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Synthesis of Some Novel Pyrazolo[3,4-*d*] pyrimidine Derivatives and Evaluation of their *In vitro* Antiproliferative and Antioxidant Activity

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ABSTRACT

*Novel substituted pyrazolo[3,4-d]pyrimidines **4-27** were synthesized starting with 1-substituted-5-amino-1H-pyrazole-4-carbonitrile **1**. Some of the prepared compounds were tested for their anticancer as well as antioxidant activities. As anticancer agents, the tested compounds were evaluated for their antiproliferative potency and their effects on the free-radical-metabolizing enzymes including superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH-Px). The levels of the oxidative stress parameters including hydrogen peroxide (H_2O_2), nitric oxide (NO) and reduced glutathione (GSH) were also investigated in vitro on human breast (MCF-7), liver (HepG2) and lung (A549) cancer cell lines comparing with the reference anticancer drug cisplatin. The antioxidant ability of the tested compounds was measured by evaluating their radical scavenging ability (RSA%) using DPPH and ABTS radical scavenging assays. Results indicated that most of the compounds exhibited remarkable cytotoxicity activity against MCF-7 and A549 cell lines. For MCF-7 cell line, the IC_{50} of N-[(9-methyl-5,6-dihydronaphtho[1',2':4,5]thieno[2,3-d]pyrimidin-11-yl)]-1H-pyrazolo[3,4-d]pyrimidin-4-yl)ethane-1,2-diamine **11** (IC_{50}: 3.60 µg/mL) was found to be more potent and efficacious than cisplatin (IC50: 4.70 µg/mL). The value of A-549 cell line IC_{50} of 1-(9-methyl-5,6-dihydronaphtho[1',2':4,5]thieno[2,3-d]pyrimidin-11-yl)-6-phenyl-1,5,-dihydropyrazolo[3,4-d]pyrimidin-4-one **22** (IC_{50} 4.80 µg/mL) is close to that of cisplatin (IC_{50}: 3.65 µg/mL). The antitumor activity of these compounds was accompanied with high activity of SOD with subsequent increase in H_2O_2 and NO production. All the selected compounds gave positive RSA % either by DPPH or ABTS assays.*

Keywords: Synthesis, Pyrazolo[3,4-*d*]pyrimidines, *N*-Acyclonucleosides, Antiproliferative, Antioxidant activity.

INTRODUCTION

In continuation of our previous work concerning designing novel chemotherapeutic agents [1-4], we report here the synthesis, anticancer effects, toxicity and antioxidant evaluation of some newly pyrazolo[3,4-*d*]pyrimidine derivatives.

The interest in the synthesis of pyrazolo[3,4-*d*]pyrimidine ring system incorporating different functionalities, is due to their versatile pharmacological activities. These activities were correlated to their similarities with purines [5,6]. Many of these derivatives were reported to show a broad spectrum of biological activities such as antimicrobial [7,8], antiviral [9,10], antiinflammatory [11], antioxidant [12], xanthine oxidase inhibitor activities [13]. Moreover, many pyrazolo[3,4-*d*]pyrimidines contributed to the quest for an ultimate antitumor chemotherapeutic agents [14-

17] especially when heterocyclic moieties which possess antitumor activity, such as thienopyrimidine [18], are incorporated into the pyrazolopyrimidine nucleus.

Although there have been great advances in the detection and treatment of cancer, it remains one of the greatest medical challenges, with the incidence of some malignancies continuing to increase [19]. For many tumor types, established treatments such as cytotoxic chemotherapy and radiotherapy provide only transient therapeutic benefits despite severe side effects [20]. Therefore, the need for better treatments has stimulated research to develop new efficient chemotherapeutic agents for management of cancer.

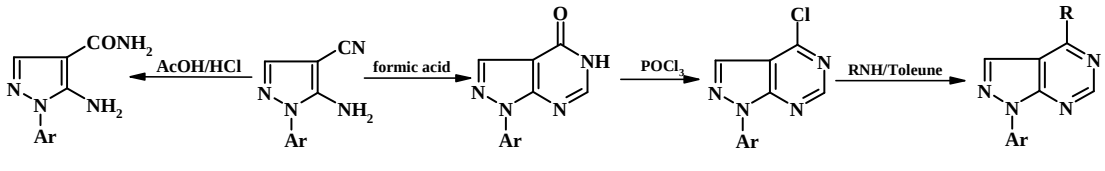
The balance of reactive oxygen species (ROS) formation and antioxidative defense level is crucial to cell survival and growth [21]. On the contrary, disturbance of this balance can produce oxidative stress. This state of oxidative stress can result in injury to all the important cellular components like proteins, DNA and membrane lipids which can cause cell death [22,23]. Tumor cells have higher levels of ROS and are more frequently deficient in most crucial antioxidative enzymes than normal cells and are therefore more vulnerable to the additional oxidative stress [24, 25]. Accordingly, a unique antitumor strategy named "oxidative therapy" was developed by delivering excess oxidative stress or disrupting antioxidative defense system in cancer cells [26]. Many conventional anticancer drugs like camptothecin, doxorubicin, cisplatin and anthracyclines exhibit antitumor activity by generating ROS [27]. Nitric oxide is an endogenous free radical species that is produced from *L*-arginine by nitric oxide synthase (NOS), a family of ubiquitous enzymes [28]. Recent evidence indicates that NO may interact with transformed, cell-derived superoxide anions and thereby generates the apoptosis inducer peroxynitrite.

Hence, the aim of the present work was to elucidate the mechanism by which the prepared compounds exert their anticancer effect on different cell lines and to examine their antiradical activity using different antiradical assays.

RESULTS AND DISCUSSION

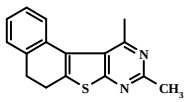
2.1. Chemistry

In continuation of our previous work in preparing various cyclic [29] and acyclic nucleosides [30] of different heterocyclic compounds, also considering the importance of pyrazolo[3,4-*d*]pyrimidine derivatives, we describe herein the preparation of some novel functionalized pyrazolo[3,4-*d*]pyrimidine and some of its corresponding *N*-nucleoside analogs. Thus, treatment of 1-substituted 5-aminopyrazole-4-carbonitrile **1** [31] with formic acid afforded pyrazolo[3,4-*d*]pyrimidin-4-one derivative **2** [32]. Heating of compound **2** in phosphorus oxychloride, gave the corresponding 4-chloropyrazolo[3,4-*d*]pyrimidine derivative **3** [29] as a key compound for further reactions. So, the reactivity of compound **3** towards *N*-nucleophiles was investigated. When compound **3** was refluxed with different amines (hydrazine hydrate, ethylamine, phenylethylamine, benzylamine, glucosamine hydrochloride, glycine, phenylhydrazine, ethylenediamine, piperidine, cyclohexylamine, morpholine or piperazine), the reaction afforded the corresponding 4-substituted amino pyrazolo[3,4-*d*]pyrimidine **4-15**, respectively (Scheme 1). The proposed structures of these compounds were elucidated on the basis of analytical and spectral data (*cf.* experimental).



R: 4, NH-NH_2; 5, NH-CH_2CH_3; 6, NH-CH_2CH_2Ph; 7, NH-CH_2Ph; 8, NH-Glucosyl; 9, NH-CH_2COOH; 10, NH-NHPh; 11, NH-$CH_2CH_2NH_2$; 12, NPiperidinyl; 13, NH-Cyclohexyl; 14, NMorpholinyl; 15, NPiperazinyl

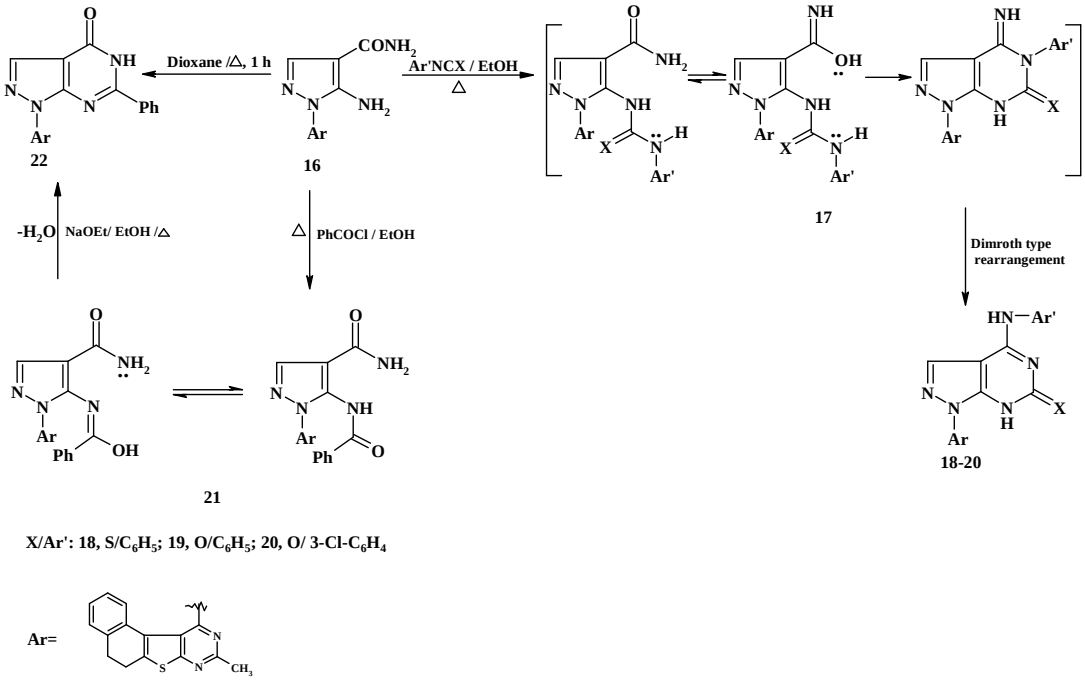
Ar=



Scheme 1: Synthesis of compounds 4-16

On the other hand, the reaction of 5-amino-1-(5,6-dihydronaphtho-[1',2':4,5]thieno[2,3-*d*]pyrimidin-11-yl)-1*H*-pyrazole-4-carbonitrile (**1)** [33] with glacial acetic acid and hydrochloric acid (2:1) gave 5-amino-1-(9-methyl-5,6-dihydronaphtho[1′,2′:4,5]thieno[2,3-*d*]pyrimidin-11-yl)-1*H*-pyrazole-4-carboxylic acid amide **16** which was used for further reactions. Treatment of compound **16** with phenyl isothiocyanate, phenyl isocyanate or 3-chlorophenyl

isocyanate afforded the cyclized 4-substituted amino-pyrazolo[3,4-*d*]pyrimidine-6-thiones **18-20** and not the imino derivatives which presumably were formed as intermediates **17** and then isomerized under reaction conditions by Dimroth rearrangement to give the corresponding more stable products **18-20**. The ^{1}H NMR spectra of compound **20** revealed the presence of two singlet peaks for 2 NH at δ12.35 and 4.14 ppm and ^{13}C NMR spectrum of **20** revealed the presence of C=O group at δ153.51 and the absence of the C=NH absorption which confirms structure **20**. The IR spectrum of compound **18** showed characteristic absorption bands at δ 3210.9, 3116.4 and 1030.77 corresponding to two –NH groups and C=S, respectively and its ^{1}H NMR spectrum showed signals exchangeable with D_2O at δ 4.47 and 11.00 ppm for 2 NH protons (*cf*. experimental).



