Biological Screening for Some Newly Synthesized Pyrazolo[3,4-*d*]Pyrimidine Derivatives

Zeinab Y. Ali [1*], Sawsan A. Mohamed [2], Mastoura M. Edrees [3]

[1.] Department of Biochemistry, National Organization for Drug Control and Research (NODCAR), Giza, Egypt.
[2.] Department of Physiology, National Organization for Drug Control and Research (NODCAR), Giza 12311, Egypt
[3.] Department of Organic Chemistry, National Organization for Drug Control and Research (NODCAR), Giza 12311, Egypt, Current address: King Khalid University, Faculty of Science, Chemistry Department, P.O. Box 9004, ABHA, KSA
zeinabyousef65@ymail.com

Abstract: In recent years, pyrazole and pyrimidine derivatives attracted organic chemists due to their widespread potential biological and chemotherapeutic activities. In this study, three novel pyrazolo[3,4-*d*] pyrimidine derivatives namely 4-(2-(furan-2-ylmethylene)hydrazinyl)-1*H*-pyrazolo[3,4-*d*]pyrimidine-6(7*H*)-thione, 6-(Benzylthio)-4-(2-(thiophen-2-ylmethylene) hydrazinyl) -1*H*-pyrazolo[3,4-*d*] pyrimidine and 6-(Benzylthio)-4-(2-(4-chlorophenyl)hydrazinyl)-1*H*-pyrazolo[3,4-*d*]pyrimidine, respectively were synthesized through different reactions of 5-Amino-1*H*-pyrazole-4-carbonitrile. Biological screening was designed to investigate their acute toxicity value, antioxidant potential and their ability to inhibit deoxyribose degradation, protein oxidation, lipid peroxidation (LPO) and xanthine oxidase (XOD) as well as, for their analgesic, antipyretic and anti-inflammatory activities. The results revealed that their median lethal intraperitoneal doses (LD_{50}) in male mice were found to be 245.8, 241.7 and 233.3 mg/100g b.w., respectively. The first compound (I) exhibited a good antioxidant activity including 1,1-diphenyl-2-picrylhydrazyl (DPPH), superoxide anion, hydrogen peroxide and nitric oxide free radicals scavenging potential and a slightly more potent inhibitor to protein oxidation, LPO and XOD. The second compound (II) showed moderate activities while the third compound (III) was less active. Furthermore, the three compounds revealed a remarkable analgesic activity in acetic acid-induced writhing response, appreciable antipyretic activity by Brewer's yeast-induced pyrexia and antiinflammatory activity reflected by their ability to reduce the Brewer's yeast- induced paw edema comparable to reference drugs.

[Zeinab Yousef Ali, Sawsan Ahmed Mohamed, Mastoura Mohamed Edrees. **Biological Screening for Some Newly Synthesized Pyrazolo[3,4-*d*]Pyrimidine Derivatives.** Nat Sci. 2012;10(7):58-69]. (ISSN: 1545-0740). http://www.sciencepub.net/nature.

Keywords: Pyrazolo[3,4-*d*]pyrimidine derivatives; LD_{50}; antioxidant; xanthine oxidase**;** analgesic**;** antipyretic; anti-inflammatory

1. Introduction

In recent years, pyrazole and pyrimidine derivatives attracted organic chemists due to their widespread potential biological and chemo-therapeutic activities (Rajendran et al., 2011). Hetero-fused pyrazolo-pyrimidines were reported to elicit central nervous system (CNS) depressant, neuroleptic, antimicrobial (Shaikh et al., 2011), anti-inflammatory, non-narcotic analgesic (Singh et al., 2011), anti-tubercular (Lauria et al., 2005) and antiviral (Radi et al., 2010). Moreover, pyrazolo[3,4-*d*] pyrimidines were identified as a general class of adenosine receptor antagonist (Antonelli et al., 2011), and they have gained an important place in anticancer therapy as dual Akt/p70S6K inhibitors (Rice et al., 2012). Therefore, this study report the design of three noval pyrazolo[3,4-*d*]pyrimidine derivatives and preliminary screening for their biological activities as antioxidant, xanthine oxidase (XOD) inhibitors, analgesic, antipyretic and anti-inflammatory activities which was eventually advanced into clinical development.

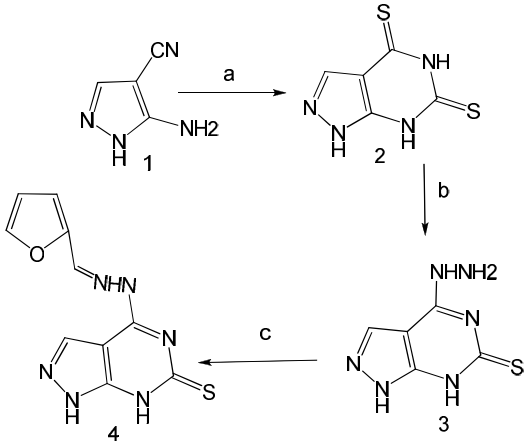
Chemistry

IR spectra were recorded for (KBr) discs on a Pye Unicam SP-1000 spectrophotometer. ^{1}H NMR spectra were measured on a Varian EM 390-200 MHz in DMSO-d_6 as solvent and using TMS as internal standard, and the chemical shifts are expressed as δ ppm. Analytical data were recorded at the Micro analytical Centre, Cairo University, Giza, Egypt.

The started compound 5-amino-1*H*-pyrazole-4-carbonitrile (compound **1**) was synthesized according to the method described by Cheng and Robins (1956). The synthesis of novel pyrazolo[3,4-*d*]pyrimidine derivatives having different substituents is shown in schemes 1 and 2 (Baraldi et al., 2003). The Reaction of **Scheme 1** with CS_2 was carried out in the presence of pyridine under reflux. After work up of the reaction mixture, the product was isolated. The product compound reacted with **2**, condensed **2** 1*H*-pyrazolo[3,4-*d*]pyrimidine-4,6(5*H*,7*H*)-dithione with hydrazine hydrate (Taylor et al., 1967) yield

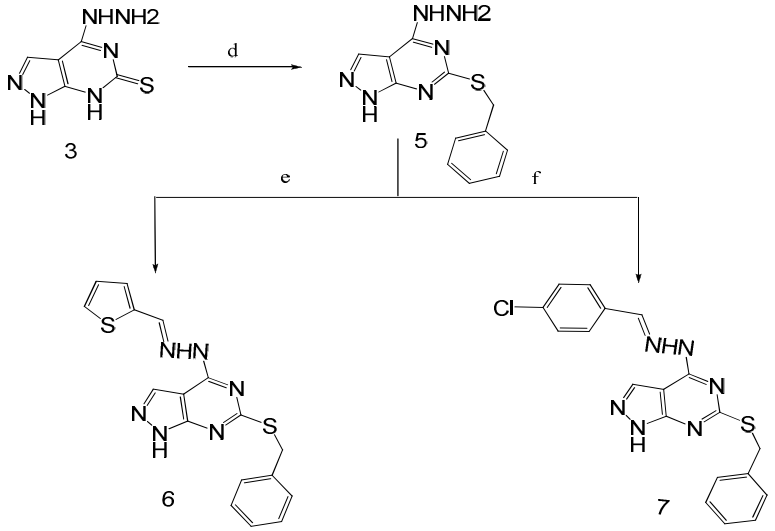
hydrazine derivatives structure **3** could be established based on IR spectrum.

