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# Synthesis, anti-inflammatory and molecular docking of some new 1,2,4-triazolobenzimidazol-3-yl acetone thiosemicarbazone cyclized derivatives as PLA-2 inhibitors

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## ABSTRACT

The present work is carried out for the synthesis and evaluation of some new 1,3,4-thiadiazolines, 1,3-thiazolines and 1,3-thiazolidin-4-ones linked to 1,2,4-triazolo[1,5-a]benzimidazole as anti-inflammatory agents. Structure elucidation of these compounds was confirmed by IR, $^1$H-NMR, and mass spectrometry along with elemental microanalyses. All new compounds were tested for their anti-inflammatory activity in comparison to indomethacin (INM) where some of them showed promising results comparable to INM at 4 hours interval. The most active anti-inflammatory compounds **(4b, 8c** and **9a)** were examined on gastric mucosa and didn't show any gastric ulcerogenic effect compared with the reference INM. Moreover, $LD_{50}$ of compounds (**4b** and **9a**) were determined in mice; they were found non toxic up to 400 mg $Kg^{-1}$ (i.p.). Also, docking simulation of some compounds into PLA2 active sites was studied.

## Indexing terms/Keywords

1,2,4-triazolobenzimidazole, thiosemicarbazones, anti-inflammatory, ulcerogenecity, PLA2 enzyme, molecular docking.

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## 1. INTRODUCTION

Interesting pharmacological properties have been associated with [1,2,4]triazolobenzimidazoles and their derivatives[1-4]. 4-Thiazolidinone derivatives have attracted continuing interest over the years because of their diverse biological activities such as antimycobacterial [5], anti-fungal [6], anticonvulsant [7], anti-inflammatory[8-10] and anti-HIV[11, 12] activities.

Also, it is well documented that thiazoline nucleus is associated with a variety of pharmacological actions, including antimicrobial [13], anti-inflammatory [14], antitumor [15] and antioxidant [16] actions. Heterocyclic thiadiazoles are widely exposed to therapeutic world, because of their known anti-inflammatory [17], anti-cancer [18], anti-tuberculosis [19], anti-convulsant [20] and anti-hypertensive [21] activities. Phospholipase A2 (PLA2) is a ubiquitous enzyme that specifically catalyzes hydrolysis of membrane phospholipids to produce lysophospholipids and free fatty acid, namely arachidonic acid, which provides substrate for eicosanoids biosynthesis. Thus, the compounds inhibiting PLA2 have been implicated as potential therapeutic agents in treatment of inflammation related diseases.

Enlightened by the aforementioned studies and data, the present work aims at the design and synthesis of some new thiosemicarbazones of 2,3-disubstituted-[1,2,4]triazolo[1,5-a]benzimidazoles along with evaluation of their in vivo anti-inflammatory activities. Also, investigation of the effect of incorporation of an extra heterocyclic rings; 4-thiazolidinones (**9a-d**), 1,3-thiazoline (**10a-d**) and 1,3,4-thiadiazolines (**11a-d**) on the biological activity compared to their opened structural analogues (**3a-d**) (which have previously prepared in our laboratory and cyclized to thiazoline (**4-7;a-f**) and thiazolidinone (**8a-d**) derivatives, Scheme 1) [22]. In addition, substantiation of the molecular docking of target compounds into active site of PLA2 enzyme will be investigated.

## 2. RESULTS AND DISCUSSION

### 2.1. Chemistry

Thiazolidinone derivatives **(9a-d)** were synthesized by refluxing thiosemicarbazones **(3a-d)** [22] in diethyl-2-bromomalonate and anhydrous sodium acetate in absolute ethanol for 20-24 h. Structures of compounds **(9a-d)** were confirmed by spectral method of analysis. IR spectra (ύ) of compounds **(9a-d)** showed a new band at 1705-1731 $cm^{-1}$ attributed to carbonyl groups and the disappearance of NH stretching bands of thiosemicarbazones. $^1$H-NMR spectra of 4-thiazolidinone series **(9a-d)** lacked signals characteristic of the two NH protons and showed a new singlet at a range of 3.60-3.80 ppm attributed to C5 proton of thiazolidinone ring. In addition to, ethyl moiety of ester group appears as triplet and quartet signals at 0.90-1.90 and 3.70-4.70 ppm, respectively. EI-mass spectrum of compound **(9a)**, revealed the molecular ion peak $M^+$ at m/z (474.00; 28.57%) (M.F., $C_{20}H_{23}N_7O_3S_2$) corresponding to molecular weight and a base peak at (m/z 95.00; 100%). Thiazoline derivatives **(10a-d**) were prepared by refluxing thiosemicarbazones **(3a-d)** in ethyl-2-chloroacetoacetate and anhydrous sodium acetate in absolute ethanol and catalytic amounts of glacial acetic acid for 22-24 h. Structures of compounds **(10a-d)** were confirmed by spectral data**.**