Scheme 2: Synthesis of compounds 18-22

Also, when compound **16** was treated with benzoyl chloride in ethanol it gave product **21** which upon treatment with sodium ethoxide in ethanol under reflux it gave 1-(9-methyl-5,6-dihydronaphtho[1',2':4,5]thieno[2,3-*d*]pyrimidin-11-yl)-6-phenyl-1,5,-dihydro-pyrazolo[3,4-*d*]pyrimidin-4-one (**22**). Also, compound **22** was obtained directly by refluxing of compound **16** with benzoyl chloride in dioxane. When compound **22** was treated with epichlorohydrin at room temperature it gave 5-(3-Chloro-2-hydroxypropyl)-1-(9-methyl-5,6-dihydronaphtho-[1',2':4,5]thieno[2,3-d]pyrimidin-11-yl)-6-phenyl-1,5-dihydro-4*H*-pyrazolo[3,4-*d*]pyrimidin-4-one 2**3.** Also, when compound **22** was treated with chloroethanol, chloracetaldehyde dimethyl acetal, chloroethyl methyl ether and 2-(2-chloroethoxy)ethanol it afforded the corresponding *N*-acyclonucleosides **24-27**, respectively (Scheme 3). The IR spectra of the latter compounds revealed the presence of the C=O absorption band and the absence of the N-H absorption band for each compound, also the ^{1}HNMR spectra indicated the absence of the NH signals and the presence of hydroxyethyl, dimethoxyethyl, methoxyethyl and hydroxymethoxy ethyl signals, respectively (*cf.* experimental).

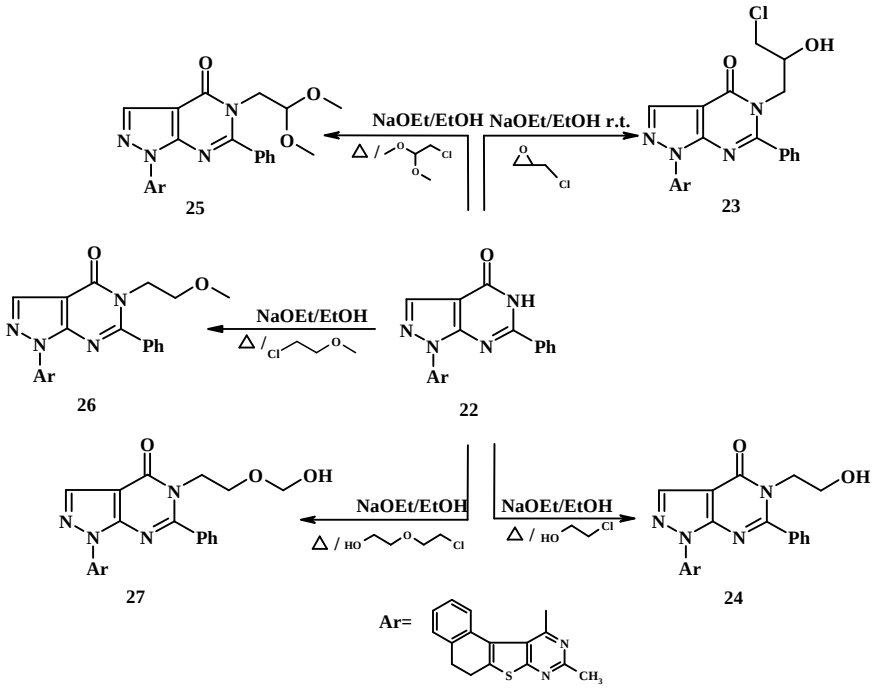
2.2. Biological activity

2.2.1. *In vitro* antiproliferative activity

The antiproliferative activity of the tested compounds was evaluated against human breast MCF-7, liver HepG2 and lung A549 cancer cell lines using Sulforhodamie B (SRB) colorimetric assay in comparison with cisplatin as reference drug.

The antiproliferative activities were expressed by median growth inhibitory concentration (IC_{50}) and provided in Table 1. From the results it is evident that although all the compounds showed no anticancer activity against HepG2 cells, four compounds displayed potent growth inhibitory activity against human breast cancer cell line MCF-7. In addition, four compounds displayed potent growth inhibitory activity against human lung cancer cell line A549 (Table 1).

Both MCF-7 and A459 cell lines showed a normal growth in our culture system. DMSO did not seem to have any noticeable effect on cellular growth. It was observed that there was gradual decrease in the viability of cancer cells with increasing the concentration of the tested compounds in a dose-dependent inhibitory effect.
The IC_{50} of the prepared compounds **(6, 10, 11 and 7)** for MCF-7 cell line were 6.80, 10.40, 3.60 and 7.00 µg/mL respectively, in particular compound **11** (IC_{50}: 3.60 µg/mL) was found to be more potent and efficacious compared to the reference drug, cisplatin (IC_{50}: 4.70 µg/mL). Similarly, the IC_{50} of the prepared compounds **(19, 81, 22 and 20)** for A459 cell line was 6.40, 7.20, 4.80 and 7.40 µg/mL respectively, it is clear that IC_{50} of compound **22** (IC_{50}: 4.80 µg/mL) is close to the value of the cisplatin (IC_{50}: 3.65 µg/mL).



Scheme 3: Synthesis of compounds 23-27

Table 1: Cytotoxicity (IC_{50}, µg/mL) of the tested compounds on human breast MCF-7, liver HepG2 and lung A549 cancer cell lines using Sulforhodamie B (SRB) colorimetric assay.

Compound	*Cell line*		
	MCF-7	**HepG2**	**A549**
Cisplatin	4.70	4.20	3.65
6	6.80	N.A.	N.A.
7	7.00	N.A.	N.A.
10	10.40	N.A.	N.A.
11	3.60	N.A.	N.A.
18	N.A.	N.A.	7.20
19	N.A.	N.A.	6.40
20	N.A.	N.A.	7.40
22	N.A.	N.A.	4.80

Values are mean ±S.E (n = 3).; N.A. = no activity.

Biochemical assays
To elucidate the mechanism by which the tested compounds exert their antitumor activities, we estimated the activities of the free-radical-metabolizing enzymes including superoxide dismutase (SOD), catalyase (CAT) and glutathione peroxidase (GSH-Px) as well as the levels of the oxidative stress parameters including hydrogen peroxide (H_2O_2), nitric oxide (NO) and reduced glutathione (GSH) in the cancer cells treated with tested compounds. Additionally, the effect of these compounds on the levels of total protein and nucleic acids was determined.

As shown in Table 2, in general treatment of the cells with different compounds (at the 1/10 of IC_{50} values) or cisplatin [34] resulted in a significant increase in the activity of SOD and level of H_2O_2 higher than those of control, accompanied with a significant decrease in the activity of CAT and GSH-Px, and depletion in GSH level, indicating an increase in the cellular levels of reactive oxygen species. This means that the antitumor activity of these

compounds was accompanied with high activity of SOD with subsequent increase in H_2O_2 production. The produced H_2O_2 should be rapidly removed through the activation of CAT and GSH-Px. The present results showed that activities of CAT and GSH-Px and the level of reduced GSH were lowered in groups treated with the prepared compounds compared to control cells. Consequently, the excess H_2O_2 produced in tumor cells with the compounds can not be removed. In other words, the accumulation of H_2O_2 and other free radicals in tumor cells should be partly the cause of tumor cell killing.

It should be mentioned that, the changes in the activities of the free-radical-metabolizing enzymes and oxidative stress parameters were in the order of compound **11** > **cisplatin** > **6** > **7** > **10** for MCF-7 and in case of A549 cell line the order was **cisplatin** > **22** > **19** > **18** > **20** which is in accordance with the order of antiproliferative activity of the tested compounds in both cell lines. The highest activity was found for the most potent antitumor compound **11** in MCF-7 and compound **22** in A549 cell lines, which resulted in the highest SOD activity and H_2O_2 and low activities of CAT and GSH-Px as well as GSH level than the other tested compounds (Table 2). The consistency between antiproliferative activity and biochemical assay results indicated that the antitumor effect of the present compounds may be exerted at least partly by production of ROS.

Moreover, results in Table 3 illustrated that, treatment of both MCF-7 and A549 cells with these compounds led to significant increase in the level of NO. These results were in accordance with that reported by Schepetkin and Quinn [35] who mentioned that polysaccharides isolated from algae have been reported to modify macrophage activity by inducing the production of cytokines and nitric oxide. There is a growing body of evidence indicating that NO is able to induce apoptosis by helping to dissipate the membrane potential of mitochondria and therefore make it more permeable [36]. In addition, the elevated level of NO was accompanied with depletion in the levels of total protein and nucleic acids compared to control. This can be explained by several cytotoxic effects that include reaction of NO with proteins and nucleic acids. The main targets of NO in proteins are the thiol group [37] and iron of active sites [38]. In the nucleus, NO has been shown to cause gene mutation [39], to inhibit DNA repair enzymes [40], and to mediate DNA strand breaks [41].

Moreover, Bienvenu *et al.* [42] reported that most chemotherapeutic agents cause cells to over generate ROS and thus, are capable of inducing apoptosis, and causing oxidative damage to DNA and proteins. The cascade of signals mediating apoptosis often involves a ROS intermediate messenger, and ROS can short circuit the pathway, bypassing the need for upstream signals for cell suicide. Latter, Huang *et al.* [43] reported that regulation of free radical-producing agents may also have important clinical applications. This mechanism for the effects of ROS generating anticancer agents is only beginning to be understood, as previously the mechanism of most anticancer agents was believed to be due mainly to direct interaction with DNA and interference with DNA regulatory machinery (*e.g.*, topoisomerases and helicases) and to the initiation of DNA damage *via* production of ROS [44].

