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Toxicological Profile, *In Vitro* and *In Vivo* Antioxidant Activities of 4-phenyl-6-(3,4,5-trimethoxyphenyl)pyrimidin-2-ylamine

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ABSTRACT

In our search for valuable therapeutics, a series of diarylaminopyrimidine (DAP) analogues have been synthesized and characterized. In this study, one of the DAPs, 4-phenyl-6-(3,4,5-trimethoxyphenyl)pyrimidin-2-ylamine, was assessed for its toxicological profile and screened for *in vitro* and *in vivo* antioxidant activities. The 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging method was used for the in *vitro* antioxidant studies and the IC_{50} values obtained were 2.37 µg/mL and 139.60 µg/mL for the standard (ascorbic acid) and DAP, respectively. According to the *in vivo* studies, 50 mg/kg DAP prevented the increase in the malondialdehyde (MDA) concentration, significantly inhibited the decrease in the level of reduced glutathione (GSH) and normalized superoxide dismutase (SOD) and catalase (CAT) activities in the blood of albino rats in the treated groups compared with those in the ethanol (negative control) group. Oral administration of synthesized DAP at 5000 mg/kg did not produce any toxic or adverse effects and no mortality was recorded after 24 hours. Our findings suggest that 4-phenyl-6-(3,4,5-trimethoxyphenyl)pyrimidin-2-ylamine, holds some promise as a potential starting material for the production of drugs that can mitigate degenerative diseases.

Keywords: Antioxidant, Catalase, Diarylaminopyrimidines, Glutathione, Malondialdehyde

INTRODUCTION

Pyrimidine is one of the most utilized heterocyclic scaffolds in synthetic organic chemistry. It is a very versatile diazine that plays a vital role in nucleic acid and protein production in humans (Amenta *et al.,* 2012). Most therapeutics are developed from pyrimidine through derivatization or other chemical transformation processes. There are several chemical pathways leading to the synthesis of pyrimidines and their derivatives (Usifoh *et al.*, 1989; Kumar *et al.*, 2011; Abbas 2015). Sharma and Sharma, (2011) synthesized 4,6-diarylaminopyrimidine analogues from the cyclo-condensation of chalcones with guanidine carbonate. This method afforded very stable compounds in good yields.

Some notable pharmacological activities attributable to compounds with pyrimidine moieties include anti-inflammatory (Nofal *et al.,* 2011), antibacterial (Bukhari *et al.,* 2011), anticancer (El-Deeb *et al.,* 2010) and cytotoxic (El-Sawy *et al.,* 2012) activities. Pyrimidine analogues have also been reported to be calcium channel blockers (Zorkun *et al.,* 2006). A number of antifolates drugs which are used in the treatment of various diseases, particularly cancers and infections, are derivatives of 2,4-diaminopyrimidines. Typical amongst these derivatives of 2,4-diaminopyrimidines are the antimalaria drug, pyrimethamine, the antibacterial drug, trimethoprim as well as methotrexate and aminopterin, both used in cancer chemotherapy (March *et al*., 2020).

The production of free radicals or specifically reactive oxygen species (ROS), as metabolic byproducts, is a continuous (and sometimes inevitable) process. Metabolic reactions such as photosynthesis, photorespiration and respiration, yield ROS as a byproduct. Reactive oxygen species (ROS) is a collective term used to describe oxygen-containing radicals that possess one or more unpaired electrons and can exist independently. Generally, the utilization of molecular oxygen by aerobic organisms inevitably leads to the production of ROS due to the partial or incomplete reduction of molecular oxygen during metabolic processes (Khorobrykh *et al.,* 2020; Li *et al*., 2020; Bhattacharjee, 2019). Some ROS are naturally very toxic, but they are, easily detoxified by certain enzymatic and nonenzymatic processes at the cellular level. This detoxification is made possible through the radical-scavenging ability of antioxidant species. When there is a considerable imbalance between the rate of ROS production and antioxidative defense, oxidative stress occurs. This is a result of the inability of both endogenous and exogenous antioxidants to effectively scavenge the free radicals produced by oxidants from circulation (Moriasi *et al.,* 2020). ROS are so reactive that they can oxidize and modify all types of cellular

components such as lipids, proteins, carbohydrates, and nucleic acids, thereby causing systemic damage (Juan *et al*., 2021; Fimognari, 2015).

ROS scavenging or the development of antioxidant principles has been a major area of research. Some enzymes involved in ROS scavenging have been chemically modified to enhance their effectiveness in free radical scavenging (Zhang *et al*., 2015; Ju *et al*., 2021; Abellanas-Perez *et al*., 2023). Disease conditions such as diabetes mellitus, Parkinson's disease and other chronic degenerative diseases, including cardiovascular and inflammatory conditions, have their pathophysiology directly linked to oxidative stress as a major causative factor (Arika *et al.*, 2019). The major ROS implicated in the pathogenesis of these diseases includes radicals, often referred to as oxygen-centred radicals such as superoxide, hydroxyl radicals, alkoxyl radicals and peroxyl radicals, and the nonradicals hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), hypochlorous acid (HOCl) and ozone (O_3) which are produced through different endogenous and exogenous pathways (Dontha, 2016).

Researchers have shown that diarylpyrimidines (DAPs) exhibit considerable antioxidant activity (Dudhe *et al.*, 2015; Himaja *et al.*, 2012). Its free radical scavenging ability is significantly increased by derivatization of the pyrimidine scaffold or by increasing its functionality. This is achieved by the introduction of substituent groups such as $-Cl^-$, $-Br^-$, $-CF_3$ and $-NO_2$ (Makula and Tabassum, 2023; Bano *et al.*, 2012). This effect is believed to increase both stability of the pyrimidine moiety and the penetration of the DAP molecule into the lipid membrane. The DAP therefore combines easily with the ROS generated by various disease conditions.

