound **4c** that was the most potent.

3.2.2 Antimalarial activity and cytotoxicity profile

The pharmacological properties of the dihydropyrimidine-phenylacetamide derivatives **(4a -4i)** were varied through the various assays undertaken to investigate their antimalarial, insecticidal and toxicological properties. Of the nine derivatives, the naphthalene derivative, **4f** (IC_{50} = 56.78±7.79 Mm) was the most active inhibiting 43% parasite growth **(Table 1),** but was not comparable to quinine which is used to successfully eliminate infections.[4] This antiplasmodial inhibition by **4f** appears to be directed against the intra-erythrocytic parasite where the derivative did not induce haemolysis, thereby not affecting the host's cell membrane by increasing permeability properties or interfering with metabolism [haemolysis: 0.12%]. The lack of toxicity of this derivative is replicated in the other toxicity assays which displayed minimal toxicity to the HEK-293 kidney epithelial cells, K562 erthryoleukemia cells, and SH-SY5Y neuroblastoma cell

line (% inhibition >65%). The minimal inhibitory effect was also observed in the larvicidal and ovicidal assays **(Table 1)**, with only 12.7% of the Artemia killed **(Table 2).**

The relative lack of activity of all the derivatives is complemented with the observation that none of the derivatives induced a significant amount of haemolysis [0.08 – 1.77%] compared to quinine [1.76%]. The SH-SY5Y neuroblastoma cell line was the most sensitive to the derivatives with an overall average of 33.8% inhibition compared to 29.12 and 0% for the K562 erythroleukemia and HEK-293 kidney epithelial cells, respectively. The most toxic of the derivatives to Artemia was the 4-fluoro, 5-chloro-benzyl derivative (**4c**); where 98.7% of the nauplii were killed compared to 99.6% by the positive control, potassium dichromate. This inhibitory effect was also observed in the ovicidal assay where only 66.8% of the Anopheles eggs hatched when treated with **4c**, which was more effective than DDT (91.2% hatched), but not as effective as 1% (v/v) formalin which prevented all eggs from hatching over a 72 hr period (Table 1). The effects of **4c** appear to be more selective for more complex multicellular organisms (Anopheles larvae, 45%; eggs, 33%; Artemia, 99%)(Table 1) than the single cells and plasmodia (0, 28, 28 and 28% for the HEK-293 kidney epithelial, K562 erythroleukemia, SH-SY5Y neuroblastoma **(Table 2)** and the NF54 *P. falciparum* parasite (Table 1)). When compared to **4e**, which contains a 4-fluoro-benzyl side chain and **4d**, with a 3-chloro benzyl side chain, the chlorine appears to contribute towards some of the toxicity of **4c** with 44.2% death of Artemia nauplii in comparison to 25.6% of the fluorine **(Table 2)**. However, their singular contribution to the effect on human cell growth **(Table 2)** and the hatching of Anopheles larvae **(Table 1)** was minimal.

Table 1: *In vitro* anti-amoebic, antimalarial and mosquito-cidal activity of dihydropyrimidine-phenylacetamide derivatives (4a -4i)

S.No.	**Compounds code**	**Anti-amoebic activity (IC_{50}; µM)**	**Antimalarial activity (% growth at 50µM)**	**Larvacidal activity at 50µM***	**% Ovicidal activity (hatch rate at 50µM)***
1	**4a**	3.25±0.01	74.85±6.66	50	94.11
2	**4b**	**0.82±0.03**	74.46±7.26	**55**	97.85

3	**4c**	**0.12±0.01**	71.56±4.72	45	**66.80**
4	**4d**	5.91±0.03	72.58±2.00	48	96.78
5	**4e**	**0.34±0.03**	77.04±13.61	50	103.22
6	**4f**	7.16±0.08	**56.78±7.79**	45	107.80
7	**4g**	**0.51±0.01**	74.69±14.31	48	102.56
8	**4h**	**1.66 ± 0.05**	84.68±3.07	48	86.45
9	**4i**	4.16 ± 0.01	73.64±7.53	**55**	92.81
10	**MTZ**	**1.8±0.01**	n.t	n.t	n.t
11	**Quinine**	n.t.	20.51±4.50	n.t	n.t
12	**DDT**	n.t	n.t	100.00	91.20
13	**Formalin**	n.t.	n.t	n.t	0.00

n.t.: not tested; *: at 72 hours

Table 2: *In vitro* toxicological activity of dihydropyrimidine-phenylacetamide derivatives (4a - 4i)

