**Synthesis of novel 2-amino-5-arylazothiazol derivatives their biological impacts: assessment of toxicity and antioxidant enzymes activities**

Mohamed Khalifa1,2\*, Mahmoud A. Mohamed1,3, Noura AlShehri4

**Supplementary file**

1. **Animals**

The male Albino rats under investigation were quarantined and allowed to acclimate to the laboratory condition for a week and were handled under standard laboratory conditions with a 12-h light/dark cycle in a temperature of 25 ± 5 °C and a relative humidity of 55 ± 5 % controlled room. The basal diet used in these studies was certified feed with appropriate analyses performed by the manufacturer and provided to research laboratories and water was available ad libitum. The Institutional Animal Care and Use Committee (IACUC) approved all protocols for the animal study according to the institutional ethical guidelines.

1. **Antioxidant potential of the synthesized compounds**

The stock solution of DPPH was prepared by dissolving 24 mg of DPPH in 100 ml methyl alcohol (MeOH), and then stored at 20 °C in the dark until needed. The working solution was obtained by diluting 10 ml of stock solution with 45 ml MeOH, to obtain an absorbance of 1.1 ± 0.1 units at 515 nm. A volume of 10 µl of different synthesized compounds concentrations (50 and 100 µg/ml), was added to 990 µl of DPPH freshly working solution up to 1 ml. Assays were continuously monitored at 515 nm over a 1 h period at 25 ºC. Changes in absorbance were minimal for all samples after 50 min. The antioxidant abilities were expressed as µM Trolox equivalents. Each sample was analyzed in triplicate. The percentage inhibition of the DPPH radical by the samples was calculated according to equation (1).

1. **Acute Toxicity studies**

Male Albino rats of 6 animals per group were administered after overnight fasting with graded doses (50-500 mg/kg body weight) injected intra peritoneal for each selected synthesized compounds suspended in DMSO. The toxicological effects were observed after 72 h of treatment in terms of mortality and expressed as LD50 and the number of animals dying during the period was noted. Others biochemical parameters determined after 20 days of different compounds administration according to the technique reported for GPT activity and for LDH activity.

1. ***In vivo* bioassays experimental design**

The animals were randomly divided into ten groups of 6 rats each. The first group served as normal control. The second group was pre-treated with standard drug Vitamin E (100 mg/ kg body weight per day) for 20 days. Group 3 to Group 10 were pre-treated with the selected compounds **2c, 3b, 3c** and **3d** at 50 and 100 mg/ kg body weight per day respectively, for 20 days. Twenty-four hours after the last administration, rats were sacrificed and blood samples were collected and centrifuged at 4000×g at 4ºC for 10 min for serums preparation.

1. **Enzymes activity measurements**
   1. ***Glutathione-S-Transferase activity (GST)***

Reaction mixture is composed of 50 mM phosphate buffer, pH 7.5, 1 mM of 1-chloro- 2, 4-dinitrobenzene (CDNB) and an appropriate volume of compound solution. The reaction was initiated by the addition of reduced glutathione GSH and formation of S-(2, 4-dinitro phenyl) glutathione (DNP-GS) was monitored as an increase in absorbance at 334 nm. The result was expressed as µmol of CDNB conjugation produced /mg protein /min.

* 1. ***Super Oxide Dismutase (SOD) activity***

SOD activity was measured through the inhibition of hydroxylamine oxidation by the superoxide radicals generated in the xanthine–xanthine oxidase system.The results were expressed in units/mg protein.

* 1. ***Glutathione Reduced (GSH-Rd) levels***

GSH in liver and kidney tissues was determined according to the Ellman method [7], which measures the reduction of 5, 5-dithio-bis (2-nitrobenzoic acid) (DTNB) (Ellman's reagent) by sulfhydryl groups to 2-nitro-5-mercaptobenzoic acid, which has an intense yellow color. The results were expressed in mg/g protein.

1. **Measurement of protein levels**

Protein levels were determined at 595 nm, using Coomassie brilliant blue G 250 as a protein binding dye and Bovine serum albumin (BSA) was used as a protein standard.

1. **Statistical analysis**

Each of the measurements described was carried out in three replicate experiments and the results are recorded as mean ± standard deviation. The significantly different calculated at the level of p≤ 0.05.