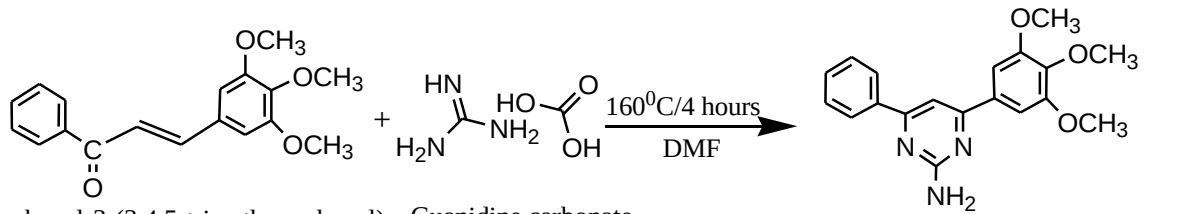
In the present study, the antioxidative capacity and toxicological profile of 4-phenyl-6-(3,4,5-trimethoxyphenyl)pyrimidin-2-ylamine was explored.

MATERIALS AND METHODS

All the reagents used for the study were of analytical grade and were used without further purification.

Synthesis of 4-phenyl-6-(3,4,5-trimethoxyphenyl)pyrimidin-2-ylamine

4-phenyl-6-(3,4,5-trimethoxyphenyl)pyrimidin-2-ylamine was synthesized and characterized as reported by Aiwonegbe *et al*., 2024. Briefly, to a 100 mL round bottom flask, 1 g (3.4 mmol) of 1-phenyl-3-(3,4,5-trimethoxyphenyl) prop-2-en-1-one and 0.61 g (3.4 mmol) of guanidine carbonate were added. The reactants were dissolved in 60 mL of dimethylformamide (DMF). An oil bath was used to heat the reaction mixture for 4 hours at 160°C under reflux. It was then cooled to room temperature, poured into crushed ice and filtered after 24 hours. The product was washed with distilled water then air-dried and characterized.



1-phenyl-3-(3,4,5-trimethoxyphenyl) prop-2-en-1-one

Guanidine carbonate

4-phenyl-6-(3,4,5-trimethoxy-phenyl) pyrimidin-2-ylamine

Experimental animals

The rats and mice used for the *in vivo* and acute toxicity studies were purchased from the Animal House of the Department of Biochemistry, University of Benin. The animals were handled according to the protocols guiding the laboratory use of animals set by the ethical committee of the Faculty of Life Sciences, University of Benin, Benin City. The ethical approval number given for the study was LS21311.

Experimental design

The method of Reddy *et al*. (2014) was adopted but the doses of the DAP were adjusted to suit the current study. Twenty-five (25) healthy male albino rats, weighing between 180 and 250 g, were divided into five groups with five animals per group (n=5). All the rats in the five groups were initially given 40 % ethanol at 1 mL/kg/day p.o. for 3 days to induce oxidative stress. The animals were thereafter treated for 14 days. Group A served as the untreated (normal control) group and the rats were given distilled water orally at 1 mL/kg. Rats in group B (positive control) were treated with 30 mg/kg/day ascorbic acid. In groups C, D and E, the rats were treated with 50, 100 and 200 mg/kg/day of the synthesized DAP, respectively. After treatment for 14 days, the rats were fasted overnight, blood samples were collected directly from the heart into EDTA sample bottles by cardiac puncture. The blood was centrifuged and the serum was used to determine superoxide dismutase (SOD), reduced glutathione (GSH), catalase (CAT) and malondialdehyde (MDA) activities. The rats were later sacrificed with a mild chloroform anaesthetic and the organs were isolated for further analysis.

Determination of in *vivo* antioxidant activity

Superoxide dismutase (SOD) activity

The superoxide dismutase (SOD) effect was determined by using the methods described by Halliwell, (1997) and Bagul *et al*., (2018) with some modifications. An aliquant mixture of plasma and 0.20 mL of the diluted microsomes was enclosed in a 2.5 mL of 0.05 M carbonate buffer. The reaction was initiated by adding 0.3 mL of 0.3 mM adrenaline. The standard was combined with 2.5 mL of 0.05 M carbonate buffer, 0.3 mL solution of 0.3 mM adrenaline and 0.20 mL of distilled water. The absorbance was measured after 30 and 150 seconds at a wavelength of 480 nm. The percentage inhibition was calculated by using equation 1.

$$\%\ Inhibition = \frac{\Delta Ac - \Delta As}{\Delta Ac} \times 100\% \quad (1)$$

where:
ΔA_c = change in the absorbance of the control
ΔA_s = change in the absorbance of the sample
One unit of SOD is the total SOD required to elicit 50% inhibition of oxidation via adrenaline to the adrenochrome in 1 minute.

$$\text{SOD Activity (U/mL)} = \frac{\%\ \text{Inhibition}}{50\%} \quad (2)$$

Glutathione peroxidase activity

Glutathione activity was measured in terms of the first-order rate constant for the decomposition of tetra-butyl hydroperoxide according to the methods described by Beckman and Koppenol (1996); Mates *et al.* (1999) and modified by Ahmend *et al*. (2023). Briefly, 500 µL of tissue homogenate was mixed with 500 µL of assay buffer (potassium phosphate 30 mM, pH 7.0), 100 µL of sodium azide (NaN_3; 10 mM), 200 µL of reduced glutathione (GSH: 4 mM), 100 µL of hydrogen peroxide (H_2O_2; 2.5 mM), and 6000 µL of distilled water. The whole reaction mixture was incubated at 37 °C for 3 min after which 0.5 mL of 10% trichloroacetic acid (TCA) was added, and the mixture was subsequently centrifuged at 3000 rpm for 5 min. Then, 1 mL of the supernatant was added to 2 mL of K_2HPO_4 (0.3 M) and 1 mL of 5,5-dithio-bis-2-nitrobenzoic acid (DTNB), and the absorbance was read at 412 nm.