Table 2. Effect of treatment with the prepared compounds on the activities of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px), as well as the levels of reduced glutathione (GSH) and hydrogen peroxide (H_2O_2) in MCF-7and A549 treated cells.

Cell lines	*Compounds*	*SOD U/mg Protein*	*CAT U/mg protein*	*GSH-Px U/mg protein*	*GSH nmol/mg protein*	*H_2O_2 nmol/mg protein*
MCF-7	**Control (DMSO)**	35.00±3. 80	7.60±0.70	9.30±1.00	40.00±5.00	15.70±1.60
	Cisplatin	135.0±13.80 [a]	2.96±0.22 [a]	4.20±0.40 [a]	18.60±2.00 [a]	65.50±7.30 [a]
	6	130.00±13.30 [a]	2.60±0.30[a]	4.80±0.60[a]	22. 50±2.60[a]	55.40±5.65[a]
	7	110.00±12.00 [a]	3.20±0.28 [a]	5.20±0.60 [a]	26. 80±2.90[a,b]	43.30±4.80 [a,b]
	10	85.20±8.60[a,b]	5.20±0.50 [a]	7.20±0.80 [a]	30. 50±3.30[a,b]	37.50±4.00 [a,b]
	11	140.0±14.50 [a]	2.26±0.22 [a]	3.30±0.38 [a]	17. 80±0.20[a]	77.50±7.80 [a]
A549	**Control (DMSO)**	39.70±4.60	8.40±0.80	13.20±1.30	42.60±5.00	14.50±1.50
	Cisplatin	220.00±24.00 [a]	2.30±0.16 [a]	4.20±0.38 [a]	13.60±1.70 [a]	65.80±6.70 [a]
	18	90.60±8.70[a,b]	4.20±0.460 [a,b]	6.50±0.60[a]	20.50±2.20 [a,b]	52.00±5.00 [a,b]
	19	120.00±13.20[a,b]	3.85±0.40 [a]	5.90±0.60[a]	18.20±1.90[a]	55.20±6.10[a,b]
	20	85.60±8.20[a,b]	4.60±0.47 [a,b]	7.00±0.80 [a,b]	26.50±2.50 [a,b]	36.80±3.60 [a,b]
	22	180.00±12.20[a,b]	3.30±0.36[a,b]	5.20±0.78 [a]	16. 80±1.80[a]	60.20±5.80[a]

Data are expressed as means ±S.E. of three separate experiments. [a] and [b] is significant difference from control and cisplatin groups respectively at ($p < 0.05$).

Table 3. Effect of the compounds on the level of total protein, nucleic acids (RNA and DNA) and nitric oxide (NO) in MCF-7 and A549 treated cells.

Cell lines	*Compounds*	*Protein ($\mu g/10^6$ cells)*	*RNA ($\mu g/10^6$ cells)*	*DNA ($\mu g/10^6$ cells)*	*NO (µmol/mg protein)*
MCF-7	**Control (DMSO)**	130.50 ± 12.80	19.60 ± 2.00	12.60 ± 1.40	2.85 ± 0.29
	Cisplatin	45.70 ± 4.25[a]	4.90 ± 0.47[a]	3.70 ± 0.32[a]	7.20 ± 0.70[a]
	6	48.60 ± 5.00[a]	5.30 ± 0.58[a]	4.90 ± 0.56[a]	6.60 ± 0.70[a]
	7	54.40 ± 6.00[a]	6.20 ± 0.66[a]	5.70 ± 0.62[a]	5.00 ± 0.55[a,b]
	10	70.00 ± 7.40[a,b]	7.80 ± 0.85[a]	6.30 ± 0.66[a,b]	4.80 ± 0.50[a,b]
	11	40.60 ± 4.00[a]	4.00 ± 0.36[a]	3.00 ± 0.35[a]	7.40 ± 0.80[a]
A549	**Control (DMSO)**	170.50 ± 16.00	22.60 ± 2.00	14.50 ± 1.50	4.20 ± 0.46
	Cisplatin	40.30 ± 6.80[a]	7.20 ± 0.65[a]	5.00 ± 0.48[a]	8.80 ± 0.90[a]
	18	60.60 ± 7.20[a,b]	12.60 ± 1.20[a,b]	7.60 ± 0.80[a,b]	6.60 ± 0.70[a,b]
	19	55.60 ± 6.00[a,b]	10.20 ± 1.28[a,b]	6.80 ± 0.70[a,b]	7.20 ± 0.80[a]
	20	60.80 ± 7.50[a,b]	12.80 ± 1.30[a,b]	9.30 ± 0.90[a,b]	6.50 ± 0.63[a,b]
	22	50.30 ± 5.20[a]	8.00 ± 0.86[a,b]	5.30 ± 0.60[a]	8.10 ± 0.80[a]

The values are expressed as mean ±SE of three sepa rate experiments.[a] and [b] is significant difference from control and cisplatin groups respectively at ($p < 0.05$).

2.2.2. *In vitro* antioxidant activity

As shown in Figure 1 all samples gave more or less antioxidant capacity (represented by radical scavenging ability; RSA%) either for $DPPH^{\bullet}$ or $ABTS^{\bullet}$.

Regarding DPPH RSA%, although all tested compounds revealing positive antiradical activity, compound **6** gave the highest RSA% (39.16%) which represented about 68% relative to that of TBHQ (RSA_{TBHQ} = 56.83 %), whereas **19** showed the least RSA% (12.64%) that represented 22.24% that of TBHQ (Table 4).

For ABTS RSA%, again all tested compounds showed positive radical scavenging ability with **6** revealing the highest value (RSA% = 50%). RSA% of **6** was higher than that of Trolox (RSA_{Trolox}% = 49.66%, Table 5).

According to Surveswaran *et. al.* [45], it is not surprising to find differences in antioxidant activity among the different assays, as each has a different mechanism of action and different reaction conditions. ABTS*-assay is more preferable because it is soluble in both aqueous and organic solvents and so can determine both hydrophilic and lipophilic antioxidant capacities [46].

Table 4. Radical scavenging activity measured by DPPH and ABTS

Sample	*Assay Type*	
	ABTS RSA%	**DPPH RSA%**
6	50	39.16
7	22.61	18.17
10	44.2	32.00
11	31.66	25.85
18	37.12	16.47
19	5.97	12.46
20	27.13	24.40
22	1.02	16.13
Trolox	49.66	-
TBHQ	-	56.83

DISCUSSION AND CONCLUSION

In conclusion, the present results suggest that some of the tested compounds possess significant antitumor activity against human breast MCF-7 cancer cell line **(**compounds **6, 10, 11** and **7)** and human A549 lung cancer cell line **(**compounds **19, 18, 22** and **20)** comparable to the activity of commonly used anticancer drug, cisplatin, where compound **11** was more potent than cisplatin in MCF-7 cells while compound **22** revealed the highest anticancer activity in A549 reach near the cisplatin effect and they exert their antitumor activities by modulating free radicals production by increasing the activity of superoxide dismutase and depletion of intracellular reduced glutathione level, catalase, glutathione peroxidase activities, accompanied with highly production of hydrogen peroxide, nitric oxide and other free radicals causing tumor cells death, as monitoring by reduction in the synthesis of protein and nucleic acids.

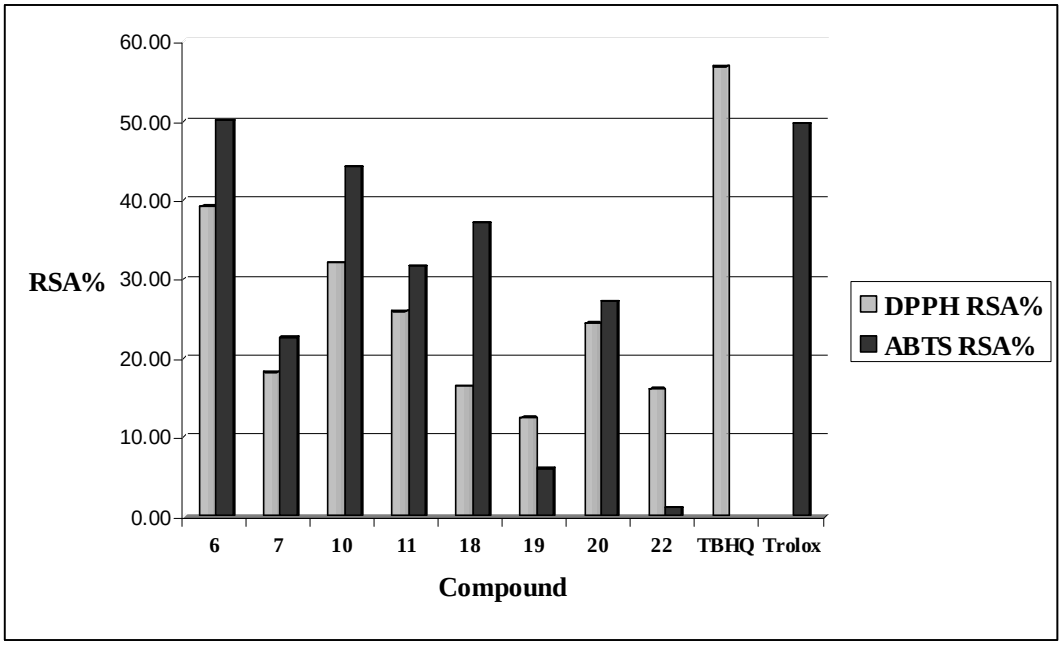


Figure 1: Radical Scavenging Activity measured by DPPH and ATBS

The antioxidant activity of the tested compounds (measured by DPPH and ABTS radical assays), on the other hand, revealed moderate to weak antioxidant activity of either compound **11** or compound **22** (RSA11= 25.85%, and 31.66%; and RSA22= 16.13 % and 1.02% for DPPH and ABTS, respectively). Interestingly, although these two compounds (**11** and **22**) revealed strong anticancer activity on MCF-7 and A549 cancer cell lines and increasing cellular reactive oxygen species (H_2O_2 and NO) but the antiradical activity of both compounds **11** and **22** was demonstrated extracellulary when it was measured by the DPPH and ABTS. This observation could be interpreted in two ways. The first is by postulating that these compounds may act by different mechanisms on the intracellular level by disturbing the antioxidant systems inside the cell to enhance radical formation which may lead to apoptosis. In agreement with this postulation, Sawicka *et al.* [47] reported that although KP972 (pyridopyrazolopyrimidine derivative) was able to decrease lipid peroxidation in erythrocytes and cisplatin showed quite the opposite effect, however, the final antiproliferative effects of the two compounds were comparable. Second, it is well known for scientists interesting in antioxidants that many antioxidants exert a paradox activity (anti-oxidation or pro-oxidation) depending on either the concentration of the antioxidant itself, the presence or absence of certain metals or co-antioxidant under conditions of oxidative stress [48, 49]. Sawicka *et al*. [47] reported that cisplatin in spite of enhancing hydroxyl radical formation from 1-50 μg/mL, but at concentration 60-70 μg/mL it behaved paradoxically and decreased hydroxyl radical formation. Moreover, the complex multistage process of carcinogenesis, in which oxidative processes may play variable and incompletely understood roles, dramatically complicates the general assessment of antioxidant benefit [50].