Showed bonds at 3483, 3220, 3112, 3018 (NH_2, 3NH) and ^{1}HNMR revealed a singlet at 4.55 ppm for NH_2. The condensation of compound **3** with furfural furnished the corresponding hydrazo derivative **4 (Scheme 1)**. The structure of compound **4** were elucidated based on the spectral data thus its IR spectrum showed bands at 3212, 3118, 3021 (3NH) the ^{1}HNMR revealed 8.25 ppm to azomethine group (CH=NH) **(Scheme 1)**. Similarly, compound **5** have been prepared via the treatment of compound **3** with benzyl chloride. Stirring at room temperature, compound **5** condensed with thiophene-2-aldehyde and p-Cl benzaldehyde yield hydrazono derivatives **6**, **7.** The structure of compounds **6** could be established based on ^{1}HNMR revealed beak at 4.44 ppm characterized for SCH_2 and 8.25 ppm revealed to (CH=N) group **(Scheme 2)**.



Scheme 1

a: CS_2, KOH; b: NH_2NH_2; c: furfural, DMF
Compound 4 represented as Compound I



Scheme 2

d: benzyl chloride,NaOH; e: thiophene-2-aldehyde,DMF; f: 4-chlorobenzaldehyde, DMF
Compounds 4 and 6 represented as Compounds II and III, respectively.

Experiment

1*H*-pyrazolo[3,4-*d*]pyrimidine-4,6(5*H*,7*H*)-dithione (2)

To a solution of 5-amino-1H-pyrazole-4-carbonitrile (1.08g, 0.01 mol) 1 in 10% alcoholic KOH (10 mL), carbon disulfide (10 mL) was added. The reaction mixture was refluxed for 2 hours, cooled, poured into cold water and neutralized with 1M HCl. The obtained solid was filtered off, washed with water, dried and recrystallized from DMF to afford **2** as a yellow powder, yield (1.29 g, 70%), m.p. 115-117^{o}C. IR (KBr) ν 3200, 3120, 3112 (3NH) cm^{-1}; ^{1}H-NMR δ 4.48 (s, NH), 8.30 (s, CH), 11.64 (d, 1H, NH), 11.97 (s, 1H, NH), MS m/z (%) 184 (M+H) $^{+.}$ Anal. Calcd for $C_5H_4N_4S_2$ (183.99): C, 32.59; H, 2.19; N, 30.41 Found: C, 32.34; H, 2.32; N, 30.54%.

2-[4-hydrazino-6-thioxo-6,7-dihydro-1*H*-pyrazolo[3,4-*d*]pyrimidin-3-yl] acetonitrile (3).

To a mixture of hydrazine hydrate (5 gm, 0.099 mol) and ethanol (5 mL) compound **2** (1.84 gm, 0.01mol) was added and the mixture was heated under reflux for 15 minutes. After the reaction was completed the precipitated crystals were collected by filtration and washed with water / ethanol to afforded the hydrazinyl derivatives .Orange crystals, yield (1.23 gm, 68%), m.p. 206-208^{o}C. IR (KBr) ν 3483, 3220, 3112, 3018 (NH_2, 3NH)cm^{-1}. ^{1}H NMR δ 4.55 (s, 2H, NH_2), 7.22 (s, H, CH), 8.53 (s, 1H, NH), 9.82 (d, 1H, NH), 11.63 (s, 1H, NH),; MS m/z (%) 182 (M+H) $^{+}$. Anal. Calcd for $C_5H_6N_6S$ (182.04): C, 32.96; H, 3.32; N, 46.12 Found: C, 32.82; H, 3.12; N, 46.20 %.

4-(2-(furan-2-ylmethylene)hydrazinyl)-1*H*-pyrazolo[3,4-*d*]pyrimidine-6(7*H*)-thione (4).

A mixture of the hydrazinyl derivatives **3** (1.82 gm, 0.01mol) and the furfural (0.96 gm, 0.01 mol) in dimethyl formamide was stirred at room temperature for 12 hrs after the reaction was completed, the solution was evaporated under reduced pressure. The separated solid was filtered and crystallized from ethanol. Reddish brown solid, Yield (2.21 gm, 85%), m.p. 215^{o}C. IR (KBr) ν 3212, 3118, 3021 (3NH) cm^{-1}.^{1}H NMR δ 7.32-8.06 (m, 3H, 5CH), 8.10 (s, 1H, CH=), 8.25 (s, 1H, CH=N), 8.52 (s, 1H, NH), 9.94 (s, 1H, NH), 12.56 (s, 1H, NH); MS m/z (%) 260 (M+H) $^{+}$. Anal. Calcd for $C_{10}H_8N_6$ OS (260.05): C, 46.15; H, 3.10; N, 32.29 Found: C, 46.23; H, 2.98; N, 32.31 %.

6-(Benzylthio)-4-hydrazinyl-1*H*-pyrazolo[3,4-*d*] pyrimidine (5).

To a stirred solution of compound **3** (1.82gm, 0.01mol), in ethyl alcohol (10 mL) at room temperature was added NaOH (15 mL, 1N) then the reaction mixture was treated with benzyl chloride (1.26 mL, 0.01 mol) drop wise with stirring. The reaction mixture was stirred for 4 hrs then the solid product was collected by filtration, washed with water and diethyl ether to give the desired product. Yellow solid, Yield (1.95 g, 72%), m.p. 190^{o}C. IR (KBr) ν 3345, 3251, 3100 (NH_2, 2NH) cm^{-1}. ^{1}H NMR δ 4.99 (s, 2H, CH_2), 5.69 (s, 2H,NH_2), 7.16-7.77 (m, 5H, ArH, CH), 8.13 (s, 1H, CH=), 8.18 (s,1H, NH), 12.54 (s,1H, NH). MS *m/z* (%) 272 (M+H)$^{+}$. Anal. Calcd for $C_{12}H_{12}N_6S$ (272.08): C, 52.92; H, 4.44; N, 30.86 Found: C,53.10; H,4.34; N,30.78 % .

6-(benzylthio)-4-(2-(thiophen-2-ylmethylene) hydrazinyl)-1*H*-pyrazolo[3,4-*d*]pyrimidine (6).

A mixture of the compound **5** (2.72 gm, 0.01 mol) and thiophene-2-aldehyde (1.2mL , 0.01 mol) in dimethyl formamide was stirred at room temperature for 2 hrs and then the reaction mixture was reflux for 3h evaporated under reduced pressure and the precipitate was crystallized from ethanol. Yellow crystals, yield (2.37 g, 65%), m.p. 210^{o}C. IR (KBr) ν 3211, 3140 (2NH) cm^{-1}.^{1}H NMR δ 4.44 (s, 2H, CH_2), 7.30-7.97 (m, 9H, ArH,thiophene H-3, CH), 8.25 (s,1H, CH), 11.71 (s,1H, NH), 12.03 (s,1H, NH); MS *m/z* (%) 366 (M+H)$^{+}$. Anal. Calcd for $C_{17}H_{14}N_6S_2$ (366.07): C, 55.72; H, 3.85; N, 22.93 Found: C,55.54; H, 3.92; N,23.10 % .