Evaluation of catalase (CAT) activity

Catalase activity was assayed by using the Halliwell and Gutteridge (1995) method. Briefly, 10 µL of tissue homogenate, containing 100-150 µg of protein, was added to a 2.8 mL solution of 50 mM potassium phosphate buffer (pH 7.0) in a 3 mL cuvette. Thereafter, 0.1 mL of freshly prepared 30 mM H_2O_2 was added to initiate the reaction. The decomposition rate of H_2O_2 was measured at a wavelength of 240 nm for 300 seconds using a spectrophotometer. A molar extinction coefficient of 0.041 $mM^{-1}cm^{-1}$ was utilized to calculate the effect of catalase on H_2O_2 mole reduction/min/mg/protein.

Estimation of malondialdehyde (MDA) activity

The malondialdehyde (MDA) activity of the synthesized DAP was estimated by using the method of Beckman and Koppenol, (1996). The treated blood sample was first incubated at 45°C for 24 hours. The mixture was then centrifuged at 3000 rpm for 20 minutes and the supernatants were isolated. A total of 1300 µL of sample was withdrawn from the microcentrifuge tube. One milliliter of supernatant was diluted ten times in Tris-HCl, and 200 µL was further used to dilute the supernatant in every culture. Then, 200 µL of distilled water was added, and the mixture was shaken together. To each of the test tubes (sample and control), 300 µL of the test reagent, R2, was added, and then shaken vigorously and incubated at 45°C for 40 minutes. After the incubation process, each tube was chilled on ice and centrifuged at 15000 rpm for 10 minutes. The absorbance of each sample was measured at 586 nm with a spectrophotometer.

Determination of *in vitro* antioxidant activity

DPPH radical scavenging assay

The scavenging effect of the synthesized DAP on DPPH radical was estimated with the method described by Jain *et al.* (2008) and Chanda *et al*., 2010 with some modifications. The incubation time was increased by five minutes such that even if the compound under investigation was slow-acting, the total antioxidant effects would still be accommodated in the test process. A 1.0 mL solution of 0.1 mM DPPH in methanol was mixed with a 3.0 mL solution containing 0.01 - 0.2 mg/mL of the synthesized DAP in methanol. The reaction mixture was shaken vigorously and incubated in the dark at room temperature for 35 minutes. After incubation, the absorbance of the mixture was measured at 517 nm in a UV spectrophotometer (Jenway Vis Spectrophotometer). Methanol was used as blank and ascorbic acid was used as the reference standard. (Aiwonegbe *et al*., 2022).

Equation 3 was used to calculate the ability to scavenge DPPH.

$$\text{DPPH radical scavenging activity (\%)} = \frac{(A_0 - A_1)}{A_0} \times 100 \quad (3)$$

where:
A_0 = absorbance of DPPH radical + methanol,
A_1 = absorbance of DPPH radical + synthesized DAP (or ascorbic acid - reference standard).
The 50% inhibitory concentration value (IC_{50}) is indicated as the effective concentration of the sample that is required to scavenge 50% of the DPPH free radical.

Acute toxicity study

The determination of acute toxicity of 4-phenyl-6-(3,4,5-trimethoxyphenyl)pyrimidin-2-ylamine) was carried out by using the modified method of Lorke (1983), with strict adherence to the Association of Economic Cooperation with Development (OECD) Instruction used for Testing Chemical No. 423 OECD, (2001). The LD_{50} was calculated based on the final results in the square root (of product) with the lowest fatal dose and highest nonfatal dose. The LD_{50} was calculated by using equation 4.

$$LD_{50} = \sqrt{(D_0 \times D_{100})} \quad (4)$$

D_0 = Highest dose with no mortality,
D_{100} = Lowest dose with mortality.

Statistical analysis

The results were analysed with GraphPad Prism version 6. The data are presented as mean ± SEM, and statistical significance was determined using one-way ANOVA, followed by Dunnett's test, where $p<0.05$ was considered to indicate statistical significance.

RESULTS

The spectroscopic characteristics of 4-phenyl-6-(3,4,5-trimethoxyphenyl)pyrimidin-2-ylamine) are shown in Figures 1 to 4 and Table 1. The specific spectroscopic data have already been reported in our previous research (Aiwonegbe *et al*., 2024).