Based on the rich scientific literature concerning cisplatin and the present work, it can be concluded that the participation of free radicals in the toxicity of cisplatin is very significant [47]. Thus it can be supposed that the prepared compounds and especially compounds **11** and **22** might be less toxic than the reference drug cisplatin. The proradical action of cisplatin results in its harmful side effects [47].

Our results encourage and leave the door open for further research regarding new non-toxic active derivatives for chemical compounds in cancer therapy with more pronounced mechanism of action.

MATERIALS AND METHODS

3. Experimental

3.1. Chemistry

All melting points are uncorrected and measured using Electrothermal IA 9100 apparatus (Shimadzu, Japan). IR spectra were recorded as potassium bromide pellets on a Perkin-Elmer IRFT 1650 spectrophotometer, National Research Center, Cairo, Egypt. The ^{1}H NMR and ^{13}C NMR spectra were determined on a Jeol-Ex-500 NMR spectrometer (National Research Center, Cairo, Egypt.), or Jeol-Ex-400 NMR spectrometer (Germany) or Jeol-Ex-

300 NMR spectrometer (Faculty of Science, Cairo University, Cairo, Egypt.). All chemical shifts (δ values) were expressed as part per million (ppm) against TMS as internal reference, and the coupling constants *J* are in Hertz. Mass spectra were recorded on EI Q1 MSLMR UPLR, National Research Center, Cairo, Egypt. Microanalyses were operated using Mario Elmentar apparatus, Organic Microanalysis Unit, National Research Center, Cairo, Egypt. Follow up of the reactions and checking the purity of the compounds were made by TLC on silica gel pre-coated aluminum sheets (Type 60 F254, Merck, Darmstadt, Germany). Compounds **1** [31], **2** [32] and **3** [29] were prepared by literature procedures.

*1-(9-Methyl-5,6-dihydronaphtho[1',2':4,5]thieno[2,3-d]-pyrimidin-11-yl)-1H-pyrazolo[3,4-d]pyrimidin-4-yl-hydrazine **(4)***

A mixture of compound **3** (0.01 mol) and hydrazine hydrate (2 ml, 99%) was refluxed in dry ethanol (20 mL) for 5 h. The reaction mixture was poured onto crushed ice and the formed precipitate was filtered off, washed several times with water, dried and recrystallized from ethanol to give compound **4**.

Pale grey powder; (yield 82%); m.p. 159-160^{o}C; IR (KBr) υ cm^{-1}: 3300, 3191, 3119 (NH_2&NH); ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm: 2.50 (s, 3H, C***H*****$_3$**), 2.70-3.10 (m, 4H, 2C***H*****$_2$**), 4.10 (s, 2H, N***H*****$_2$**, D_2O exchangeable), 6.50 (s, 1H, N***H***, D_2O exchangeable), 7.10-7.40 (m, 4H, 3Ar-***H***+pyrazole-***H***), 8.21 (s, 1H, pyrimidine-***H***), 8.50 (d, 1H, , *J* = 8 Hz, Ar-***H***); ^{13}C NMR (400 MHz, DMSO-d_6) δ ppm: 19.58 (CH_3), 23.37 (CH_2-C5'), 29.07 (CH_2-C6'), 118.30, 126.20, 126.54, 126.60, 126.98, 127.23, 127.36, 127.74, 127.87, 128.36, 130.46, 130.95, 134.39, 135.13, 154.77, 158.11, 164.62 (17 sp2 carbon atoms); MS, *m/z* (%): 401.00 (M^++1, 20.35), 100.00 (M^+, 16.15), 80.00 (100.00); Anal. calcd. for $C_{20}H_{16}N_8S$ (400.46) (%): C, 59.98; H, 4.03; N, 27.98; S, 8.01. Found (%): C, 59.83; H, 4.14; N, 28.10; S, 7.87.

*General procedure for the preparation of compounds **5-15***

A solution of compound **3** (0.02 mol) in dry toluene (20 mL) was treated with equimolar amount (0.02 mol) of different amines (ethylamine, phenylethylamine, benzylamine, glucosamin hydrochloride, glycine, phenylhydrazine, ethylenediamine, piperidine, cyclohexylamine, morpholine or piperazine). The reaction mixtures were refluxed for 3-5 h, and the reaction was monitored with TLC [chloroform/ methanol (9:1)] till the reaction was completed then the product was filtered off while hot, washed several times with hot water and ethanol, air dried and recrystallized from dioxane to give compounds **5-15,** respectively.

*N-Ethyl-1-(9-methyl-5,6-dihydronaphtho[1',2':4,5]thieno[2,3-d]pyrimidin-11-yl)pyrazolo[3,4-d]pyrimidin-4-amine **(5)***

Pale brown powder; (yield 69%); m.p. 161-162^{o}C; IR (KBr) υ cm^{-1}: 3407.60 (-NH), 1642.09 (C-N); ^{1}H NMR (500 MHz, DMSO-d_6) δ ppm: 1.75 (t, 3H, *J* = 2.5 Hz, CH_2-C***H*****$_3$**), 2.39 (q, 2H, , *J* = 2.5 Hz, C***H*****$_2$**-CH_3), 2.51 (s, 3H, C***H*****$_3$**), 2.87-2.98 (m, 4H, 2C***H*****$_2$**), 6.27 (br, 1H, N***H***, D_2O exchangeble), 7.18-7.31 (2m, 4H, 3Ar-H+pyrazole-***H***), 8.01 (s, 1H, pyrimidine-***H***), 8.42 (d, 1H, , *J* = 8 Hz, Ar-***H***); ^{13}C NMR (400 MHz, DMSO-d_6) δ ppm: 15.48 (CH_3), 20.55 (CH_3), 23.39 (CH_2), 29.27 (CH_2), 36.05 (CH_2), 118.33-135.15 (17 sp^2 carbon atoms), 154.80, 158.15, 164.65; MS, *m/z* (%): 414.30 (M^++1, 0.39), 411.30 (M^+-1, 0.53), 369.30 (M^+-[HN-CH_2CH_3], 1.29), 280.10 (18.80), 268.05 (100.00); Anal. calcd. for $C_{22}H_{19}N_7S$ (413.49) (%): C, 63.90; H, 4.63; N, 23.71; S, 7.75. Found (%): C, 63.79; H, 4.78; N, 23.85; S, 7.92.

*N-(2-Phenylethyl)-1-(9-methyl-5,6-dihydronaphtho[1',2':4,5]thieno[2,3-d]pyrimidin-11-yl)-pyrazolo[3,4-d]pyrimidin-4-amine **(6)***

Pale yellow powder; (yield 70%); m.p. 320-323^{o}C; IR (KBr) υ cm^{-1}: 3410.49 (-NH); ^{1}H NMR (500 MHz, DMSO-d_6) δ ppm: 2.34 (s, 3H, C***H*****$_3$**), 2.70 (t, 3H, *J* = 17.69 Hz, CH_2-C***H*****$_2$**-Ph), 2.81-2.88 (2m, 4H, 2C***H*****$_2$**), 3.43 (m, 2H, HN-C***H*****$_2$**-CH_2), 5.89 (br, 1H, N***H***, D_2O exchangeable), 7.15-7.31 (2m, 9H, 8Ar-***H***+pyrazole-***H***), 8.38 (d, 1H, *J* = 7.5 Hz, Ar-***H***), 8.55 (s, 1H, pyrimidine-***H***); ^{13}C NMR (400 MHz, DMSO-d_6) δ ppm: 20.57 (CH_3), 23.38 (CH_2), 29.27 (CH_2), 34.74 (CH_2), 42.42 (CH_2), 118.33-139.76 (15 sp2 carbon atoms), 154.86, 158.19, 161.00, 164.65, 166.11; MS, *m/z* (%): 489.20 (M^+, 0.12), 413.15 (M^+-Ph, 0.02), 399.15 (M^+-CH_2Ph, 0.04) 385.10 (M^+-CH_2CH_2Ph , 0.12), 105.10 (100); Anal. calcd. for $C_{28}H_{23}N_7S$ (489.59) (%): C, 68.69; H, 4.74; N, 20.03; S, 6.55. Found (%): C, 68.56; H, 4.84; N, 19.87; S, 6.44.

*N-Benzyl-1-(9-methyl-5,6-dihydronaphtho[1',2':4,5]thieno[2,3-d]-pyrimidin-11-yl)-pyrazolo[3,4-d]pyrimidin-4-amine **(7)***

Pale grey powder; (yield 69%); m.p. 245-246^{o}C; IR (KBr) υ cm^{-1}: 3431.71 (-NH); ^{1}H NMR (300 MHz, DMSO-d_6) δ ppm: 2.35 (s, 3H, C***H*****$_3$**), 2.83-2.90 (2m, 4H, 2C***H*****$_2$**), 3.89 (s, 2H, -C***H*****$_2$**-Ph), 6.03 (br, 1H, N***H***, D_2O exchangeable), 7.21-7.45 (2m, 9H, 8Ar-***H***+pyrazole-***H***), 8.12 (d, 1H, *J* = 7.6 Hz, Ar-***H***), 8.40 (s, 1H, pyrimidine-***H***); MS, *m/z* (%): 475.00 (M^+, 0.01), 286.05 (100), 224.00 (19.86); Anal. calcd. for $C_{27}H_{21}N_7S$ (475.56) (%): C, 68.19; H, 4.45; N, 20.62; S, 6.74. Found (%): C, 68.07; H, 4.34; N, 20.78; S, 6.65.