6-(benzylthio)-4-(2-(4-chlorobenzylidene) hydrazinyl)-1*H*-pyrazolo[3,4-*d*]pyrimidine (7).

A mixture of the compound **5** (2.72 gm, 0.01 mol) and 4-chlorobenzaldehyde (1.31 mL, 0.01 mol) in dimethyl formamide was stirred at room temperature for 2 hrs and then the reaction mixture was reflux for 3h evaporated under reduced pressure and the precipitate was crystallized from ethanol.. Orange solid, yield (2.36 gm, 60%), m.p. 154^{o}C. IR (KBr) ν 3230, 3213 (2NH) cm^{-1}.^{1}H NMR δ 4.23 (s, 2H, CH_2), 7.04-7.78 (m, 9H, ArH,thiophene H-3, CH), 8.33 (s,1H, CH), 11.42 (s,1H, NH), 12.01 (s,1H, NH); MS *m/z* (%) 394 (M+H)$^{+}$. Anal. Calcd for $C_{19}H_{15}ClN_6S$ (394.08): C, 57.79, H, 3.83, N, 21.28 Found: C,58.02, H, 3.74, N,21.35 % .

Biological Screening

Animals

Male albino Sprague-Dawley mice weighting 25 ± 5 gm and rats weighting 150 ± 10 gm were obtained from National Organization For Drug Control And Research (NODCAR), Giza, Egypt. Animals were housed in standard cages with controlled temperature (20 - 25^{o}C) and 12 hours light dark cycle with free access to water and standard

pellets diet (protein 18.8%, barely 37%, corn 15 %) and water.

Chemicals

All other chemicals used were of analytical grade supplied by Fluka Co. (Buchs, Switzerland) or Sigma Co. (St. Louis, USA). **Drugs:** Allopurinol (No-Uric, EIPICO, Egypt), Acetylsalicylate (Aspirin), Paracetamol (Bento pharmaceuticals**),** Diclofenac sodium **(**Voltarin, Novartis Pharma, Egypt) and Brewer's yeast (MEPACO. Pharma, Egypt)

Preliminary Acute Toxicity Test

Preliminary acute toxicity experiments were carried out using 18 groups (6 mice/each). Each compound was i.p injected in progressively increasing doses (100-350 mg/100 g b.w) to find out the range of doses which cause zero and 100% mortality of animals after 24 hrs. The medium lethal dose LD_{50} was calculated using the arithmetic method of Aliu and Nwude (1982).

Assessment of Antioxidant Activity

1,1-Diphenyl-2-picrylhydrazyl radical scavenging activity

The ability of three newly synthesized pyrozolo-pyrimidine derivatives to act as hydrogen donors were measured *in vitro* by stable free radical as 1,1-diphenyl-2-picryl-hydrazyl ($DPPH^{\bullet}$) as described by Blois (1958). Antioxidant reacts with DPPH (deep violet color, a stable free radical), converts it to 1,1-diphenyl-2-picrylhydrazine (colourless, nonradical $DPPH_2$). The degree of discoloration indicates the scavenging potential of the test compound. The reaction mixture contained 1 mL of $DPPH^{\bullet}$ solution (0.3 mM in methanol) and 2.5 mL of the test compounds or butylated hydroxyanisole (BHA) at different concentrations (0–120 µg/mL). The mixture was shaken and allowed to stand at room temperature for 30 min. then the absorbance was measured at 517 nm using UVspectrophotometer (Unicam, Japan) and calculated as the percentage of inhibition.

Superoxide anion scavenging activity

Superoxide anion ($O_2^{\bullet-}$) scavenging activity of the three newly synthesized pyrozolo-pyrimidine derivatives was measured by the reduction of NBT according to a previously reported method (Fontana et al., 2001). The non-enzymatic phenazine methosulfate-nicotinamide adenine dinucleotide (PMS/NADH) system generates superoxide radicals, which reduce nitro blue tetrazolium (NBT) to a purple formazan. In this assay, the 1 mL reaction mixture contained phosphate buffer (20 mM, pH 7.4), NADH (73 µM), NBT (50 µM) and various concentrations (0–120 µg/mL) of test compounds. The reaction was started by adding 1 mL of PMS solution (10 µM) to the mixture. After incubation for 5 min at ambient temperature, the absorbance at 562 nm was measured in a spectrophotometer (Unicam, Japan) against an appropriate blank and calculated as the percentage of inhibition. BHA was used as positive control.

Hydrogen peroxide radical scavenging activity

The ability of the three newly synthesized pyrozolo-pyrimidine derivatives to scavenge hydrogen peroxide was determined according to the method of Ruch et al., (1989). The three newly synthesized pyrozolo-pyrimidine derivatives or BHA at different concentrations (0–120 µg/mL) in phosphate buffer solution (PBS, pH 7.4) were added to hydrogen peroxide solution (40 mM) in PBS (pH 7.4). Absorbance of H_2O_2 at 230 nm was recorded spectrophotometrically after 10 min and calculated as the percentage of inhibition.

Nitric oxide scavenging activity

Nitric oxide radical scavenging was estimated on the basis of Griess Illosvoy reaction (Garratt, 1964). At physiological pH, nitric oxide generated from aqueous sodium nitroprusside (SNP) solution interacts with oxygen to produce nitrite ions that quantified by the Griess Illosvoy reaction. The reaction mixture contained 10 mM SNP, phosphate buffered saline (PBS) (pH 7.4) and various doses of the three newly synthesized pyrozolo-pyrimidine derivatives or standard solution (BHA) (0–120 µg/mL) in a final volume of 3 mL. After incubation for 150 min at 25°C, 1 mL sulfanilamide (0.33% in 20% glacial acetic acid) was added to 0.5 mL of the incubated solution and allowed to stand for 5 min. Then 1 mL of napthylethylenediamine dihydro-chloride (NED) (0.1% w/v) was added and the mixture was incubated for 30 min at 25°C. The pink chromophore generated during diazotization of nitrite ions with sulphanilamide and subsequent coupling with NED was measured spectrophotometrically at 540 nm against a blank sample and calculated as the percentage of inhibition.

Hydroxyl radical scavenging activity (Inhibition of deoxyribose degradation)

The ability of the three newly synthesized pyrozolo-pyrimidine derivatives to prevent oxidative degradation of deoxyribose by hydroxyl radical was assayed as described by Chung et al., (1997). The assay is based on quantification of the degradation product of 2-deoxyribose by condensation with

thiobarbituric acid (TBA). The hydroxyl radicals were generated by the Fe3+-ascorbate-EDTA-H2O2 system (Fenton reaction). The reaction mixture in a final volume of 1 mL contained 2-deoxy-2-ribose (2.8 mM); KH2PO4-KOH buffer (20 mM, pH 7.4); $FeCl_3$ (100 µM); EDTA (100 µM); H_2O_2 (1.0 mM); ascorbic acid (100 µM) and various concentrations (0–120 µg/mL) of the test sample or reference compound (BHA). After incubation for 1 h at 37°C, 1mL of 2.8% TCA and 1mL of 1% TBA were added and placed in a boiling water bath for 10min to develop the pink colour. Then, the resultant mixture was allowed to cool and centrifuged at 395 ×g for 5 min. The absorbance was recorded at 532 nm in a UV-VIS spectrophotometer and calculated as the percentage of inhibition.