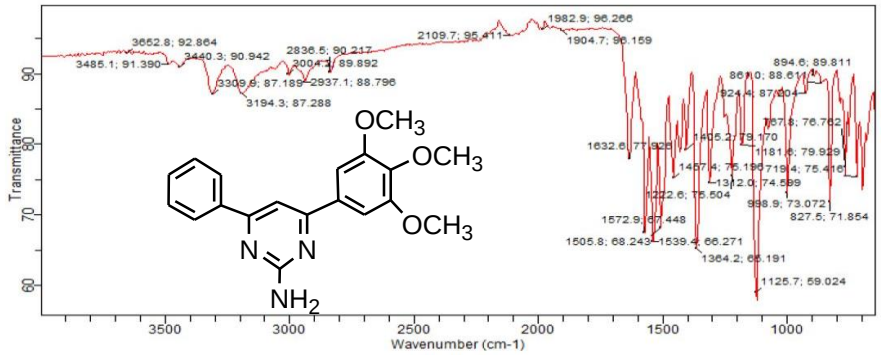


Figure 1: IR spectrum of 4-phenyl-6-(3,4,5-trimethoxyphenyl)pyrimidin-2-ylamine

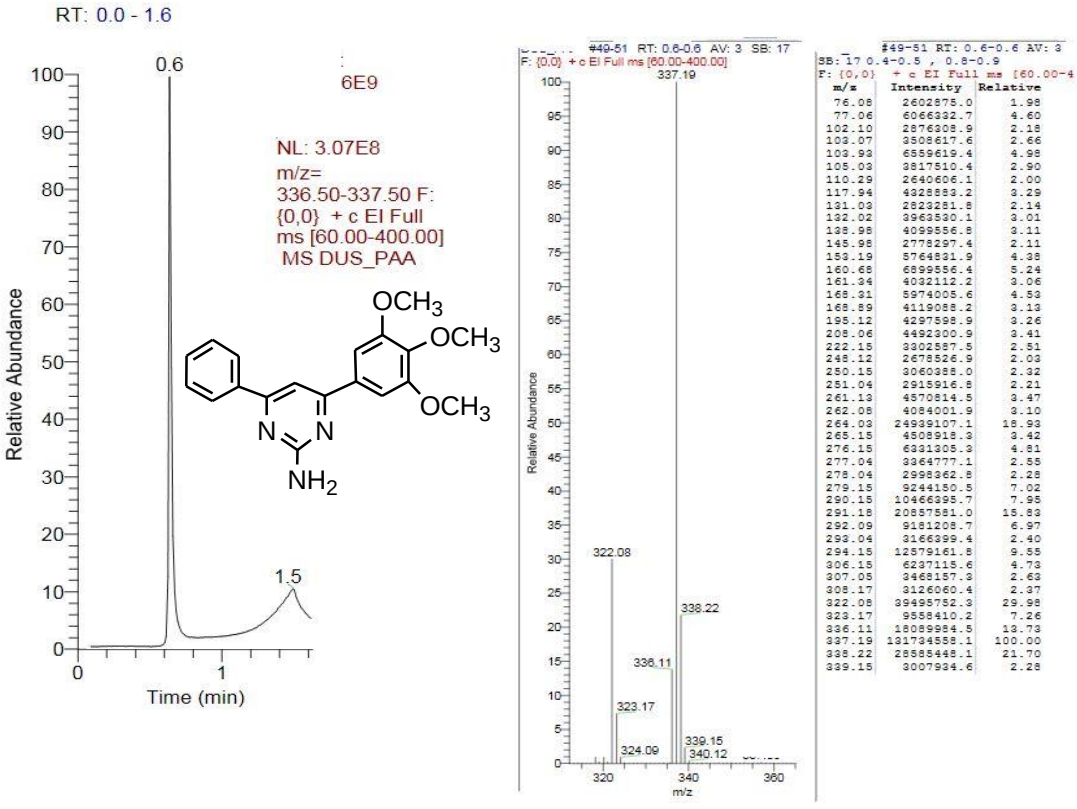


Figure 2: GC- MS spectra of 4-phenyl-6-(3,4,5-trimethoxyphenyl)pyrimidin-2-ylamine

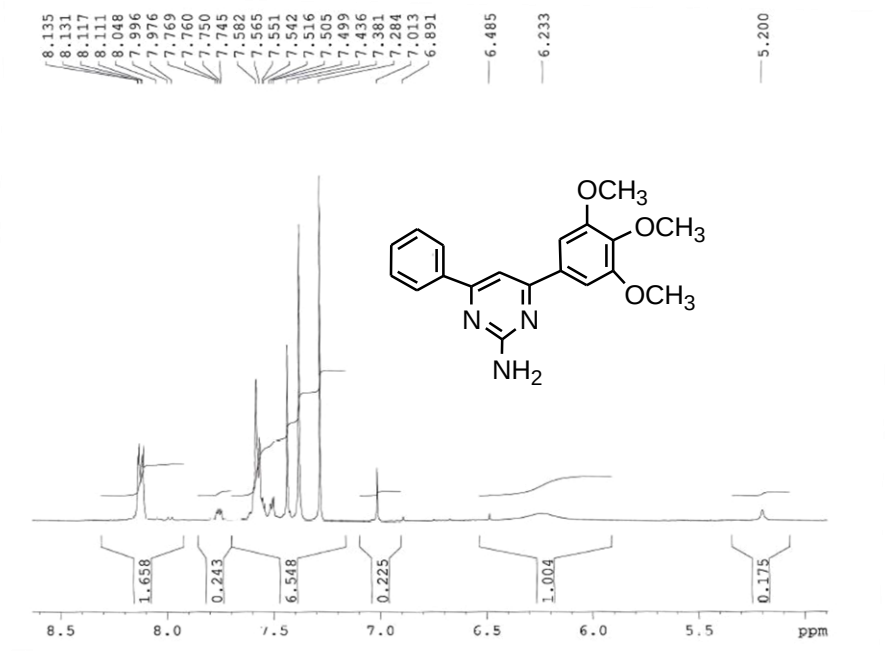


Figure 3: 1H NMR spectrum of 4-phenyl-6-(3,4,5-trimethoxyphenyl)pyrimidin-2-ylamine