N-[1-(9-Methyl-5,6-dihydronaphtho[1',2':4,5]thieno[2,3-d]pyrimidin-11-yl)-1H-pyrazolo[3,4-d]pyrimidin glucosamine ***(8)***
Off white powder; (yield 58%); m.p. 195-197^{o}C; IR (KBr) υ cm^{-1}: 3377.71 (-OH), 3290.93 (-NH), 2923.56 (CH_2), 2856.06 (CH), 1660.41 (C=N); ^{1}H NMR (500 MHz, DMSO-d_6) δ ppm: 2.43 (s, 1H, C***H*****$_3$**), 2.46-2.51 (m, 4H, 4C***H***), 2.82-2.90 (2m, 4H, 2C***H*****$_2$**), 3.55-3.57 (m, 2H, C***H*****$_2$**O), 4.41 (m, 2H, O***H***, D_2O exchangeable), 4.67 (m, 2H, O***H***, D_2O exchangeable), 4.92-5.00 (m, 1H, OH, D_2O exchangeable), 5.33 (m, H, COC***H***), 7.14-7.31 (2m, 5H, 4Ar-***H***+Pyrazole-***H***), 8.35 (d, 1H, *J* = 7.6 Hz, Ar-***H***), 8.58 (s, 1H, pyrimidine-***H***), 12.39 (1H, N***H***, D_2O exchangeable); MS, *m/z* (%): 548.00 (M^++1, 25), 547.7 (M^+, 20.8), 459.6 (25.0), 162.00 (Glucosamine unite-[NH+H], 25.00), 63.80 (100.00); Anal. calcd. for $C_{26}H_{25}N_7O_5S$ (547.58) (%): C, 57.03; H, 4.60; N, 17.91; S, 5.86. Found (%): C, 56.88; H, 4.72; N, 18.02; S, 5.70.

N-[1-(9-Methyl-5,6-dihydronaphtho[1',2':4,5]thieno[2,3-d]pyrimidin-11-yl)-1H-pyrazolo[3,4-d]pyrimidin-4-ylamino)-acetic acid ***(9)***
Pale yellow powder; (yield 60%); m.p. 267-269^{o}C; IR (KBr) υ cm^{-1}: 3844.4-2949.59 (broad, -OH), 1735.34 (C=O), 1569.77 (C=N); ^{1}H NMR (300 MHz, DMSO-d_6) δ ppm: 2.45 (s, 3H, C***H*****$_3$**), 2.73-2.89 (2m, 4H, 2C***H*****$_2$**), 4.25 (s, 2H, N-C***H*****$_2$**-), 5.96 (br, 1H, N***H***, D_2O exchangeable), 7.29-7.51 (2m, 9H, 8Ar-***H***+pyrazole-***H***), 8.10 (d, 1H, *J* = 7.6 Hz, Ar-***H***), 8.35 (s, 1H, pyrimidine-***H***), 12.5 (s, 1H, COO***H***, D_2O exchangeable); MS, *m/z* (%): 443.00 (M^+, 1.28), 268.05 (100.00), 251.05 (0.01), 192.05 (0.86), 74.05 (2.32); Anal. calcd. for $C_{22}H_{17}N_7O_2S$ (443.48) (%): C, 59.58; H, 3.86; N, 22.11; S, 7.23. Found (%): C, 59.43; H, 3.74; N, 21.94; S, 7.10.

1-(9-Methyl-5,6-dhydronaphtho[1',2':4,5]thieno[2,3-d]pyrimidin-11-yl)-4-(2-phenylhydrazino)-1H-pyrazolo[3,4-d]pyrimidine ***(10)***
Yellowish orange powder; (yield 66%); m.p. 266-267^{o}C; IR (KBr) υ cm^{-1}: 3418.21 (-NH), 3208.00 (-NH), 3046.01-3004.55 (Ar-H), 2926.45 (aliphatic-H), 1590.02 (C=N); ^{1}H NMR (500 MHz, DMSO-d_6) δ ppm: 2.39 (s, 3H, C***H*****$_3$**), 2.84-2.95 (2m, 4H, 2C***H*****$_2$**), 5.10 (br, 2H, D_2O-exchangeable), 6.93-7.45 (2m, 9H, 8Ar-***H***+pyrazole-***H***), 8.00 (d, 1H, *J* = 7.6 Hz, Ar-***H***), 8.35 (s, 1H, pyrimidine-***H***); ^{13}C NMR (400 MHz, DMSO-d_6) δ ppm: 20.54 (CH_3), 23.37 (CH_2), 30.65 (CH_2), 118.32-135.14 (17 sp^2 carbon atoms), 154.80, 158.16, 164.64; MS, *m/z* (%): 476.00 (M^+, 0.04), 475.00 (M^+-1, 0.03), 474.00 (M^+-2, 0.04), 383.00 (M^+-[-NH-Ph], 0.02), 370.00 (M^+-[-NH-NH-Ph],0.03), 268.00 (100.00), 224.00 (22.77), 105.00 $(PhN_2)^+$, 14.41), 92.00 (49.98), 77.00 (21.66); Anal. calcd. for $C_{26}H_{20}N_8S$ (476.55) (%): C, 65.53; H, 4.23; N, 23.51; S, 6.73. Found (%): C, 65.42; H, 4.35; N, 23.31; S, 6.84.

N-[(9-Methyl-5,6-dihydronaphtho[1',2':4,5]thieno[2,3-d]pyrimidin-11-yl)]-1H-pyrazolo[3,4-d]pyrimidin-4-yl)ethane-1,2-diamine ***(11)***
Brown powder, (yield 61%), m.p. 300-302^{o}C; IR (KBr) υ cm^{-1}: 3407.6 (-NH_2), 3296.71 (-NH), 2920.66 (Ar-H), 2853.17 (H-aliphatic), 16527 (C=N); ^{1}H NMR (DMSO-d_6) δ ppm: 2.33 (s, 3H, C***H*****$_3$**), 2.46 (m, 2H, -CH_2C***H*****$_2$**NH_2), 2.84 (m, 4H, 2C***H*****$_2$**), 3.31 (m, 2H, -C***H*****$_2$**CH_2NH_2), 5.00 (br, 2H, N***H*****$_2$**, D_2O-exchangeble) 7.21-7.60 (m , 4H, 3Ar-***H***+pyrazole-***H***), 8.02 (d, 1H, *J* = 7.6 Hz, Ar-***H***), 8.41 (s, 1H, pyrimidine-***H***), 11.55 (br, 1H, N***H***, D_2O-exchangeable); ^{13}C NMR (500 MHz, DMSO-d_6) δ ppm: 20.57 (CH_3), 23.38 (CH_2), 29.27 (CH_2), 118.30-139.80 (15 sp2 carbon atoms), 154.86, 158.09, 161.00, 164.85; MS, *m/z* (%): 429.10 (M^++1, 0.14), 368.10 (M^+-NH$(CH_2)_2NH_2$, 0.13), 268.05 (100.00), 251.05 (24.41); Anal. calcd. for $C_{22}H_{20}N_8S$ (428.51) (%): C, 61.66; H, 4.70; N, 26.15; S, 7.48. Found (%): C, 61.48; H, 4.82; N, 26.02; S, 7.32.

1-[(9-Methyl-5,6-dihydronaphtho[1',2':4,5]thieno[2,3-d]pyrimidin-11-yl)]-4-piperidin-1-yl-1H-pyrazolo[3,4-d]pyrimidine ***(12)***
Brownish yellow powder, (yield 71%), m.p. 139-141^{o}C; IR (KBr) υ cm^{-1}: 2924.52 (Ar-H), 2856.06 (aliphatic-H), 1625.7 (C=N); ^{1}H NMR (500 MHz, DMSO-d_6) δ ppm: 0.80 (2m, 2H, C***H*****$_2$**), 1.13-1.19 (2m, 4H, 2C***H*****$_2$**), 2.56 (s, 3H, C***H*****$_3$**), 2.69-2.71 (m, 4H, -C***H*****$_2$**-N-C***H*****$_2$**), 2.81-2.85 (2m, 4H, 2C***H*****$_2$**), 7.06-7.39 (m , 4H, 3Ar-***H***+pyrazole-***H***), 8.05 (d, 1H, *J* = 7.0 Hz, Ar-***H***), 8.57 (s, 1H, pyrimidine-***H***); MS, *m/z* (%): 454.40 (M^++1, 0.21), 453.35 (M^+, 1.79), 369.30 (M^+-pipridinyl, 1.49), 251.15 (1.42), 85.10 [(pipridinyl)$^+$, 18.82], 80.00 (100.00); Anal. calcd. for $C_{25}H_{23}N_7S$ (453.56) (%): C, 66.20; H, 5.11; N, 21.62; S, 7.07. Found (%): C, C, 66.03; H, 4.99; N, 21.80; S, 6.94.

N-Cyclohexyl-1-[(9-Methyl-5,6-dihydronaphtho[1',2':4,5]thieno[2,3-d] pyrimidin-11-yl)]-1H-pyrazolo[3,4-d]pyrimidin-4-amine ***(13)***
Pale brown powder, (yield 79%); m.p. 250-252^{o}C; IR (KBr) υ cm^{-1}: 3418.21 (N-H), 2925.48 (Ar-***H***), 2858.95 (aliphatic-H), 1599.66 (C=N); ^{1}H NMR (500 MHz, DMSO-d_6) δ ppm: 0.83 (m, 2H, H3a+H5a), 1.16-1.17 (2m, 2H, H4a+H4b), 1.49 (m, 2H, H3b+H5b), 1.57-1.62 (m, 2H, H2a+H6a), 1.79-1.90 (2m, 2H, H2b+H6b), 2.21-2.27 (m, 1H, HN-C***H***), 2.50 (s, 3H, C***H*****$_3$**), 2.89-2.96 (2m, 4H, 2C***H*****$_2$**), 5.59 (s, 1H, -N***H***, D_2O-exchangeble), 7.12-7.33 (m , 4H, 3Ar-***H***+pyrazole-***H***), 8.33 (d, 1H, *J* = 7.6 Hz, Ar-***H***), 8.90 (s, 1H, pyrimidine-***H***); MS, *m/z* (%): 467.35 (M^+, 027), 369.30 (M^+-[H-N-cyclohexyl], 5.57), 98.10 ([H-*N*-cyclohexyl]$^+$, 24.86), 71.10 (100.00); Anal. calcd. for $C_{26}H_{25}N_7S$ (467.58) (%): C, 66.78; H, 5.39; N, 20.97; S, 6.86. Found (%): C, 66.59; H, 5.19; N, 20.87; S, 6.98.