Inhibition of Fe^{2+}-Ascorbate-Induced Protein Oxidation:

Determination of protein carbonyl content

The effects of the three newly synthesized pyrozolo-pyrimidine derivatives on protein oxidation were carried out according to the slightly modified method of Levine et al. (1994). Bovine serum albumin (BSA) was oxidized by a Fenton-type reaction. The reaction mixture (1.2 mL), containing sample solution or reference compound (BHA) (0–120 µg/mL), potassium phosphate buffer (20 mM, pH 7.4), BSA (4 mg/mL), $FeCl_3$ (50 mM), H_2O_2 (1 mM) and ascorbic acid (100 mM) was incubated for 30 min at 37°C. For determination of protein carbonyl content in the samples, 1 mL of 10 mM 2,4-dinitrophenylhydrazine (DNPH) in 2 M HCl was added to the reaction mixture. Samples were incubated for 30 min at room temperature. Then, 1 mL of cold TCA (10%, w/v) was added to the mixture and centrifuged at 3000 ×g for 10 min. The protein pellet was washed three times with 2 mL of ethanol/ethyl acetate (1:1, v/v) and dissolved in 1 mL of guanidine hydrocholoride (6 M, pH 2.3). The absorbance of the sample was read at 370 nm in a UV-VIS spectrophotometer and calculated as the percentage of inhibition.

Inhibition of Fe^{2+}-ascorbate-induced lipid peroxidation

The extent of lipid peroxidation was measured by estimation of the thiobarbituric acid reactive substances (TBARS) in liver, kidney and heart homogenates. Malondialdehyde (MDA), produced by oxidation of polyunsaturated fatty acids, reacts with two molecules of thiobarbituric acid (TBA) yielding a pinkish red chromogen with an absorbance maximum at 535 nm (Fernandez et al., 1997). Rats were anesthetized with ether and sacrificed by decapitation. The tissues (liver, heart and kidney) were quickly removed, washed and homogenized in cold 100 mM Tris-HCl buffer pH 7.4 (1 : 10 w/v) in a Teflon glass homogenizer. The homogenates were centrifuged at 800 ×g for 10 min at 4°C. The reaction mixtures contained 0.5 mL of supernatant, 0.1 mL of 150 mM Tris–HCl buffer (pH 7.2), 0.05 mL of 400 mM ascorbic acid, 0.05 mL of 400 mM $FeCl_2$ and 0.05 mL of different concentrations of the three newly synthesized pyrozolo-pyrimidine derivatives or α-tocopherol incubated at 37°C for 1 h. After the incubation, 0.9 mL of distilled water and 2 mL of 0.6% TBA were added and then heated for 60 min in a boiling water bath and cooled drown to room temperature. Five milliliter of n-butanol was added and the mixture shaken vigorously. The n-butanol layer was separated by centrifugation at 3000 ×g for 10 min. The organic layer was taken and its absorbance at 532 nm was measured and calculated as the percentage of inhibition.

Xanthine oxidase inhibitory activity

The inhibitory effect of the three newly synthesized pyrozolo-pyrimidine derivatives on xanthine oxidase (XOD, EC 1.1.3.22) was determined spectro-photometrically by following the increase in the absorbance at 295 nm which indicated the formation of uric acid (Kong et al., 2000). The reaction mixture consisted of 400 µl of 200 mM phosphate buffer solution (PBS) (pH7.5), 200 µl of 0.6 mM xanthine, 20 µl of sample solution or reference compound as allopurinol (1,5-dihydro-4*H*-pyrazolo[3,4-*d*]pyrimidin-4-one) at different concentrations (0–50 µg/mL). The reaction was initiated by adding 200 µl of xanthine oxidase (0.1 U) solution and incubated at 25°C for 30 min. The reaction was stopped by addition of 1N hydrochloric acid and the absorbance was read at 295 nm and calculated as the percentage of inhibition.

Calculation of 50% inhibition concentration (IC_{50})

All determinations were performed in triplicate. The % radical scavenging activity of each three newly synthesized pyrozolo-pyrimidine derivatives or reference drugs at different concentrations was calculated from a control measurement of the reaction mixture without the test sample using the following formula:

Radical scavenged % = 100 X ((Absorbance $_{control}$ - Absorbance $_{sample}$) / Absorbance $_{control}$).

Then the activity was expressed as IC_{50} values determined from dose response curve. IC_{50} is the concentration of the sample in µg/mL required to

scavenge 50% of free radicals. The IC_{50} value is inversely related to the activity.

Analgesic activity (*Acetic acid induced writhing test*)

Abdominal writhing (abdominal constriction and extension of hind limbs) was induced in mice by intraperitoneal injection of 3% (v/v) acetic acid solution at a dose of 0.1 mL/10 gm b.wt. as described by Turner (1965). Thirty mice were divided into eight groups each of six, Group 1 severed as positive control and received saline, Group 2 received Asprin at dose of 200 mg/kg b.wt., Groups 3 and 4 received first compound at doses of 245.8 mg/kg (1/10 LD_{50}) and 122.9 mg/kg (1/20 LD_{50}) receptively, Groups 5 and 6 received second compound at doses of 24.17 mg/kg (1/10 LD_{50}) and 120.85 mg/kg (1/20 LD_{50}) receptively and Groups 7 and 8 received third compound at doses of 233.3 mg/kg (1/10 LD_{50}) and 116.7 mg/kg (1/20 LD_{50}) by intraperitoneal injection 30 minute before acetic acid treatment. The mice were placed individually and allowed to elapse. The number of writhes was counted for 20 min. For scoring purposes, a writhe is indicated by stretching of the abdomen with simultaneous stretching of at least one hind limb.

Antipyretic Activities (*Brewer's yeast-induced pyrexia*)

The antipyretic activity of the three compounds was evaluated using Brewer's yeast-induced pyrexia in fasted rats (Loux et al., 1972). Pyrexia were induced in rats by subcutaneously injection below the nape of the neck with 20% Brewer's yeast suspended in normal saline at a dose of 20 mL/kg b.wt. Rectal body temperature was recorded by digital thermometer immediately before and 18 h after Brewer's yeast injection. Thirty animals showing an increase of rectal temperature > 0.5°C were selected and divided into five equal subgroups, each composed of 6 rats. The 1st group received normal saline. The three newly synthesized pyrozolo-pyrimidine derivatives were given interaperitoneally in a dose of 150 mg / kg b.wt to the 2nd, 3rd and 4th groups receptively. The 5th group was given paracetamol (as reference drug) at a dose of 150 mg/Kg b. wt. Body temperature was recorded after half an hour, then every hour for 2 hours after pyrexia induction

Anti-Inflammatory Activity (*Brewer's yeast induced paw oedema*)

Acute inflammation was induced in rats by injecting Brewer's yeast (0.1 mL of 20% suspension in 0.9% saline) below the plantar aponeurosis of the hind paw as described by Hore et al. (1997). Thirty male albino mice were divided into five equal groups, each composed of 6 rats. The first group was i.p injected by saline. The three compounds at doses of 30 mg/Kg were i.p. injected to groups 2, 3and 4, respectively while, group five was i.p. injected by diclofenac sodium (as reference drug) in a dose of 30 mg/kg. Four hours after injection of phlogistic agent, the increase in paw thickness was measured using a digital skin caliber and recorded as index of inflammation (expressed in mm). The paw skin was recorded again by skin caliber 30, 60, 90 and 120 minute hours after the drug administration.