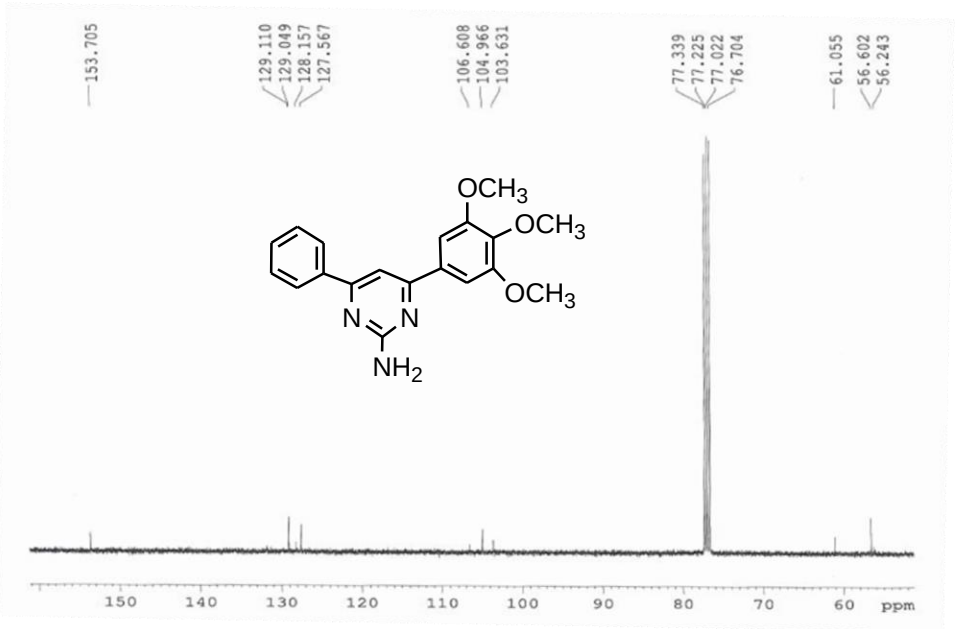


Figure 4: 13C NMR spectrum of 4-phenyl-6-(3,4,5-trimethoxy-phenyl)pyrimidin-2-ylamine

Table 1: Cartesian coordinates of 4-phenyl-6-(3,4,5-trimethoxy-phenyl)pyrimidin-2-ylamime in the gas phase and chloroform

4-phenyl-6-(3,4,5-trimethoxy-phenylpyrimidin-2-ylamine: $C_{19}H_{19}N_3O_3$	
C,0,-2.4002132027,0.9391262635,1.1035403389	C,0,-2.3995568081,0.9630900532,1.094121402
C,0,-1.0325819101,0.7089392718,1.0334523945	C,0,-1.0303626173,0.7362369276,1.0301380405
C,0,-0.3775044234,0.7176565785,-0.2001567267	C,0,-0.3745240347,0.7150433392,-0.2037206154
C,0,-1.1191437902,0.9403535643,-1.3625324088	C,0,-1.1158399775,0.9076495424,-1.3725008941
C,0,-2.4895192215,1.1607823971,-1.2918712941	C,0,-2.4877006938,1.1243298343,-1.307632774
C,0,-3.1324050487,1.1663611907,-0.0583496647	C,0,-3.1319503124,1.1575433951,-0.0743852682
H,0,-2.8971262824,0.9402402566,2.0664468409	H,0,-2.8966116783,0.9880260365,2.056617453
H,0,-0.452422944,0.522565102,1.9283382426	H,0,-0.453088636,0.5791883849,1.9326215844
H,0,-0.6329018529,0.9145931058,-2.330617459	H,0,-0.6291353083,0.863428543,-2.3396569144
H,0,-3.0560360487,1.3218165158,-2.2012576623	H,0,-3.053815006,1.2615651474,-2.2211312903
C,0,1.0896876416,0.4870737374,-0.2529681093	C,0,1.0935051215,0.4878766781,-0.2527360943
C,0,1.8720288894,0.9885373775,-1.2929270855	C,0,1.8735101735,0.9827343967,-1.2977150972
C,0,3.2379667474,0.7101763766,-1.2558642311	C,0,3.2408606838,0.7135594576,-1.2543117828
H,0,1.4356751857,1.5650087521,-2.0938804553	H,0,1.4346161916,1.5471710062,-2.1060951182
C,0,2.9427517729,-0.4252611524,0.6879194558	C,0,2.9518850557,-0.4086690896,0.7020548705
C,0,4.1624535087,1.1862583048,-2.3178775077	C,0,4.1625588108,1.1860335801,-2.3205901191
C,0,5.3751867051,0.524927483,-2.5158960159	C,0,5.3697439532,0.517618876,-2.5309456386
C,0,3.8418001841,2.2853240865,-3.1155810669	C,0,3.8436270022,2.2913622269,-3.1109083872
C,0,6.2414011859,0.941034108,-3.5152556738	C,0,6.232941164,0.9323726609,-3.5343092684
H,0,5.6424825612,-0.3224322337,-1.8983237239	H,0,5.638065255,-0.334810452,-1.9203966075
C,0,4.7095760504,2.7071102593,-4.1147571476	C,0,4.7085946322,2.7112604692,-4.113571702
H,0,2.9261593548,2.8464595435,-2.9770231141	H,0,2.9326268081,2.8575518059,-2.9611533125
C,0,5.9155980735,2.0330008691,-4.3276982168	C,0,5.9094481664,2.0308978471,-4.3398717334
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N,0,3.7757222404,-0.0108424361,-0.2717303928	N,0,3.7831139819,0.0078648254,-0.2605317253
N,0,3.4990079635,-1.114368685,1.7265962086	N,0,3.510229994,-1.0822386235,1.7477719619
H,0,2.8706580411,-1.6273395524,2.3221281119	H,0,2.8861518578,-1.6012568612,2.3444631624
H,0,4.4308094566,-1.4696688229,1.5922884077	H,0,4.4398204058,-1.4472178318,1.6155028104
O,0,4.351406988,3.7521617136,-4.9187489445	O,0,4.3568322169,3.7661100924,-4.9085505369
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O,0,7.4009543331,0.2511822901,-3.7452088702	O,0,7.386871639,0.2356343582,-3.7712716892
C,0,5.1275521317,4.9322070861,-4.7233574999	C,0,5.1208117864,4.9509213374,-4.6718378431
H,0,5.0101437312,5.2929177962,-3.6969790071	H,0,4.9701621038,5.2934064751,-3.6443300873
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H,0,5.634147401,1.7463186973,-6.9163128928	H,0,5.635020779,1.7349028374,-6.9332499052
H,0,7.3674241964,2.0506350376,-7.2161086224	H,0,7.3650957567,2.0588835695,-7.2288076204
C,0,8.5734037602,0.9436473683,-3.3217854612	C,0,8.5681699178,0.9158863078,-3.3396053592
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H,0,9.4162333489,0.2986796714,-3.563265609	H,0,9.4061729931,0.2692201404,-3.5912895481
H,0,8.6714454613,1.8961876843,-3.8469518972	H,0,8.6701027341,1.8736813515,-3.8531498145
H,0,-4.200289757,1.3416596519,-0.0031799259	H,0,-4.2005374723,1.3299945902,-0.0242538827

Superoxide dismutase (SOD) activity

The effect of 4-phenyl-6-(3,4,5-trimethoxyphenyl)pyrimidin-2-ylamine (DAP) on SOD activity in the test animals is presented in Figure 5. DAP mitigated the depletion of SOD in a similar manner to that in the positive control (Group B). However, the greatest effect was displayed in Group C, which was treated with 50 mg/kg DAP, while the least effect was shown in Group E, which was treated with 200 mg/kg DAP.