S.No.	**Compounds code**	**Cytotoxicity profile (% Cell growth at 100µM)**			**% *Artemia* inhibitory activity at 50µM**
		HEK-293	**K562**	**SH-SY5Y**	
1	**4a**	93.14±8.16	63.02±2.41	54.24±1.73	28.89
2	**4b**	117.19±14.51	92.18±1.30	86.84±4.67	44.17
3	**4c**	108.21±10.55	72.02±6.18	71.63±4.94	**98.67**
4	**4d**	82.41±4.34	73.89±3.75	**53.34±2.15**	17.40
5	**4e**	103.04±6.69	**61.08±4.38**	58.47±12.91	25.58
6	**4f**	120.21±3.74	72.96±3.82	68.36±3.64	12.67
7	**4g**	80.87±8.94	68.85±1.30	58.68±2.57	51.30
8	**4h**	107.14±4.63	72.74±5.03	93.48±5.80	11.22
9	**4i**	79.22±5.44	70.91±3.09	60.15±2.02	39.13
10	**Camptothecin**	0.13±0.11	0.13±0.04	0.53±0.40	n.t
11	**Potassium dichromate**	n.t	n.t	n.t	99.44

3.3. Molecular docking study of 4-aryl-5-cyano-2-methylthio-6-oxo-1,6-dihydropyrimidine derivatives

Molecular docking is a computational study to check the interaction between ligand and protein. These compounds were interacted with the enzyme *O-acetyle-l-serine sulfohydrolase (EhOASS)* of *E.histolytica*. PDB id of this enzyme is 3BM5 that the PDB format was directly downloaded from protein data bank (www. rcsb.org/pdb). Heteroatoms and water molecules, present in the crystal structure of protein were deleted, and followed by energy minimization in AutoDock tools then saved in PDBQT format. The structure of compounds was drawn in ChemDraw 16.0 and then 3D structure was prepared in chem 3D. PDBQT file of the ligand was prepared in through AutoDock tool. Docking was performed by AutoDock vina 4.2. The docking interaction of output file was visualized in PyMOL.

The molecular docking results indicate that all the dihydropyrimidine-phenylacetamide derivatives **(4a -4i)** had binding free energy in range of -6.3 to -8.0. Compounds 4c and 4f show the strongest binding affinity with binding free energies of -8.0 kcal/mol, suggesting to form the most stable ligand-protein complex. Compounds 4a, 4b, and 4g also demonstrate strong binding affinities (ranging from -7.9 to -7.5 kcal/mol) with multiple hydrogen bonds and moderate bond distances. Compound 4i has the weakest binding affinity with a binding free energy of -6.3 kcal/mol, possibly due to weaker hydrogen bonds as indicated by the longer distances. Docking images of compounds **4c** and **4f** showed the bonding interactions. The 3D images A and B indicated donor and acceptor regions. In 2D structure, Both the compounds **4c** and **4f showed two** hydrogen bonding, interacting with amino acids GLU 28 and ILE 44 in case of 4c, and SER 7and SER 8 amino acids in case of compounds **4f. (Table 3) (Fig-4)** Overall, compounds **4c** and **4f** are the most promising candidates for further development based on their strong binding interactions.

Table 3: Binding affinity of dihydropyrimidine-phenylacetamide derivatives **(4a -4i)**

S.No	Compound Code	Binding Free Energy (kcal/mol)	Hydrogen bonds	
			Amino acids residues	Distance in Å
1	**4a**	-7.5	LYS 58, ASN 88	2.25, 2.59

2	**4b**	-7.5	TYR 319, LYS 271	2.09, 2.90
3	**4c**	**-8.0**	GLU 28, ILE 44	2.77, 2.33
4	**4d**	-7.4	ASN 158	2.02
5	**4e**	-7.3	ARG 10	2.65
6	**4f**	**-8.0**	SER 7, SER 8	2.68, 2.54
7	**4g**	**-7.9**	TYR 14, SER 8, ARG 12, ARG 10, SER 7	2.30, 2.41, 2.67, 2.74, 2.85, 3.08
8	**4h**	-7.3	SER 8¸ SER 7, TYR 14	2.10, 2.69, 2.90
9	**4i**	-6.3	ASP 244, SER 84	2.77, 3.36