IR spectra (ύ) of compounds **(10a-d)** showed a new band at 1682-1698 $cm^{-1}$ attributed to the ester carbonyl group and the disappearance of NH stretching bands of thiosemicarbazones **(3a-d)**. $^1$H-NMR spectra of these series lacked signals characteristic of the two NH protons and showed a new singlet at a range of 2.35-2.55 ppm attributed to methyl group at C4 of thiazoline ring. Also, they showed introduction of ethyl group of $COOCH_2CH_3$; as triplet for $CH_3$ at δ 1.10-1.90 ppm and quartet for $CH_2$ at δ 4.35-4.70 ppm. EI-mass spectrum of compound **(10c)**, revealed the molecular ion peak $M^+$ at m/z (525.00; 92.31%), corresponding to molecular weight and a base peak at (m/z 226.00; 100%) (M.F.$C_{25}H_{31}N_7O_2S_2$). Another cyclization of thiosemicarbazones **(3a-d)** by refluxing with acetic anhydride for 10-15 h afforded the target thiadiazoline derivatives **(11a-d)**. Structures of thiadiazoline derivatives **(11a-d)** were confirmed by spectral methods of analysis. IR spectra (ύ) of compounds **(11a-d)** showed a new band at 1669-1659 $cm^{-1}$ attributed to the amidic carbonyl group of acetyl moiety and disappearance of NH stretching bands of thiosemicarbazones. Their $^1$H-NMR spectra showed two singlet signals due to methyl groups of diacetyl moieties (2 $CH_3CO$) at 2.10-2.35 ppm. EI-mass spectrum of compound **(11a)**, revealed the molecular ion peak $M^+$ (also the base peak) at m/z (446.00; 100%) (M.F., $C_{19}H_{23}N_7O_2S_2$).