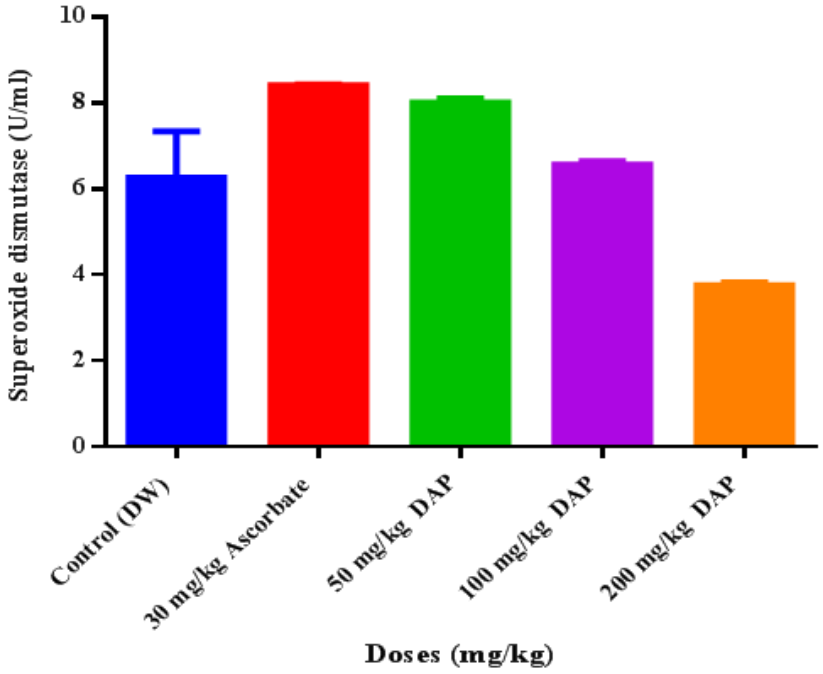

Figure 5: Effect of DAP on superoxide dismutase (SOD)

Estimation of glutathione peroxidase activity

The effect of 4-phenyl-6-(3,4,5-trimethoxyphenyl)pyrimidin-2-ylamine (DAP) on glutathione peroxidase activity is presented in Figure 6. The peroxidase activity displayed an inverse relationship with the graded doses of the compound in the treated groups. The highest activity was observed in animals treated with 50 mg/kg DAP.

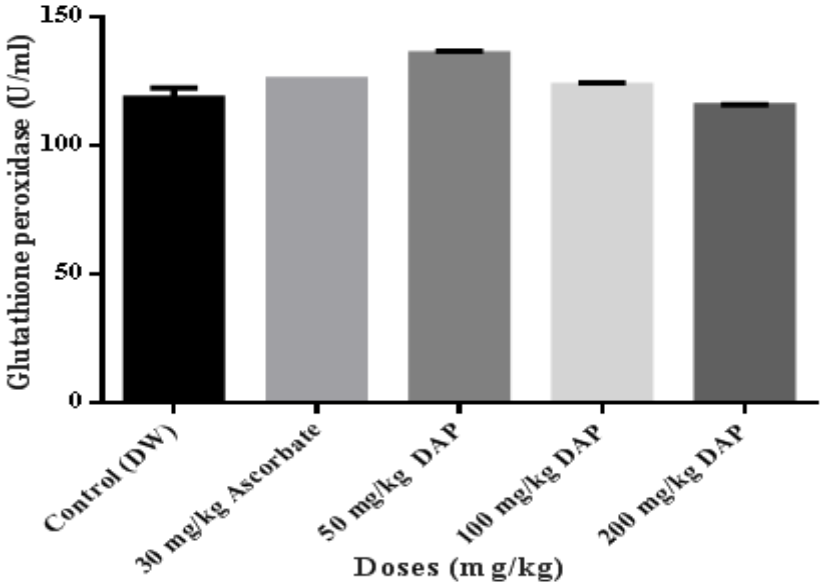

Figure 6: Effect of DAP on glutathione peroxidase activity

Evaluation of catalase (CAT) activity

The results of the catalase activity assay are presented in Figure 7. The catalase activity in the treated groups increased in a dose-dependent manner, with the highest activity occurring in group treated with DAP (50 mg/kg). This activity was comparable to the catalase activity in the positive control group.