Statistical Analysis

Values reported are the mean ± S.E. The statistical comparisons were done using one way analysis of variance (ANOVA) (using SPSS 17 statistical software) and the different between means were compared by Duncan's multiple range test (DMRT). $P < 0.05$ were considered as significant. For a single comparison in the analgesic, antipyretic and anti-Inflammatory data, the significance of differences between means was determined by the student's *t*-test. A level of $P<0.05$ was accepted as statistically significant.

RESULTS AND DISCUSSION

Pharmaceutical chemistry is devoted to the discovery and development of new agents for treating diseases. Chemistry of pyrimidine and fused pyrimidine derivatives has been of increasing interest, since molecules based on the pyrazolo[3,4-*d*] pyrimidine ring system exhibit a multitude of interesting pharmacological properties (Antonelli et al., 2011). Preliminary biological screening methods could provide the needed observations necessary to select compounds with potentially useful properties for further chemical and pharmacological investigations. Therefore, the current study is directed to synthesis three novel pyrazolo[3,4-*d*] pyrimidine derivatives and screening for their biological activities in different model systems, comparing with reference drugs.

Preliminary acute toxicity test

The acute toxicity of the three test compounds in mice indicated their good safety profiles; their median lethal intraperitoneal doses (LD_{50}) values were found to be 245.8, 241.7 and 233.3 mg/100g b.w., respectively.

Evaluation of Antioxidant Activity

Formation of reactive oxygen species (ROS) is characteristic of aerobic organisms, including superoxide radicals, singlet oxygen and hydroxyl radicals are often generated as by-products of

biological reactions (Cui et al., 2012). Some of them play a positive role in the energy production, phagocytosis, regulation of cell growth and intercellular signaling or synthesis of biologically important compounds. Also, during the inflammatory process, phagocytes generate the superoxide anion ($O_2^{\bullet-}$) radical at the inflamed site and this is connected to other oxidizing species as hydroxyl radicals ($OH^{\bullet}$) are among the most ROS and are considered to be responsible for some of the tissue damage occurring in inflammation (Poljsak, 2011). Antioxidants can terminate the chain reactions by removing radical intermediates and can inhibit other oxidation reactions by being oxidized themselves. So, we need antioxidants to ensure our defence mechanism (Saha and Tamrakar, 2011). Therefore, the current research is directed to screening for the radical scavenging potential of the three noval novel pyrazolo[3,4-*d*] pyrimidine derivatives against important reactive radicals as DPPH and ROS. The results in **Table 1** illustrated the inhibitory effect of three newly synthesized pyrozolo-pyrimidine derivatives on $DPPH^{\bullet}$, superoxide anion ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), nitric oxide ($NO^{\bullet}$) and hydroxyl radicals ($OH^{\bullet}$) scavenging activity in comparable to reference synthetic antioxidant compound as butylated hydroxyanisole (BHA).

Table 1. Inhibitory effect of the three newly synthesized pyrozolo-pyrimidine derivatives on different radicals in comparable to butylated hydroxyanisole (BHA)

Treatments	Concentration required for 50% inhibition (µg/mL)				
	$DPPH^{\bullet}$	$O_2^{\bullet-}$	H_2O_2	$NO^{\bullet}$	$OH^{\bullet}$
Compound I	26.9 ± 0.12 [a]	53.5 ± 1.12 [b]	30.2 ± 1.16 [b]	23.7 ± 1.32 [a]	20.3 ± 0.83 [a]
Compound II	38.2 ± 1.52 [b]	62.1 ± 1.31 [c]	41.0 ± 1.01 [c]	35.6 ± 1.41 [b]	22.6 ± 1.21 [ab]
Compound III	50.1 ± 0.96 [c]	74.0 ± 2.96 [d]	45.8 ± 1.07 [d]	39.1 ± 1.13 [b]	25.5 ± 1.19 [b]
BHA	24.7 ± 0.69 [a]	43.2 ± 0.66 [a]	24.1 ± 1.06 [a]	22.6 ± 0.98 [a]	21.0 ± 1.04 [a]

The data are representative of three experiments and expressed as mean of % inhibition ± S.E
Values within a column followed by different letters are significant different (P <0.05) by DMRT.
Compound I, II and III are that of no. 4, 6 and 7, respectively as represented in Scheme 1 and 2.

$DPPH^{\bullet}$ is a stable free radical at room temperature. It accepts an electron or hydrogen radical to form a stable molecule $DPPH_2$. $DPPH^{\bullet}$ radical assay is a commonly used substrate for fast evaluation of antioxidant activity because of its stability in the radical form and simplicity of the assay. This assay depends on the quantitatively measurement of the colour change of DPPH solution from purple into yellow by spectrophotometer absorbance at 517 nm as the radical is quenched by the antioxidant (Blois, 1958). The results in **Table 1** revealed that as BHA, the first compound exhibited the highest $DPPH^{\bullet}$ radical scavenging activity followed by second and third compound with IC_{50} values of 26.9 ± 0.12, 38.2 ± 1.52 and 50.1 ± 0.96 µg/mL, respectively.

Both superoxide anion ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) are only moderately reactive molecules. Their cellular damage appears to be due to their conversion into more reactive species as hydroxyl radical ($^{\bullet}OH$) through Fenton reaction or peroxynitrite ($ONOO^-$) through reaction of nitric oxide with superoxide (Cui et al., 2012). In the present study, both $O_2^{\bullet-}$ and H_2O_2 scavenging activity of the three newly synthesized pyrozolo-pyrimidine derivatives was decreased in the order of BHA > compound I > compound II > compound III with IC_{50} values of 43.2 ± 0.66, 53.5 ± 1.12, 62.1 ± 1.31 and 74.0 ± 2.96 µg/mL for superoxide anion and 24.1 ± 1.06, 30.2 ± 1.16, 41.0 ± 1.01 and 45.8 ± 1.07 µg/mL for hydrogen peroxide, respectively. In this study, the non-enzymatic PMS/NADH system was used to generate superoxide radicals. These radicals reduce NBT into a purple colored formazan which was measured at 562 nm.

In the present study, nitric oxide ($NO^{\bullet}$) was generated from sodium nitroprusside in aqueous solution at physiological pH, which interacts with oxygen to produce nitrate ions that can be estimated by use of Greiss reagent. Scavengers of nitric oxide complete with the oxygen, leading to reduced production of nitric oxide (Halliwell and Gutteridge 2007). The results showed that compound 1 exhibited a good $NO^{\bullet}$ scavenging activity in comparable with that of standard agent, BHA (Table 1). This activity decreased in the order of BHA ≥ compound I > compound II ≥ compound III with IC_{50} values of 22.6 ± 0.98, 23.7 ± 1.32, 35.6 ± 1.41 and 39.1 ± 1.13 µg/mL, respectively.