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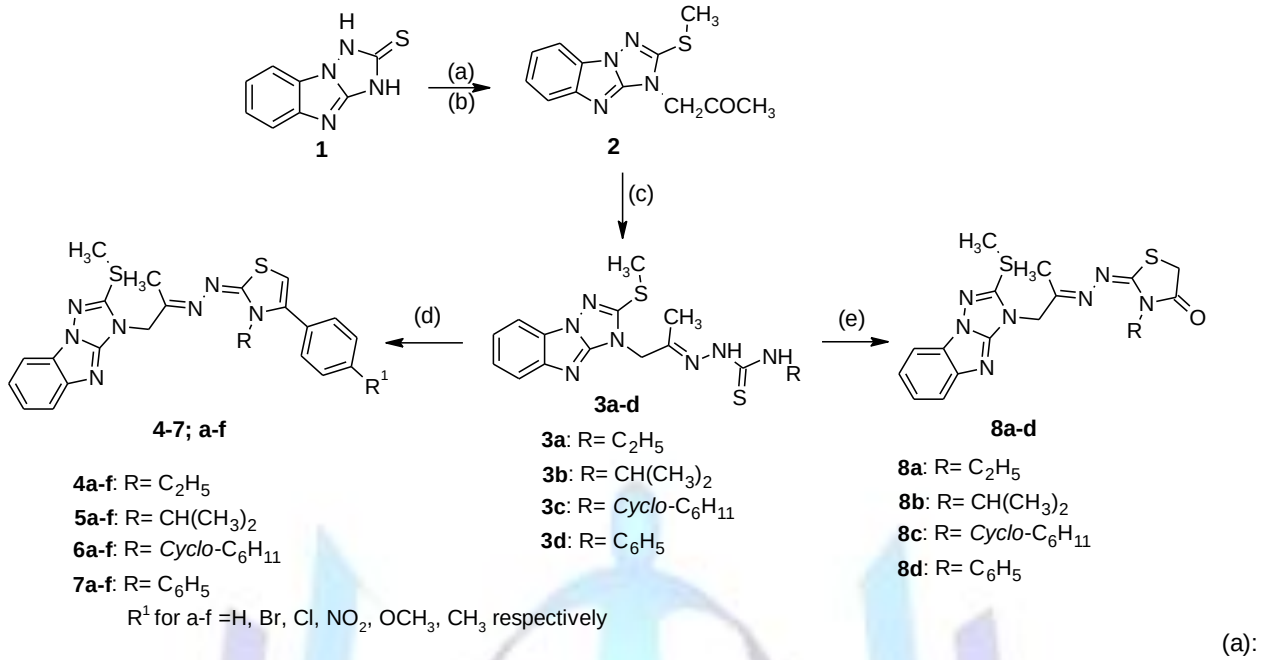
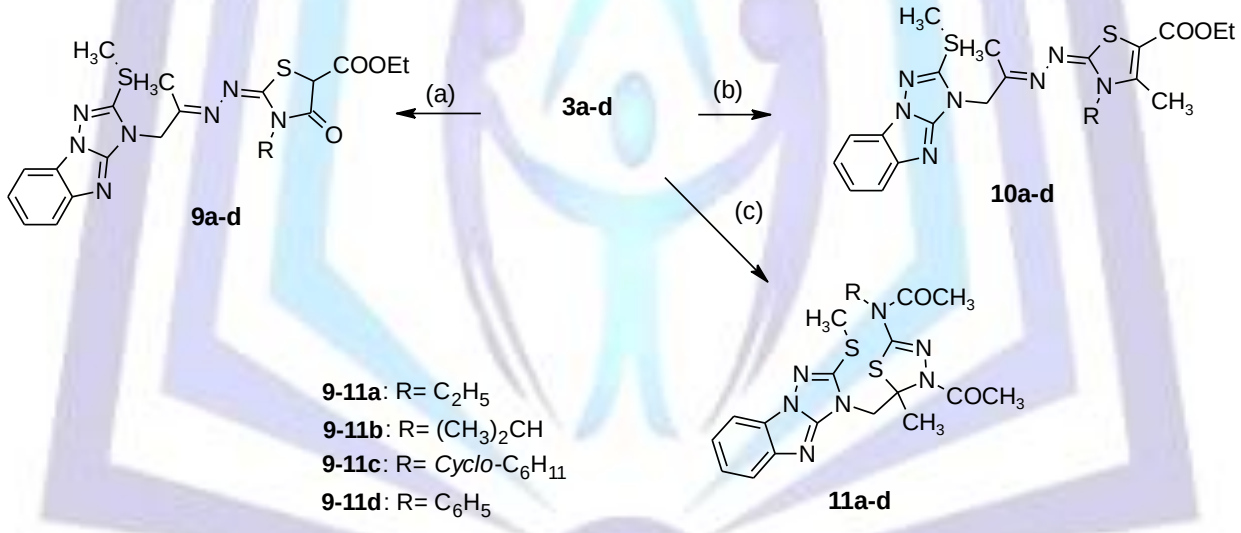



(a): $CH_3I$, $K_2CO_3$, Acetone, (b): $ClCOCH_2CH_3$, $K_2CO_3$, Acetone, (c): $NH_2NH(C=S)NHR$, Ethanol, drops of glacial acetic acid, Reflux, (d): 4-(un)substituted-$C_6H_4$-$COCH_2Br$, NaOAc, Ethanol, Reflux (e): $BrCH_2COOEt$, NaOAc, Ethanol, Reflux.

**Scheme 1**. Synthetic pathway of compounds **(2**, **3a-d**, **4-7**;**a-f** and **8a-d)**[22]




(a): $BrCH(COOEt)_2$, NaOAc, Ethanol, Reflux (b): $CH_3COCHClCOOC_2H_5$, NaOAc, Ethanol, Acetic acid, reflux (c): $CH_3COOCOCH_3$, Reflux.

**Scheme 2**. Synthetic pathway of compounds **(9-11a-d)**

## 2.2. Biological screening

### 2.2.1. Anti-inflammatory activity

In the present work, 29 newly synthesized compounds (**3a-d**, **4a-f**, **5d**, **6d**, **7d**, **8-11;a-d)** were evaluated for their in vivo anti-inflammatory effect by the carrageenan induced paw edema bioassay in rats using INM as a reference drug. Results were presented as the time course dependent size of edema (thickness of right paw) and percentage of edema inhibition at a dose of 0.02 mmole/Kg at time intervals 0.5, 1.0, 2.0, 3.0 and 4.0h, **Table 1**.