1-[(9-Methyl-5,6-dihydronaphtho[1',2':4,5]thieno[2,3-d]pyrimidin-11-yl)]-4-morpholin-4-yl-1H-pyrazolo[3,4-d]pyrimidine ***(14)***
Brown powder, (yield 60%); m.p. 270-271^{o}C; IR (KBr) υ cm^{-1}: 2920.66 (Ar-H), 2851.24 (Aliphatic-H), 1661.37 (C=N), 1593.88 (C=C); ^{1}H NMR (500 MHz, DMSO-d$_6$) δ ppm: 2.50 (s, 3H, C***H*****$_3$**), 2.78-2.85 (2m, 4H, 2C***H***$_2$), 3.07 (m, 4H, -N(C***H*****$_2$**)$_2$), 3.77 (m, 4H, O(C***H*****$_2$**)$_2$), 7.07-7.41 (m, 4H, 3Ar-***H***+pyrazole-***H***), 7.98 (d, 1H, *J* = 7.8 Hz, Ar-***H***), 8.29 (s, 1H, pyrimidine-***H***); ^{13}C NMR (400 MHz, DMSO-d$_6$) δ ppm: 20.53 (CH$_3$), 23.46 (CH$_2$), 29.33 (CH$_2$), 48.02, 64.18, 117.86-135.27 (12 sp^2 carbon atoms), 156.70, 157.67, 162.59; MS, *m/z* (%): 455.30 (M$^+$, 0.02), 369.30 (M$^+$-morpholine, 1.58), 251.20 (0.59), 86.10 (1.19), 69.10 (100.00); Anal. calcd. for $C_{24}H_{21}N_7OS$ (455.53) (%): C, 63.28; H, 4.65; N, 21.52; S, 7.04. Found (%): C, 63.14; H, 4.53; N, 21.37; S, 7.24.

1-[(9-Methyl-5,6-dihydronaphtho[1',2':4,5]thieno[2,3-d]pyrimidin-11-yl)]-4-piperazin-1-yl-1H-pyrazolo[3,4-d]pyrimidine ***(15)***
Pale brown powder, (yield 67%); m.p. 235-237^{o}C; IR (KBr) υ cm^{-1}: 3007.44 (N-H), 2049 (C-N-C), 1597.73 (C=N), 1504.20 (C=C); ^{1}H NMR (500 MHz, DMSO-d$_6$) δ ppm: 2.35 (s, 3H, C***H*****$_3$**), 2.59 (m, 4H, N-H(C***H*****$_2$**)$_2$), 2.75 (2m, 4H, 2C***H*****$_2$**), 3.22 (m, 4H, N(C***H*****$_2$**)$_2$), 7.11-7.53 (m, 4H, 3Ar-***H***+pyrazole-***H***), 8.03 (d, 1H, *J* = 7.6 Hz, Ar-***H***), 8.40 (s, 1H, pyrimidine-***H***); MS, *m/z* (%): 454.30 (M$^+$, 0.01), 453.30 (M$^+$-1, 0.04), 369.30 (M$^+$-piperazine, 0.82), 255.75 (47.58), 85.10 (piperazine, 4.17), 63.95 (100.00); Anal. calcd. for $C_{24}H_{22}N_8S$ (454.55) (%): C, 63.42; H, 4.88; N, 24.65; S, 7.05. Found (%): C, 63.24; H, 5.00; N, 24.76; S, 6.92.

5-Amino-1-(9-methyl-5,6-dihydronaphtho[1',2':4,5]-thieno[2,3-d]pyrimidin-11-yl)-1H-pyrazole-4-carboxilic acid amide ***(16)***
To compound **1 [16]** (100 mmol) was added 50 mL of glacial acetic acid and 25 mL hydrochloric acid, and then the reaction mixture was heated under reflux for 5 h. The reaction mixture was poured onto water and the formed precipitate was collected by filtration and washed several times with hot water and air dried, then recrystallized from ethanol/dioxane to give compound **16**. Pale buff crystals; (yield 73%); m.p. 198-199^{o}C; IR (KBr) υ cm^{-1}: 3395-3230 br (2NH$_2$), 1630 (C=O); ^{1}H NMR (500 MHz, DMSO-d$_6$) δ ppm: 2.35 (s, 3H, C***H*****$_3$**), 2.81-2.86 (m, 4H, 2C***H*****$_2$**), 4.01-4.50 (br s, 2H, CON***H*****$_2$**, D$_2$O exchangeable), 6.34 (br s, 2H, N***H*****$_2$**, D$_2$O exchangeable), 7.07-7.31 (m, 4H, 3Ar-***H***+pyrazole-***H***), 8.11 (d, 1H, *J* = 7.7 Hz, Ar-***H***); MS, *m/z* (%): 377.10 (M$^+$+1, 3.17), 376.10 (M$^+$, 3.17), 332.10 (M$^+$-CONH$_2$, 3.61), 316.10 (3.28), 279.10 (4.48), 250.10 (3.61), 69.00 (100.00); Anal. calcd. for $C_{19}H_{16}N_6OS$ (376.43) (%): C, 60.62; H, 4.28; N, 22.33; S, 8.52. Found (%): C, 60.77; H, 4.07; N, 22.19; S, 8.61.

General procedure for the synthesis of compounds ***18, 19 and 20***
To compound **16** (0.01 mol) was added an equivalent amount (0.01 mol) of the respective phenyl isothiocyanate, phenyl isocyanate or 3-chlorophenyl isocyanate in 30 mL dry ethanol, and the reaction mixture was refluxed for 2, 3 and 5h, respectively. The reaction mixture was poured onto crushed ice and the deposited solid was collected by filtration, dried and recrystallized from ethanol to give compounds **18**, **19** and **20** in 89%, 73% and 68% yield, respectively.

1-(9-Methyl-5,6-dihydronaphtho[1',2':4,5]thieno[2,3-d]pyrimidin-11-yl)-4-phenylamino-1,7-dihydropyrazolo[3,4-d]pyrimidine-6-thione ***(18)***
Yellow crystals; (yield 89%); m.p. 135-136^{o}C; IR (KBr) υ cm^{-1}: 3210.9 (-NH-), 3116.4 (=N-H), 1030.77 (C=S). ^{1}H NMR (400 MHz, DMSO-d$_6$) δ ppm: 2.46 (s, 3H, C***H*****$_3$**), 3.38-3.45 (m, 4H, 2C***H*****$_2$**), 4.47 (s, 1H, N***H***, D$_2$O exchangeable), 7.10-7.39 (2m, 4H, 3Ar-***H***+pyrazole-***H***), 7.58 (br, 1H, Ar-***H***), 11.00 (s, 1H, N***H***, D$_2$O exchangeable); MS, *m/z* (%): 493 (M$^+$, 0.01), 495 (M$^+$+2, 0.03) 181 (40.95), 93.05 (100); Anal. calcd. for $C_{26}H_{19}N_7S_2$ (493.60) (%): C, 63.26; H, 3.88; N, 19.86; S, 12.99. Found (%): C, 63.11; H, 3.76; N, 19.97; S, 12.89.

1-(9-Methyl-5,6-dihydronaphtho[1',2':4,5]thieno[2,3-d]pyrimidin-11-yl)-4-phenylamino-1,7-dihydropyrazolo[3,4-d]pyrimidin-6-one ***(19)***
Pale white powder; (yield 73%); m.p. 205-207^{o}C; IR (KBr) υ cm^{-1}: 3316 (-NH-), 1708.62 (C=O); ^{1}H NMR (500 MHz, DMSO-d$_6$) δ ppm: 2.33 (s, 3H, C***H*****$_3$**), 2.46-2.85 (m, 4H, 2C***H*****$_2$**), 4.09 (s, 1H, N***H***, D$_2$O exchangeable), 7.11-7.19 (m, 6H, 5Ar-***H***+pyrazole-***H***), 7.20-7.22 (m, 2H, Ar-***H***), 8.36 (d, 1H, *J* = 7.7 Hz, Ar-***H***), 12.36 (s, 1H, N***H***, D$_2$O exchangeable); ^{13}C NMR (400 MHz, DMSO-d$_6$) δ ppm: 20.53 (CH$_3$), 23.57 (C-5'), 29.39 (C-6'), 116.47, 118.33, 121.90, 125.88, 126.20, 126.59, 127.11, 127.90, 128.83, 130.34, 130.97, 132.45, 133.25, 134.38, 135.18, 137.80, 138.20, 140.79, 141.32, 153.37, 154.75, 158.13, 164.64 (C=O); MS, *m/z* (%): 477.20 (M$^+$, 0.1), 352.20 (19.98), 296.10 (42.43), 268.10 (100); Anal. calcd. for $C_{26}H_{19}N_7OS$ (477.54) (%): C, 65.39; H, 4.01; N, 20.53; S, 6.71. Found (%): C, 65.51; H, 4.16; N, 20.64; S, 6.51.

4-(3-Chlorophenylamino)-1-(9-methyl-5,6-dihydronaphtho[1',2':4,5]-thieno[2,3-d]pyrimidin-11-yl)-1,7-dihydropyrazolo[3,4-d]pyrimidin-6-one ***(20)***

Yellow powder; (yield 68%); m.p. 212-214^{o}C; IR (KBr) υ cm^{-1}: 3415.31 (-NH-), 1661.37 (N-C=O). ^{1}H NMR (500 MHz, DMSO-d$_6$) δ ppm: 2.37 (s, 3H, C***H*****$_3$**), 2.46-2.91 (m, 4H, 2C***H*****$_2$**), 4.14 (s, 1H, N***H***, D$_2$O exchangeable), 7.00-7.29 (m, 4H, 3Ar-***H***+pyrazole-***H***), 8.05-8.31 (m, 4H, *p*-Cl-Ar-***H***), 8.43 (d, 1H, *J* = 7.7 Hz, Ar-***H***), 12.35 (s, 1H, N***H***, D$_2$O exchangeable). ^{13}C NMR (400 MHz, DMSO-d$_6$) δ ppm: 20.53 (CH$_3$), 23.38 (CH$_2$), 29.39 (CH$_2$), 116.47-158.09 (22Ar-C), 153.51 (C=O); MS, *m/z* (%): 513.20 (M$^+$+2, 0.01),511.20 (M$^+$, 0.02), 269.10 (31), 268.10 (100). Anal. calcd. for $C_{26}H_{18}ClN_7OS$ (511.98) (%): C, 60.99; H, 3.54; Cl, 6.92; N, 19.15; S, 6.26. Found (%): C, 61.10; H, 3.68; Cl, 6.81; N, 19.28; S, 6.06.