Cells have developed antioxidant mechanisms to quench these radicals but when their generation exceeds the scavenging capacity of the cell, they can attack proteins, lipids and DNA result in lipid peroxidation and DNA mutation that leading

to several diseases as cancer, atherosclerosis, cardiovascular diseases, ageing and inflammatory diseases. Thus a potent scavenger of these species may serve as a possible preventive intervention for free radical-mediated human diseases (Poljsak, 2011; Cui et al., 2012).

DNA is a sensitive bio-target for radical-mediated oxidative damage. Among ROS, hydroxyl radical ($^{\bullet}OH$), the most reactive radical formed in biological systems, can induce chemical modifications in DNA. It can attack DNA at its sugar (deoxyribose) or the base. Any damage to the DNA can result in changes in the encoded proteins that may lead to malfunctions or complete inactivation of the encoded proteins (Halliwell and Gutteridge 2007; Saha and Tamrakar, 2011). In the current study, $^{\bullet}OH$ are generated within a Fe^{3+}/ascorbate/EDTA/H_2O_2 system (Fenton reaction) eventually attack deoxyribose, degrading it into a series of fragments (malondialdehyde-like products). Quantification of these degradation products carried out by condensation with TBA upon heating, at low pH, yielding a pink chromogen. Whenever, $^{\bullet}OH$ scavenger is added to the reaction mixture, it competes with deoxyribose for $^{\bullet}OH$ radicals and consequently inhibit deoxyribose degradation (Giậo et al., 2008). The $^{\bullet}OH$ scavenging activity showed significant protection of deoxyribose that decreased in the order of BHA ≥ compound I ≥ compound II ≥ compound III with IC_{50} values of 20.3 ± 0.83, 21.0 ± 1.04, 22.6 ± 1.21 and 25.5 ± 1.19, respectively.

In addition, ROS can react readily with a wide range of biological substrates as protein, and lipid result in their oxidative deterioration (Cui et al., 2012). In this concept, the data in **Table 2** revealed the inhibitory effect of three newly synthesized pyrozolo-pyrimidine derivatives on protein oxidation (expressed as protein carbonyl), lipid peroxidation in different tissues (expressed as malondialdehyde, MDA) and xanthine oxidase in comparable to corresponding reference agent.

Table 2. Inhibitory effect of the three newly synthesized pyrozolo-pyrimidine derivatives on protein oxidation (expressed as protein carbonyl) lipid peroxidation and xanthine oxidase in comparable to reference agents.

Treatments	Concentration required for 50% inhibition (µg/mL)				
	Protein carbonyl	*Lipid peroxidation*			*Xanthine oxidase*
		Liver	*Kidney*	*Heart*	
Compound I	40.5 ± 1.56 [b]	43.5 ± 2.12 [b]	33.3 ± 1.33 [a]	27.7 ± 1.39 [a]	21.4 ± 0.90 [a]
Compound II	31.2 ± 0.69 [a]	48.6 ± 0.64 [b]	34.8 ± 0.74 [a]	26.9 ± 1.35 [a]	27.8 ± 1.40 [b]
Compound III	47.3 ± 1.35 [c]	66.1 ± 1.52 [c]	44.6 ± 1.39 [b]	35.5 ± 1.46 [b]	32.6 ± 1.25 [c]
Reference drug	27.5 ± 1.14 [a]	35.3 ± 2.02 [a]	35.6 ± 1.30 [a]	26.1 ± 1.79 [a]	18.7 ± 1.57 [a]

The data are representative of three experiments and expressed as mean ± S.E
Values within a column followed by different letters are significant different (P<0.05) by DMRT.
Compound I, II and III are that of no. 4, 6 and 7, respectively as represented in Scheme 1 and 2.

Inhibition of Fe^{2+}-Ascorbate-Induced Protein Oxidation

Protein oxidation is defined as covalent modification of a protein induced by ROS. Most types of protein oxidations are essentially irreversible, whereas, a few involving sulfur-containing amino acids are reversible. Protein carbonylation is widely used marker of protein oxidation. The oxidation of a number of protein amino acids particularly Arg, His, Lys, Pro, Thr and Trp give free carbonyl groups which may inhibit or alter their activities and increase susceptibility towards proteolytic attack (Basle´ et al., 2010; Saha and Tamrakar, 2011). Oxidative protein damages play a significant role in the aetiology and/or progression of several human diseases as aging, Alzheimer's disease, chronic lung disease, chronic renal failure and diabetes (Dalle-Donne et al., 2003; Cui et al., 2012). The results in **Table 2** revealed that the highest inhibition was detected in both BHA and compound II followed by compound I and II with IC_{50} values of 27.5 ± 1.14, 31.2 ± 0.69, 40.5 ± 1.56 and 47.3 ± 1.35 µg/mL, respectively.

Inhibition of Fe^{2+}/ascorbate-induced lipid peroxidation in liver, kidney and heart homogenates

Lipid peroxidation is an important mediator of pathophysiological events in liver, kidney and heart disorders. When lipids react with free radicals, they undergo per-oxidation to form lipid peroxides, which decompose to form numerous products mainly malondialdehyde (MDA). Therefore, MDA level indicated the extent of lipid peroxidation in biological samples was measured *in vitro*. The MDA level was estimated as TBARS. It was reported that liver,

kidney and heart have difference in polyunsaturated fatty acid content, so they different in their sensitivity and susceptibility to free radical oxidative degradation (Gutiérrez et al., 2010). α-Tocopherol (vitamin E) is the most important and widely used as natural, lipid-soluble, chain-breaking reference antioxidant agent. In the present study, lipid peroxidation was non-enzymaticaly induced by Fe^{2+}/ascorbate in rats liver, kidney and heart tissue homogenates. The obtained results in **Table 2** demonstrated that the ability of different treatments to inhibit the lipid peroxidation in liver was decreased in the order of α-tocopherol > compound I ≥ compound II > compound III with IC_{50} values of 35.3 ± 2.02, 43.5 ± 2.12, 48.6 ± 0.64 and 66.1 ± 1.52, respectively. As compared to α-tocopherol, both compounds I and II showed good protection for kidney and heart against lipid peroxidation, while compound III was less active.

Xanthine oxidase inhibitory activity

A xanthine oxidase inhibitor is any substance that inhibits the activity of xanthine oxidase (XOD), which is an enzyme that catalyzes the oxidation of hypoxanthine to xanthine and can further catalyze the oxidation of xanthine to uric acid (Soni et al., 2011). The accumulation of uric acid in the body is responsible for the formation of several diseases and thus it plays a vital role in producing hyperuricemia and gout (Umamaheswari et al., 2011). Pyrazolo[3,4-*d*]pyrimidine derivatives use as the core structure in many drugs, covering wide range of pharmacological applications such as allopurinol (1,5-dihyroxu-4*H*-pyrazolo [3,4-*d*] pyrimidin-4-one) which consider the only clinically available xanthine oxidase inhibitors for the treatment of hyperuricemia and gouty arthritis (Farghaly et al., 2011). As shown in **Table 2**, all the three newly synthesized pyrozolo-pyrimidine derivatives displayed a significant XOD inhibitory activity decreased in the order of allopurinol ≥ compound I > compound II > compound III with IC_{50} values of 18.7 ± 1.57, 21.4 ± 0.90, 27.8 ± 1.40 and 32.6 ± 1.25 µg/mL, respectively

Analgesic activity

The effect of three newly synthesized pyrozolo-pyrimidine derivatives on acetic induced writhing in mice was given in **Table 3**. The present study revealed that all the three compounds showed a significant analgesic effect (P<0.001) at both doses (1/10 and 1/20); they were able to reduced pain induced by acetic acid writhing responses in dose dependant manner as compared to positive control group (untreated group).