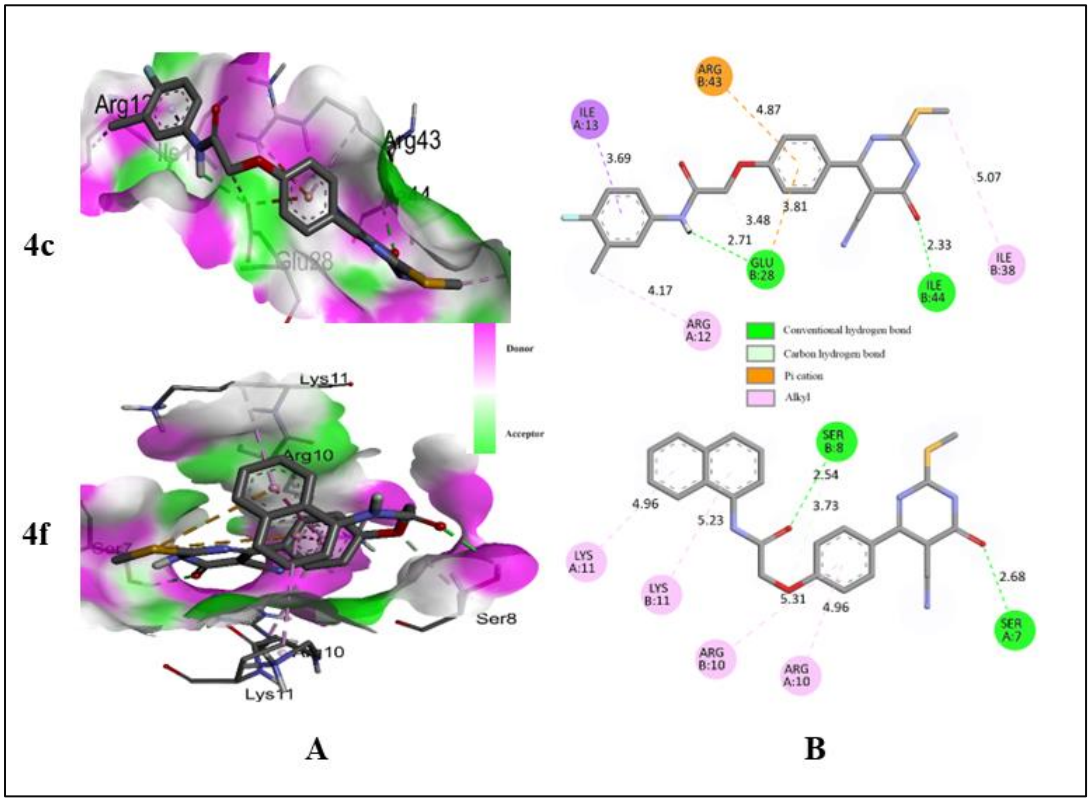


Figure 4: image **A** indicating H bond-donar (pink) and H-bond acceptor (green) region of **4c** and **4f**. image **B** shows 2D structure of **4c** and **4f.**

ADME Study of final dihydropyrimidine-phenylacetamide derivatives (4a -4i)

To evaluate the physiochemical properties of these compounds, we need to consider several key factors based on the data provided, such as molecular weight, rotatable bonds, hydrogen bond

acceptors and donors, and TPSA (Topological Polar Surface Area). All the compounds have molecular weights between 392.43 and 471.33 which is less than 500. The compounds have 7 or 8 rotatable bonds, 5 to 6 hydrogen bond acceptors and 2 hydrogen bond donors. The TPSA values range from 133.17 to 150.24. Given that these compounds have TPSA values slightly below from 140 (except for **4g**, which is even higher with 150.24), they might have good oral bioavailability. **(Table 4)** So, these compounds are considered favorable for drug-like properties according to Lipinski's rule of five and better for oral bioavailability. Further, through the application of a decision tree, Toxtree online version (https://toxtree.sourceforge.net/) and Chemicalize (https://chemicalize.com), *In silico* toxic hazards characteristics predicted that all the compounds were unlikely to permeate through the blood-brain-barrier and the negative Kp values indicated a reduced skin permeation potential **(Table 5)** In contrast, the predictive toxicological profile of the derivatives indicated that all the compounds have a high potential risk of being toxic in humans (Class III) as determined by the revised Cramer rules. In addition, there is a high risk of multiple drug interactions where all the compounds were predicted to be CYP3A4 inhibitors, a hepatic metabolic pathway commonly involved in the metabolism of clinically administered drugs. Similarly, all the compounds were predicted to be CYP2C9 inhibitors, but not CYP2D6 **(Table 5)**.