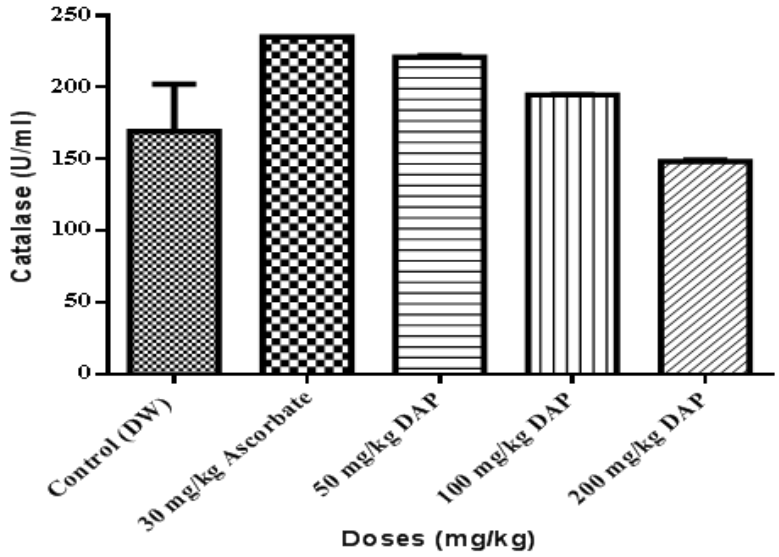

Figure 7: Effects of DAP on *in vivo* catalase activity

Estimation of malondialdehyde (MDA) activity

Figure 8 presents the effects of DAP on the malondialdehyde activity in the serum of the experimental animals. At a dose of 50 mg/kg, DAP sufficiently mitigated oxidative stress in the test animals to a level that was slightly greater than that of ascorbic acid. This is evident in the marked reduction in their serum MDA level. The least reduction in MDA was elicited by the highest dose of 200 mg/kg.

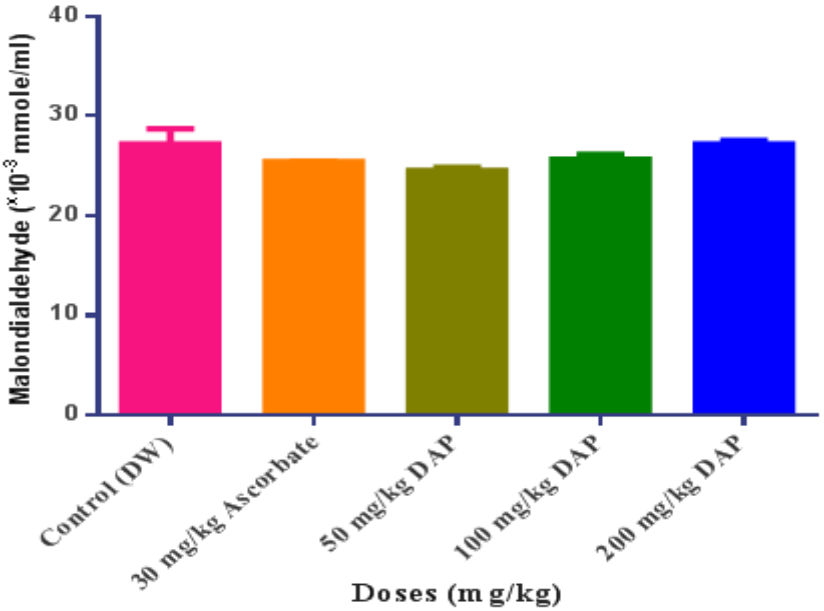

Figure 8: Effect of DAP on malondialdehyde activity

DPPH radical scavenging assay

The results of the antiradical activity of 4-phenyl-6-(3,4,5-trimethoxyphenyl)pyrimidin-2-ylamine (DAP) and the standard (ascorbic acid) against DPPH is presented in Table 2. The scavenging effect on the substrate was expressed as the 50% inhibitory concentration, (IC_{50}). The IC_{50} is the concentration of an antioxidant at which 50% inhibition of free radical activity is observed. The lower the IC_{50} value is, the greater the overall effectiveness of the antioxidant. The IC_{50} values obtained at the end of the experiment were 139.6 µg/mL and 2.37 µg/mL for the synthesized DAP and ascorbic acid respectively.

Table 2: DPPH free radical scavenging activity of DAP

Concentration (µg/mL)	Free Radical Scavenging (%)	
	Diarylaminopyrimidine (DAP)	(Ascorbic acid)
1.00	1.70	23.40
2.00	6.90	35.30
5.00	7.00	76.60
10.00	8.81	85.20
25.00	9.30	98.20
50.00	34.70	98.40
100.00	42.40	99.00
200.00	76.40	99.10

Acute toxicity study

The results of the acute toxicity study of 4-phenyl-6-(3,4,5-trimethoxyphenyl)pyrimidin-2-ylamine (DAP) are presented in Table 3. The study was performed in two phases for the compound. Observations were focused on parameters such as piloerection, sensitivity to sound and touch, locomotion, aggressiveness, the appearance of the faeces, salivation, urination, convulsions, coma and death.

Table 3: Acute toxicity of DAP in albino mice

Treatment	Doses (mg/kg)	Number of lethality	Percentage mortality	Adverse effect/ Mortality
DAP	10	0/5	0	Absent
DAP	100	0/5	0	Absent
DAP	1000	0/5	0	Absent
DAP	1600	0/5	0	Absent
DAP	2900	0/5	0	Absent
DAP	5000	0/5	0	Absent
Control	Distilled water	0/5	0	Absent

At the end of the study, the administration of up to 5000 mg/kg DAP stimulated no observable signs of toxicity or death, and the compound was deemed safe. This is as stipulated by the Globally Harmonized System (GHS) for the classification of chemicals (OECD, 1998). Additionally, the internal organs (lungs, liver, kidneys, spleen, stomach, intestine, and ovaries) of the sacrificed animals were found to be normal in weight and physiology.

DISCUSSION

Ethanol was used to induce oxidative stress in the animal models in this study. The cellular metabolism of ethanol leads to an increase in malondialdehyde (MDA) activity, and hydroxyethyl radical (HER) levels, a decrease in glutathione (GSH) levels and a general decrease in antioxidant activity (Das and Vasudevan, 2007). Consequently, reactive oxygen species (ROS) and reactive nitrogen species (RNS), which are the precursors of oxidative stress, are produced (Das and Vasudevan, 2005). When there is an excess of reactive oxygen species (ROS), a type of oxidative stress is initiated resulting in profound cellular oxidative damage. This damage is the major cause of certain life-threatening degenerative disease conditions such as myocardial infarction, obesity, hypertension, diabetes mellitus, various types of cancers, neurological and immune disorders, renal failure, and ageing (Young *et al*., 2001).