Table 3. Effect of the three newly synthesized pyrozolo-pyrimidine derivatives on acetic acid induced writhing in mice in comparable to aspirin

Treatment (mg / Kg)		No. of writhing (counts / 20 min)	% of Inhibition
Control		97.0 ± 1.10	--
Aspirin		29.0 ± 0.36***	70.1
Compound I	1\10	15.0 ± 0.36***	84.5
	1\20	43.7 ± 1.10***	54.9
Compound II	1\10	36.0 ± 0.36***	62.9
	1\20	39.0 ± 0.36***	57.9
Compound III	1\10	43.3 ± 0.76***	55.3
	1\20	43.7 ± 1.10***	54.9

Values are expressed as means ± S.E. (n=6) ***P < 0.001 VS. Control
Compound I, II and III are that of no. 4, 6 and 7, respectively as represented in Scheme 1 and 2.

Antipyretic activity

Yeast- induced pyrexia is called pathogenic fever (Tomazetti et al 2005). Antipyretics are the agents that reduce the elevated body temperature. Antipyretic activity is generally exhibited by the nonsteroidal anti-inflammatory drugs, due their inhibitory effect on prostaglandin biosynthesis in the central nervous system (Howard 1993). Antipyretic activity was observed up to 2 hours after administration of paracetamol and test compounds. As shown in **Table 4** the experimental rats exhibited a marked increase in rectal temperature at 18 hours after Brewer's Yeast injection. As paracetamol, the test compounds at dose of 150 mg/kg showing effective antipyretic activity after 30 minute compared with control.

Table 4. Effect of the three pyrozolo-pyrimidine derivatives on Brwer's Yeast- induced Pyrexia in rats in comparable to paracetamol

Groups	Initial	18 hrs after Yeast injection	30 min	60 min	90 min	120 min
Control	36.26 ± 0.02	36.77 ± 0.03	36.93 ± 0.04	37.06 ± 0.09	37.16 ± 0.01	37.23 ± 0.01
Compound I	36.26 ± 0.02	37.53 ± 0.08***	37.36 ± 0.02***	36.67 ± 0.02**	36.56 ± 0.02***	36.50 ± 0.04***
Compound II	36.25 ± 0.02	36.9 ± 0.04***	36.66 ± 0.04**	36.66 ± 0.02**	36.63 ± 0.02**	36.60 ± 0.04***
Compound III	36.23 ± 0.02	37.07 ± 0.02**	36.70 ± 0.04**	36.66 ± 0.02**	36,66 ± 0.02**	36.33 ± 0.02***
Paracetamol	36.27 ± 0.02	37.56 ± 0.08***	36.60 ± 0.08*	36.47 ± 0.04**	36.47 ± 0.04***	36.33 ± 0.02***
Values are expressed as means ± S.E. (n=6),*P<0.05, **P <0 .01, ***P < 0.001 vs control Compound I, II and III are that of no. 4, 6 and 7, respectively as represented in Scheme 1 and 2						

Anti-inflammatory

Inflammation is a complex reaction to injurious agents including microbes. It involves vascular responses such as activation and migration of leukocytes and systemic reactions (Ismail et al., 2006). The three newly synthesized pyrozolo-pyrimidine derivatives were screened for anti-inflammatory activity using yeast induced rat hind paw edema method. Anti-inflammatory activity of test compounds and reference drug (Diclofenac sodium) at different assessment times after yeast injection are shown in **Table 5**. The results revealed that all test compounds exhibited anti-inflammatory activity. Compound I was more effective in the inhibition of paw edema than diclofenac sodium during all experimental periods. After 2 hours, this activity was decreased in the order of compound I > diclofenac sodium > compound II ≥ Compound III.

Table 5. Effect of pyrozolo-pyrimidine derivatives on paw edema induced by yeast in rats

Groups	Paw volume (mL)				% of inhibition			
	30 min	60 min	90 min	120 min	30 min	60 min	90 min	120 min
Control	0.42 ± 0.001	0.44 ± 0.002	0.45 ± 0.003	0.48 ± 0.003	--	--	--	--
Compound I	0.40 ± 0.001***	0.38 ± 0.004***	0.37 ± 0.001***	0.36 ± 0.003***	4.80	13.6	17.7	25.0
Compound II	0.41 ± 0.001***	0.40 ± 0.003***	0.38 ± 0.002***	0.35 ± 0.002***	2.40	9.10	15.5	20.8
Compound III	0.43 ± 0.006*	0.42 ± 0.004***	0.40 ± 0.005***	0.38 ± 0.003***	---	4.50	11.1	20.8
Diclofenac sodium	0.41 ± 0.001***	0.40 ± 0.004***	0.38 ± 0.003***	0.37 ± 0.004***	2.40	9.10	15.5	22.9
Values are expressed as means ± S.E. (n=6) *P<0.05, ***P<0.001 Compound I, II and III are that of no. 4, 6 and 7, respectively as represented in Scheme 1 and 2								

Conclusion

Compound I exhibited a market antioxidant activity and potent inhibiton to protein oxidation, lipid peroxidation and XOD inhibition. Compound II showed moderate activities while compound III was less active. Furthermore, these three compounds revealed a remarkable analgesic activity in acetic acid-induced writhing response, appreciable antipyretic activity by Brewer's yeast-induced pyrexia and antiinflammatory activity reflected by their ability to reduce the Brewer's yeast-induced paw edema in comparable to reference drugs.