Table 4. Physiochemical properties of target compounds.

S. No	Code	Mol. Weight	Rotatable Bonds	H-bond acceptors	H-bond donors	TPSA	Lipinski violations
1	**4a**	392.43	7	5	2	133.17	0
2	**4b**	426.88	7	5	2	133.17	0
3	**4c**	444.87	7	6	2	133.17	0
4	**4d**	420.48	7	5	2	133.17	0
5	**4e**	410.42	7	6	2	133.17	0
6	**4f**	442.49	7	5	2	133.17	0
7	**4g**	420.44	8	6	2	150.24	0
8	**4h**	471.33	7	5	2	133.17	0
9	**4i**	406.46	7	5	2	133.17	0

Table 5: *In silico* predicative toxicological properties of 2-(4-(5-cyano-2-(methylthio)-6-oxo-1,6-dihydropyrimidin-4-yl)phenoxy)-N-phenylacetamide derivatives.

S.No.	Compound code	Log *K*p (cm/s)	CYP1A2 inhibitor	CYP2C19 inhibitor	CYP2C9 inhibitor	CYP2D6 inhibitor	CYP3A4 inhibitor	CYP1A2 inhibitor
1	**4a**	-6.81	Yes	Yes	Yes	No	Yes	Yes
2	**4b**	-6.58	Yes	Yes	Yes	No	Yes	Yes
3	**4c**	-6.61	No	Yes	Yes	No	Yes	No
4	**4d**	-6.46	No	Yes	Yes	No	Yes	No
5	**4e**	-6.84	Yes	No	Yes	No	Yes	Yes
6	**4f**	-6.22	No	Yes	Yes	No	Yes	No
7	**4g**	-7.29	No	Yes	Yes	No	Yes	No
8	**4h**	-6.8	Yes	Yes	Yes	No	Yes	Yes
9	**4i**	-6.64	Yes	Yes	Yes	No	Yes	Yes

Conclusion

The newly synthesized dihydropyrimidine-phenylacetamide derivatives **(4a -4i)** were synthesized and characterized by different spectroscopic and spectrometric techniques like IR, NMR and mass. Further, all these derivatives were tasted for their biological potential. The anti-amoebic screening study indicated that dihydropyrimidine-phenylacetamide derivatives had greater potential against *E. histolytica,* used strain HM1: IMSS. Where, five compounds: **4b** (IC_{50} = 0.82 µM), **4c** (IC_{50} = 0.12 µM), **4e** (IC_{50} = 0.34 µM), **4g** (IC_{50} = 0.51 µM), and **4h** (IC_{50} = 1.66 µM) were exhibited better anti-amoebic activity that was less than the reference MNZ (IC_{50} =1.80 µM). Compound **4f** had lowest activity with IC_{50} of 7.16±0.08 µM. Antimalarial screening against *P. falciparum* of these compounds showed low activity but compared to compound **4f** with IC_{50} value of **56.78±7.79** µM as the most active against the malaria parasite among all these compounds, followed by **4c** having IC_{50} value 71.56±4.72 µM. Further, *in silico* docking study indicated that these compounds had good binding affinity in range of -6.3 to -8.0 kcal/mol when interacted with the enzyme *O-acetyle-l-serine sulfohydrolase (EhOASS)* of *E. histolytica* (PDB

id: 3BM5). Pharmacological and pharmacokinetic study also showed good oral bioavailability of these derivatives. The above study concluded that compound **4f** which was least active as anti-amoebic, was found to have some efficacy against malaria strain among all the derivatives. Compound **4c** showed lowest IC_{50} **(0.12 µM)** against amoebic strain HM1: IMSS. Overall, dihydropyrimidine-phenylacetamide derivatives **(4a -4i)** exerted promising anti-amoebic activity and could be used for further development.

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