It is believed that one of the major ways to prevent, slow down or disrupt the mechanism of oxidative damage is through the intake of exogenous antioxidants. This may eventually lead to the neutralization of ROS, by scavenging or decomposing them (Dontha, 2016).

Malondialdehyde (MDA) activity is measured via a nonenzymatic antioxidant assay. MDA is the major oxidation product of the peroxidation of polyunsaturated fatty acids (PUFAs). An increase in the level of malondialdehyde is particularly indicative of lipid oxidation. The malondialdehyde assay is based on the principle that the adduct (MDA-TBA2) formed MDA reacts with thiobarbituric acid (TBA), and has a strong absorbance at 532 nm (León and Borges, 2020; Merás *et al*., 2020). The synthesized compound markedly reduced MDA activity to an extent comparable to that of the standard. The mechanism of action of the compound in the reduction of MDA seems to be mostly favoured at a dosage of 50 mg/kg. At 100 and 200 mg/kg, the MDA level increased to a level comparable to that recorded in Group A, suggesting that 50 mg/kg was the optimum dose for the assay.

SOD is an enzymatic antioxidant that scavenges superoxide radicals by converting them to H_2O_2, accompanied by the release of molecular oxygen. SOD was assayed by measuring the absorbance of haematein (an auto-oxidation product of haematoxylin) at 560 nm. The rate of

auto-oxidation is proportional to the haematoxylin concentration. SOD inhibits auto-oxidation to between 90-95% below pH 7.8 (Martin *et al*., 1987; Kono, 2022).

The principle of the assay is represented by the following equation:

$O_2 + HTH_2 + SOD \rightarrow H_2O_2 + HT (Abs560\uparrow)$

Where HTH_2 = haematoxylin; HT = haematein

A pronounced increase in SOD enzyme activity in the blood is considered a significant biomarker of a metabolic disorder (Puppel *et al*., 2022; Tripathi *et al*., 2015). SOD enzyme activity in the control group was not significantly different from that in Group C, which was treated with 50 mg/kg synthesized DAP.

Catalase is an enzymatic antioxidant that is found in all animal tissues, including red blood cells and the liver. Catalase enzyme system protects the tissues from highly reactive hydroxyl radicals by scavenging H_2O_2 and breaking it down to molecular oxygen and water (H_2O_2 + Catalase → $H_2O + 2O_2$) (Sandamalika *et al*., 2021; Alfonso-Prieto *et al*., 2009).

The catalase activity of DAP was estimated based on the rate of decomposition of hydrogen peroxide, which is directly proportional to the decrease in absorbance at $\lambda = 240$ nm. In this study, moderate catalase activity was exhibited by DAP. In a related peroxide assay on synthesized heterocyclic compounds, Bellam *et al*., (2017) reported mild to moderate antioxidant activity for synthesized N-alkylated benimidazoles.

The glutathione (GSH) level (especially in the liver) is a near-accurate estimate of the antioxidant capacity in various disease conditions (such as cancer) and healthy individuals. GSH is a cosubstrate for glutathione peroxidase (GPx), and the GHS/GPx enzymatic antioxidant system scavenges reactive oxygen species (ROS), reactive nitrogen species (RNS) and lipid hydroperoxides (Gökce and Dag, 2023). Glutathione peroxidase also plays a role in the removal and detoxification of mutagenic and genotoxic substances in the body (Flohé *et al*., 2011; Sies *et al.*, 1997).

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) antioxidant model is nonenzymatic and is based on the spectrophotometric determination of the depletion of DPPH free radical by a known concentration of an antioxidant (Gabriel and Idu, 2021). The synthesized compound (DAP) inhibited 50% of DPPH free radicals at a concentration (IC_{50}) of 139 µg/mL. However, this concentration was too high compared to the IC_{50} value of 2.37 µg/mL for the standard, ascorbic acid. The result obtained from this model of antioxidant study may be due to the low solubility of the synthesized compound in the solvent (methanol) used for the DPPH antiradical study (Alneyadi *et al*., 2020). The antioxidant activities in the in *vivo* assays suggested that the compound had significant and reliable antioxidative properties. A similar result was obtained by Makula and Tabassum (2023) for the DPPH scavenging activity of benzamide derivatives of DAP.

The synthesized compound (DAP) was well tolerated by the albino mice used for the acute toxicity test. Acute toxicity is often characterized by adverse effects that occur following oral or dermal administration of a single dose of a substance. An acute toxicity study of synthesized diarylpyrimidine (DAP) suggested that it is safe or not toxic to experimental animals since no mortality was recorded at the highest dose of 5000 mg/kg (Bulus *et al*., 2011).

CONCLUSION

The effect of the diaryl amino pyrimidine (DAP), 4-phenyl-6-(3,4,5-trimethoxyphenyl)pyrimidin-2-ylamine on ethanol-induced oxidative stress in albino rats was examined. The study was carried out *in vitro* and *in vivo*. Enzymatic and nonenzymatic antioxidants were assayed for free radical scavenging by determining the levels of malondialdehyde (MDA), the activities of superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH) in the serum of the rats and the inhibition of DPPH free radicals by the synthesized compound in *vitro*. The results showed that there was a significant reduction in MDA concentration and that the depletion of SOD, GSH and CAT was inhibited in the treated groups compared with the standard group. The optimum dose established for the synthesized compound was 50 mg/kg. The compound may have some characteristics that can eliminate free radicals or stimulate enzymatic antioxidants to scavenge reactive oxygen species (ROS) or other harmful radicals, thereby mitigating the adverse effects of oxidative stress.

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