5-*(Benzoylamino)-1-(9-methyl-5,6-dihydronaphtho[1',2':4,5]thieno[2,3-d]pyrimidin-11-yl)-1H-pyrazole-4-carboxamide* ***(21)***

To 0.01 mol of compound **16** in 20 mL of cold ethanol in ice bath, was added an equivalent amount of benzoyl chloride and the reaction mixture was gently heated for about an hour. The reaction mixture was poured onto crushed ice and the deposited solid was collected by filtration, dried and dissolved in chloroform then re-precipitated with petroleum ether 60/80, filtered off and the solvent was evaporated to give compound **21**. Pale yellow fine crystals (yield 43%); m.p. 106-108^{o}C; IR (KBr) υ cm^{-1}: 3432.67 (broad –NH$_2$+NH), 1648.84 (HN-[C=O]-Ph), 1607.38 (HN-C=O); ^{1}H NMR (300 MHz, DMSO-d$_6$) δ ppm: 2.37 (s, 3H, C***H*****$_3$**), 2.85-2.90 (m, 4H, 2C***H*****$_2$**), 3.97 (br, 2H, -N***H*****$_2$**, D$_2$O exchangeable), 7.14-7.27 (m, 3H, Ar-***H***), 7.50-7.63 (m, 4H, Ar-***H***), 7.91-8.15 (m, 2H, Ar-***H***+pyrazole-***H***), 8.41 (d, 1H, *J* = 7.6 Hz, Ar-***H***), 12.39 (s, 1H, N***H***, D$_2$O exchangeable); MS, *m/z* (%): 480.50 (M$^+$, 31.00), 90.10 (100), 76.10 (39.41); Anal. calcd. for $C_{26}H_{20}N_6OS$ (480.54) (%): C, 64.98; H, 4.20; N, 17.49; S, 6.67. Found (%): C, 65.10; H, 4.31; N, 17.58; S, 6.52.

1-(9-Methyl-5,6-dihydronaphtho[1',2':4,5]thieno[2,3-d]pyrimidin-11-yl)-6-phenyl-1,5,-dihydropyrazolo[3,4-d]pyrimidin-4-one ***(22)***

Method 1:

To 0.01 mol of compound **16** in 30 mL of dry dioxane, was added an equivalent amount of benzoyl chloride and the reaction mixture was refluxed for 7 h. The reaction mixture was poured onto crushed ice and the deposited solid was collected by filtration, dried and recrystallized from ethanol/dioxane to give compound **22** in 68% yield.

Method 2:

To a freshly prepared solution of sodium ethoxide (0.01 mol of sodium metal in 30 mL dry ethanol), a 0.01 mol of compound **21** was added and the reaction mixture was refluxed for 2 h. After the reaction was completed, the reaction mixture was poured onto crushed ice and the deposited solid was collected by filtration, washed several times with hot water, dried and recrystallized from ethanol/dioxane to give compound **22** in 89% yield.

Pale brown powder, m.p. 286-288^{o}C; (products from methods 1 & 2 are identical in all aspects; m.p. & mixed m.p., TLC and IR) IR (KBr) υ cm^{-1}: 3432 (-NH-), 1648 (HN-C=O); ^{1}H NMR (500 MHz, DMSO-d$_6$) δ ppm: 2.37 (s, 3H, C***H*****$_3$**), 2.81-2.93 (m, 4H, 2C***H*****$_2$**), 7.13-7.27 (m, 3H, Ar-***H***), 7.57-7.62 (m, 4H, Ar-***H***), 7.98-8.10 (m, 2H, Ar-***H***+pyrazole-***H***), 8.39 (d, 1H, *J* = 7.7 Hz, Ar-***H***), 12.38 (s, 1H, N***H***, D$_2$O exchangeable); ^{13}C NMR (400 MHz, DMSO-d$_6$) δ ppm: 20.57 (CH$_3$), 23.38 (CH$_2$), 29.27 (CH$_2$), 118.31-135.12 (19 sp^2 carbon atoms), 154.82 (C=O), 158.19, 164.64; MS, *m/z* (%): 464.20 (M$^+$+2, 30.88), 462.20 (M$^+$, 51.00), 194.00 (94.41), 90.10 (100), 76.10 (39.41); Anal. calcd. for $C_{26}H_{18}N_6OS$ (462.52) (%): C, 67.52; H, 3.92; N, 18.17; S, 6.93. Found (%): C, 67.72; H, 4.07; N, 18.27; S, 7.05.

5-(3-Chloro-2-hydroxypropyl)-1-(9-methyl-5,6-dihydronaphtho-[1',2':4,5]thieno[2,3-d]pyrimidin-11-yl)-6-phenyl-1,5-dihydro-4H-pyrazolo[3,4-d]pyrimidin-4-one ***(23)***

A solution of compound **22** (0.462 g, 0.01 mol) in sodium ethoxide (0.01 mol Na metal in 20 mL dry ethanol) was stirred for about 1 h at room temperature. The reaction was monitored with TLC [chloroform / methanol (9:1)] to ensure the formation of the salt, then an equivalent amount (0.01 mol) of epichlorohydrin was added and the reaction mixture was stirred at room temperature for 2 h. The reaction was monitored with TLC till the reaction completed, then the reaction mixture was poured onto crushed ice and extracted with chloroform, dried with anhydrous sodium sulfate and concentrated to be treated with petroleum ether 60-80. The formed precipitate was filtered off and the remained solvent was evaporated to give compound **23**. Pale white fine crystals; (yield 48%); m.p. 109-110^{o}C; IR (KBr) υ cm^{-1}: 3408.57 (-OH), 2923.56 (CH-aliphatic), 2855.10 (CH-aromatic), 1599.66; ^{1}H NMR (300 MHz, DMSO-*d*$_6$) δ ppm: 2.50 (s, 3H, C***H*****$_3$**), 2.76-2.86 (m, 4H, 2C***H*****$_2$**), 3.65 (dd, 1H, Ha), 3.85 (dd, 1H, Hb), 4.25 (dd, 1H, Ha), 4.28 (dd, 1H, Hb), 5.59 (m, 1H, -C***H***-OH), 6.41 (d, 1H, -O***H***), 7.23-7.50 (m, 6H, Ar-***H***), 7.54-7.85 (m, 3H, 2Ar-***H***+pyrazole-***H***), 8.37 (d, 1H, *J* = 7.6 Hz, Ar-***H***); MS, *m/z* (%): 555.10 (M$^+$, 0.61), 553.10 (M$^+$-2, 1.25), 552.10 (M$^+$-3, 1.33), 551.10 (M$^+$-4, 1.79), 550.10 (M$^+$-5, 1.19), 488.10 (M$^+$-[CH$_2$Cl+OH], 1.08), 383.10 (1.83), 394.15 (20.44), 369.10 (2.56), 365.10 (35.38), 266.05 (100.00), 251.05 (9.11); Anal. calcd. for

$C_{29}H_{21}ClN_6O_2S$ (555.05) (%): C, 62.75; H, 4.18; Cl, 6.39; N, 15.14; S, 5.78. Found (%): C, 62.59; H, 4.05; Cl, 6.65; N, 15.020; S, 5.97.

General procedure for preparation of ***24, 25, 26 and 27***
To a solution of compound **22** (0.462 g, 0.01 mol) in sodium ethoxide (0.01 mol Na metal in 20 mL dry ethanol) an equivalent amounts (0.01 mol) of chloroethanol, chloroacetaldehyde dimethyl acetal, chloroethyl methyl ether, or 2-(2-chloroethoxy)ethanol were added respectively and the reaction mixtures were slowly heated up to 50-60^oC for 2-4 h. The reaction mixture was monitored with TLC [chloroform/ methanol (9:1)] till the reaction was complete, then the reaction mixture was poured onto crushed ice and then extracted with chloroform and concentrated to be passed through a filter funnel containing silica gel using chloroform/methanol mixture (8:2). The solvent was evaporated to give compounds **24**, **25**, **26,** and **27,** respectively.

5-(2-Hydroxyethyl)-1-(9-methyl-5,6-dihydronaphtho[1',2':4,5]thieno[2,3-d]pyrimidin-11-yl)-6-phenyl-1,5-dihydro-4H-pyrazolo[3,4-d]pyrimidin-4-one ***(24)***
Pale brown powder; (yield 63%); m.p. 248-250^oC; IR (KBr) υ cm^{-1}: 3378.67 (OH), 1592.91 (N-C=O); ^1H NMR (300 MHz, DMSO-d_6) δ ppm: 2.77 (s, 3H, C***H*****$_3$**), 2.81-2.93 (2m, 4H, 2C***H*****$_2$**), 3.67-3.88 (m, 4H, N-C***H*****$_2$**-C***H*****$_2$**-OH), 5.00 (bs, 1H, O***H***, D_2O exchangeable), 7.20-7.85 (m, 9H, 8Ar-***H***+pyrazole-***H***), 8.30 (d, 1H, *J* = 7.6 Hz, Ar-***H***); MS, *m/z* (%): 507.30 (M^++1, 0.91), 506.30 (M^+, 0.70), 503.30 (M^+-3H, 0.65), 424.30 (M^+-[Ph+5H], 1.03), 396.30 (M^+-[Ph+2H+CH_2OH], 1.03), 383.30 (M^+-[Ph+CH_2CH_2OH], 0.75), 292.20 (0.75), 251.20 (2.36); Anal. calcd. for $C_{28}H_{22}N_6O_2S$ (506.57) (%): C, 66.39; H, 4.38; N, 16.59; S, 6.33. Found (%): C, 66.26; H, 4.49; N, 16.45; S, 6.49.