Corresponding Author:
Zeinab Yousef Ali (Ph D)
Department of Biochemistry, National Organization for Drug Control and Research , 35521 Giza, Egypt.
E-mail: zeinabyousef65@ymail.com
Fax: 002-02-35855582

References

1. Aliu YO, Nwude N. Veterinary pharmacology and toxicology Experiments. 1st edn., Baraka Press, and publishers Ltd Nigeria 1982, pp:104-110.
2. Antonelli A, Bocci G, La Motta C, Ferrari S, Fallahi P, Fioravanti A, Sartini S, Minuto M, Piaggi S, Corti A, Alì G, Berti P, Fontanini G, Danesi R, Da Settimo Fand Miccoli P. Novel pyrazolopyrimidine derivatives as tyrosine kinase inhibitors with antitumoral activity in vitro and in vivo in papillary dedifferentiated thyroid cancer. Endocrinology 2011;152(1): 332–335.
3. Baraldi PG, Tabrizi MA, Bovero A, Avitabile B, Preti D, Fruttarolo F. Recent developments in the field of A_{2A} and A_3 adenosine receptor antagonists. Eur J Med Chem 2003;38:367-382.
4. Basle´ E, Joubert N, Pucheault M. Protein chemical modification on endogenous amino acids. Chemistry & Biology 2010;17:213-227.
5. Blois MS. Antioxidant determinations by the use of a stable free radical, Nature 1958;181:1199-1200.
6. Cheng CC, Robins RK. Potential Purine Antagonists. VI. Synthesis of 1-Alkyl- and 1-Aryl-4-substituted Pyrazolo[3,4-d] pyrimidines. J Org Chem 1956; 21(11):1240–1256.
7. Chung SK, Osawa T, Kawakishi S. Hydroxyl radical scavenging effects of spices and scavengers from brown mustard (*Brassica nigra*). Bioscience Biotechnology Biochemistry 1997;61:118–123.
8. Cui H, Kong Y, Zhang H. Oxidative stress, mitochondrial dysfunction, and aging. Journal of Signal Transduction, Volume 2012 (2012), Article ID 646354, 13 pages. doi:10.1155/2012/ 646354.
9. Dalle-Donne I, Giustarini D, Colombo R, Rossi R, Milzani A. Protein carbonylation in human diseases. TRENDS in Molecular Medicine 2003; 9(4):169-176.
10. Farghaly TA, Abass IM, Abdalla MM, Mahgoub OA. Synthesis and pharma-cological activities of fused pyrimidinones. World J Chemistry 2011; 6(1): 08-18.
11. Fernandez J, Perez-Alvarez JA, Fernandez-Lopez JA. Thiobarbituric acid test monitoring lipid oxidation in meat. Food Chemistry 1997; 99:345-353.
12. Fontana M, Mosca L, Rosei MA. Interaction of enkephalines with oxyradicals. Biochem Pharmacol 2001;61:1253-1257.
13. Garratt DC. The quantitative analysis of Drugs. Volume 3. Chapman and Hall ltd, Japan 1964:456-458.
14. Giậo MS, Sanjose G, Muniz P, Perez R, Kosinska M, Pintado ME, Malcata FX. Protection of deoxyribose and DNA from degradation by using aqueous extracts of several wild plants. J Sci Food Agric 2008; 88:633–640.
15. Gutiérrez AM, Gavazza M, Marmunti M, Palacios A. Non-enzymatic lipid peroxidation from liver, heart and kidney of chinchilla and rat. InVet. 2010;12(1): 43-51.
16. Halliwell B, Gutteridge JMC. Free radicals in biology and medicine, 4th ed., Oxford: Clarendon Press, 2007.
17. Hore SK. Dumka VK, Tandan SK, Tripathi HC, KumarD. Modification of Brewer's yeast-hiduced inflammation and nocicepetion in rats centrally administered prostaglandins and their inhibitors. Indian Journal of Pharmacology 1997;29:416-419.
18. Kong LD, Cai Y, Huang WW, Cheng CH and Tan RX. Inhibition of xanthine oxidase by some Chinese medicinal plants used to treat gout. J.Ethnophar 2000;73:199-207.
19. Howard, M. Fever and consequences. Neurosci. Biobehhav 1993;17(3):237-269.
20. Ismail MM, Ghorab MM, Noaman E, Ammar YA, Heiba HI, Sayed MY. Arzneimittel Forschung. Drug Research, 2006, 301.
21. Lauria A, Bruno M, Diana P, Barraja P, Montalbane A, Cirrincione G. Annelated pyrrolo-pyrimidines from amino-cyanopyrroles and BMMAs as leads for new DNA-interactive ring systems. Bioorg Med Chem 2005;13:1545-53.
22. Levine RL, Williams JA, Stadman ER, Shacter E. Carbonyl assay for determination of oxidatively modified proteins. Methods Enzymol. 1994;233:346-57.
23. Loux JJ, Depalma, Yankell SL. Antipyretic testing of asprin in rats. Toxicol. Appl. Pharmacol 1972;22: 672-675.
24. Poljsak B. Strategies for reducing or preventing the generation of oxidative stress. Oxidative Medicine and Cellular Longevity, vol. 2011, article ID 194586, 15 pages, 2011. doi:10.1155/2011/194586
25. Radi S, Salhi S; Radi A. Synthesis and preliminary biological activity of some new pyrazole derivatives as acvclonucleoside analogues. Letters in Drug Design & Discovery 2010;7:27-30.
26. Rajendran A, Raghupathy D, Priyadarshini M. Green synthesis of biologically active

pyrazolopyrimidine derivatives using an ionic liquid 2-methyl-3-butylimidazolium chloride. Int J Chem Tech Research 2011; 3(1):293-297.
27. Rice KD, Kim MH, Bussenius J, Anand NK, Blazey CM, Bowles OJ, Canne-Bannen L, Chan DS, Chen B, Co EW, Costanzo S, Defina SC, Dubenko L, Engst S, Franzini M, Huang P, Jammalamadaka V, Khoury RG, Klein RR, Laird AD, Le DT, Mac MB. Pyrazolopyrimidines as dual Akt/p70S6K inhibitors. Bioorg Med Chem Lett 2012; 22(8):2693-2697.
28. Ruch RJ, Cheng SJ, Klaunig JE. Prevention of cytotoxicity and inhibition of intercellular communication by antioxidant catechins isolated from Chinese green tea. Carcinogenesis 1989;10:1003-1008.
29. Saha D, Tamrakar A. Xenobiotics, oxidative stress and free radicals vs. antioxidants: dance of death to heaven's life. Asian J Res Pharm Sci 2011;1(2):36-38.
30. Shaikh BM, Konda SG, Chobe SS, Mandawad GG, Yemul OS, Dawane BS. PEG-400: prompted eco-friendly synthesis of some novel pyrazolo [1, 5-a] pyrimidine derivatives and their in vitro antimicrobial evaluation. J. Chem. Pharm. Res., 2011, 3(2):435-443.

1/26/2012

31. Singh S, Sharma PK, Dudhe R, Kumar N. A review: Pyrazolopyrimidine moiety. J Pharma science monitor, 2011;0976-7908:1224-1237.
32. Soni JP, Parmar DR, Sen DJ. Febuxostat: The New Generation novel xanthine oxidase Inhibitors. Internationale Pharmaceutica Sciencia 2011;1(1):107-115.
33. Taylor EC, McKillop A, Warrener RN. Heterocyclic syntheses from o-aminonitriles-XXVII: A one-step synthesis of fused pyrimidine dithiones. Tetrahedron 1967;23(2): 891-896.
34. Tomazetti J, Avila DL, Ferreria AP, Martins JS, Souza FR, Royer C. Baker yeast- induced fever in young rats: Characterization and validation on an animal model for antipyretics screening. J. Neurosci Methods 2005;147:29-35.
35. Turner RA. Screening methods in pharma-cology. New York, London, Academic Press. 1965;PP. 113.
36. Umamaheswari M, Madeswaran A, Asokkumar K, Sivashanmugam T, Subhadradevi V, Jagannath P. Discovery of potential xanthine oxidase inhibitors using in silico docking studies. Der Pharma Chemica, 2011;3(5):240-247