*5-(2,2-Dimethoxyethyl)-1-(9-methyl-5,6-dihydronaphtho[1',2':4,5]thieno[2,3-d]pyrimidin-11-yl)-6-phenyl-1,5-dihydro-4H-pyrazolo[3,4-d]pyrimidin-4-one (***25***)*
Pale brown oil; (yield 51%); IR (neat) υ cm^{-1}: 2922.59 (Ar-H), 2854.13 (=C-H), 1664.27 (N-C=O); ^1H NMR (300 MHz, DMSO-d_6) δ ppm: 2.53 (s, 3H, C***H*****$_3$**), 2.81-3.00 (2m, 4H, 2C***H*****$_2$**), 3.51-3.79 (m, 8H, CH(OC***H*****$_3$)$_2$**+N-C***H*****$_2$**), 4.86 (t, 1H, *J* = 7.5 Hz, C***H***$(OCH_3)_2$), 7.16-7.31 (m, 9H, 8Ar-***H***+pyrazole-***H***), 8.22 (d, 1H, *J* = 7.6 Hz, Ar-***H***); MS, *m/z* (%): 552.10 (M^++2, 4.83), 551.10 (M^++1, 0.05), 550.10 (M^+, 5.43), 380.10 (6.08), 368.10 (3.31), 316.10 (5.48), 278.00 (1.47), 251.00 (1.15), 105.05 (100.00), 88.00 (2.44); Anal. calcd. for $C_{30}H_{26}N_6O_3S$ (550.63) (%): C, 65.44; H, 4.76; N, 15.26; S, 5.82. Found (%): C, 65.34; H, 4.89; N, 15.06; S, 6.01.

5-(Methoxyethyl)-1-(9-methyl-5,6-dihydronaphtho[1',2':4,5]thieno[2,3-d]pyrimidin-11-yl)-6-phenyl-1,5-dihydro-4H-pyrazolo[3,4-d]pyrimidin-4-one ***(26)***
Pale orange oil; (yield 58%); IR (neat) υ cm^{-1}: 2920.66 (Ar-H), 2854.13, 1660.41 (N-C=O); ^1H NMR (300 MHz, DMSO-d_6) δ ppm: 2.53 (s, 3H, C***H*****$_3$**), 2.71-2.85 (m, 4H, 2C***H*****$_2$**), 3.25 (s, 3H, OC***H*****$_3$**), 3.68 (t, 2H, *J* = 8.1 Hz, C***H*****$_2$**), 4.01 (t, 2H, *J* = 8.1 Hz, C***H*****$_2$**), 7.20-7.51 (2m, 9H, 8Ar-***H***, pyrazole-***H***), 8.21 (d, 1H, *J* = 8.6 Hz, Ar-***H***); MS, *m/z* (%): 523.30 (M^++3, 0.16), 522.30 (M^++2, 0.30), 521.30 (M^++1, 0.30), 520.30 (M^+, 1.30), 428.30 (M^+-[CH_3+Ph], 1.18), 384.30 (M^+-[$CH_2CH_2OCH_3$+Ph], 1.21), 251.05 (0.59), 105.05 (100.00); Anal. calcd. for $C_{29}H_{24}N_6O_2S$ (520.60) (%): C, 66.90; H, 4.65; N, 16.14; S, 6.16; Found (%): C, 66.79; H, 4.78; N, 16.29; S, 6.02.

5-[2-(Hydroxymethoxy)ethyl]-1-(9-methyl-5,6-dihydronaphtho-[1',2':4,5]thieno[2,3-d]pyrimidin-11-yl)-6-phenyl-1,5-dihydro-4H-pyrazolo[3,4-d]pyrimidin-4-one ***(27)***
Orange oil; (yield 61%); IR (neat) υ cm^{-1}: 3370.00 (br, -OH), 3057.58 (Ar-H), 1604.48 (N-C=O); ^1H NMR (300 MHz, DMSO-d_6) δ ppm: 2.39 (s, 3H, CH_3), 2.21 (t, 2H, *J* = 5.5 Hz, N-C***H*****$_2$**), 2.45-2.74 (2m, 4H, 2C***H*****$_2$**), 2.90-2.94 (m, 2H, -C***H*****$_2$**-C***H*****$_2$**-O), 3.6-3.8 (br, 1H, OH, D_2O exchangeable), 5.10 (s, 2H, O-C***H*****$_2$**-OH), 7.3-7.8 (m, 9H, 8Ar-***H***+pyrazole-***H***), 8.2 (d, 1H, *J* = 7.6 Hz, Ar-***H***); MS, *m/z* (%): 538.50 (M^++2, 44.84), 537.50 (M^++1, 40.81), 536.50 (M^+, 42.82), 535.50 (M^+-1, 27.96), 396.10 (2.52), 381.10 (14.61), 316.30 (4.53), 251.20 (7.30), 108.10 (100.00); Anal. calcd. for $C_{29}H_{24}N_6O_3S$ (536.60) (%): C, 64.91; H, 4.51; N, 15.66; S, 5.98. Found (%): C, 64.79; H, 4.64; N, 15.47; S, 5.82.

3.2. Materials and methods

3.2.1. Chemicals

Dimethyl sulphoxide (DMSO), cisplatin and Sulfo-Rhodamine-B stain (SRB) were purchased from Merck (Darmstadt, Germany). All other chemicals and reagents used in this study were of analytical grade and purchased from Sigma-Aldrich chemical Co. (St. Louis, MO, USA).

3.2.2. Cell lines and culturing

Anticancer activity screening for the tested compounds utilizing 3 different human tumor cell lines including human breast MCF-7; liver HepG2 and lung A549cancer cell lines were obtained from the American Type Culture Collection (Rockville, MD, USA). The tumor cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat inactivated fetal calf serum (GIBCO), penicillin (100 U/mL) and

streptomycin (100 µg/mL) at 37°C in humidified atmosphere containing 5% CO_2. Cells at a concentration of 0.50 x 10^6 were grown in a 25 cm^2 flask in 5 mL of complete culture medium.

3.2.3. *In vitro* antiproliferative assay

The antiproliferative activity was measured *in vitro* using the Sulfo-Rhodamine-B stain (SRB) assay according to the previous reported standard procedure [51]. The concentration required for 50% inhibition of cell viability (IC_{50}) was calculated and the results are given in Table 1.

3.2.4. Biochemical assays

The cells in culture medium were treated with 20 µL of 1/10 of IC_{50} values of the compounds or the standard reference drug, cisplatin, then incubated for 24 h at 37 ºC, in a humidified 5% CO_2 atmosphere. The cells were harvested and homogenates were prepared in saline using a tight pestle homogenizer until complete cell disruption for further biochemical analysis. The supernatants obtained after centrifugation of cell homogenates were used for determination of following parameters. The activities of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px) were determined as described by Aebi [52] and Marklund and Marklund [53], respectively**.** The levels of hydrogen peroxide (H_2O_2), nitric oxide (NO) and reduced glutathione (GSH) were determined by the methods of Wolf [54], Montgomery and Dymock [55] and Ellman [56], respectively. Nucleic acids (DNA and RNA) and total protein were precipitated and measured in cell homogenates. Total DNA was extracted and assayed according to the method described by Zhou *et al.* [57], total RNA was extracted and assayed according to the method adopted from the method provided by Hybaid/AGS (Germany), and total cellular protein was assayed by the method of Lowry *et al.* [58].

3.3. Antiradical activity of the prepared compounds

3.3.1. Chemicals

1,1-Diphenyl-2-picrylhydrazyl (DPPH, approximately 90%), 2,2-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid, ABTS), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), and tert- butyl hydroquinone (TBHQ) were purchased from Sigma (St. Louis, MO, USA).

The samples that were tested for the antioxidant activity by measuring their radical scavenging ability (RSA) using two assays, namely DPPH-assay and ABTS-assay are as follow (**18**, **19**, **20**, **22**, **6**, **7**, **10**, and **11**).

3.3.2. Radical scavenging activity (RSA %)

3.3.2.1. DPPH Assay

RSA % was assessed by the stable 2,2`-diphenyl-1-picrylhydrazyl free radical (DPPH*) scavenging method as described by Hamed *et al.* [59]. In details, 150µL (DMSO solution; 100 ppm final concentration) of each sample (or TBHQ as a reference antioxidant) was added to 850 µL pure methanol and 2 mL of freshly prepared 0.13 mM DPPH* solution in methanol. For control sample 850 µL methanol and 150 µL dimethyl sulfoxide (DMSO) were added to 2 mL DPPH* solution The sample solutions were vigorously shaken on a vortex for 30 seconds and then immediately placed in a UV/VIS spectrophotometer (T80 UV/VIS Spectrometer, PG Instruments Ltd, UK). The absorbance was measured at 516 nm against pure methanol after 30 min incubation. RSA was calculated according to the following equation:

$$RSA\ \% = [(Abs_c – Abs_s) / Abs_c] \times 100$$

Where: Abs_c= absorbance of control DPPH sample without added antioxidant at beginning; Abs_s = absorbance of sample after 30 min.

3.3.2.2. ABTS assay

For ABTS assay, the procedure followed the method of Arnao *et al.* [60] with some modifications. The stock solutions included 7.4 mM $ABTS^{*+}$ solution and 2.6 mM potassium persulfate solution. The working solution was then prepared by mixing the two stock solutions in equal quantities and allowing them to react for 12 h at room temperature in the dark. The solution was then diluted by mixing 1mL ABTS* working solution with 60 mL methanol to obtain an absorbance of 1.1±0.02 units at 734 nm using the spectrophotometer. 150µL (DMSO' solution; 100 ppm final concentration) of each sample (or Trolox as a reference antioxidant) were allowed to react with 2850 µL of the ABTS* working solution for 2 h in a dark condition. Then the absorbance was taken at 734 nm using the spectrophotometer. Control sample was prepared by adding 150 µL DMSO to 2850 µL working ABTS* solution. Fresh ABTS* working solution was prepared for each assay.

RSA was calculated according to the following equation:

$$RSA\ \% = [(Abs_c – Abs_s) / Abs_c] \times 100$$

Where: Abs_c= absorbance of control ABTS sample without added antioxidant at beginning; Abs_s = absorbance of sample after 120 min.

3.3.3. Statistical analysis

The results are reported as Mean ± Standard error (S.E.) for at least three times experiments. Statistical differences were analyzed according to that followed by one way ANOVA test followed by student's *t* test wherein the differences were considered to be significant at $p < 0.05$.

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