The obtained anti-inflammatory activity of the tested compounds could be correlated to structural variations and modifications, taking the edema protection after 4 h time interval as a criterion for comparison relative to INM. The anti-inflammatory activity results revealed that all the test compounds showed a gradual increase of the anti-inflammatory activity up to its maximum at 4 h.

Results of anti-inflammatory activity of key precursors, thiosemicarbazones **(3a-d)** displayed moderate anti-inflammatory activity relative to INM (59.56–64.28 %). Compound **3a** with the smallest substituent (R= $C_2H_5$) was the most

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potent thiosemicarbazone exhibited 64.28% at 4 h interval of the anti-inflammatory activity relative to INM. On the other hand, cyclization of the most potent thiosemicarbazone **(3a)** (R= $C_2H_5$) to thiazoline derivatives **(4a-f)** led to a significant improvement in the anti-inflammatory activity. As noticed, thiazoline derivatives **(4a-f)** exhibited anti-inflammatory (activity relative to INM ranges from (64.28- 92.85%), and maximum inhibition was shown by compound **4b** (R = $C_2H_5$, $R^1$ = Br) (% activity relative to INM = 92.85).

To study the effect of R group at 3-position of thiazolines on the anti-inflammatory activity of substituted thiazoline derivatives, compounds **5d**, **6d** and **7d** (R= i-Pr, cyclo-$C_6H_{11}$, $C_6H_5$, respectively; $R^1$= $NO_2$) were selected as representative examples for the anti-inflammatory assay and compared the results with thiazoline derivative **4d** (R = $C_2H_5$, $R^1$ = $NO_2$); (% of the anti-inflammatory activity relative of INM =66.70 %). Best result was shown when (R = i-Pr); (% of the anti-inflammatory activity relative of INM =73.28 %), which indicates that the steric factor plays an important role in edema inhibition in this series. On the other hand, increasing bulkiness of R group (compounds **6d** and **7d** (R= cyclo-$C_6H_{11}$, $C_6H_5$, respectively); decreases the activity markedly (% of the anti-inflammatory activity relative of INM =62.28, 59.56 %, respectively).

Another cyclization of thiosemicarbazones **(3a-d)** to the thiazoline derivatives **(10a-d)** did not improve the anti-inflammatory activity, and this may attributed to steric factor. The first thiazolidinone derivatives; **(8a-d)** displayed significant anti-inflammatory activity compared to uncyclized parent thiosemicarbazones **(3a-d)**. They exhibited activity relative to INM ranging from (59.56-85.71%), and thiazolidinone series **(8b-d)** (R= i-Pr, cyclo-$C_6H_{11}$, $C_6H_5$, respectively) were the most active.

The anti-inflammatory activity enhanced markedly in case of thiazolidinone derivatives **(9a-d)** compared to the uncyclized thiosemicarbazones **(3a-d)**. The best results were exhibited by compounds **9a-b** (R= $C_2H_5$, i-Pr), showing activity relative to INM, 88.13% and 73.85%, respectively)..While, cyclization to the corresponding thiadiazoline derivatives **(11a-d)** decreased the anti-inflammatory activity (activity relative to INM ranging from 34.28-41.42%).

As a general conclusion, all thiosemicarbazone derivatives **(3a-d)** showed the same activity. The anti-inflammatory effect enhanced in case of thiazoline derivatives **(4;a-f)** more than in thiazoline derivatives **(10a-d)**. Cyclization to the corresponding thiadiazoline **(11a-d)** decreased the anti-inflammatory effect. But cyclization to thiazolidinone derivatives **(8a-d)** and **(9a-d)** enhanced the activity markedly.

**Table 1: Percentage of edema inhibition of compounds (3a-d, 4a-f, 5d, 6d, 7d, 8-11;a-d) and indomethacin.**

| Compd. No. | Edema inhibition (%) ± S.E. | | | | | Activity relative to INM (%) after 4 hrs |
|---|---|---|---|---|---|---|
| | 0.5 h | 1 h | 2 h | 3 h | 4 h | |
| Negative Control | -- | -- | -- | -- | -- | |
| Indomethacin | 36.17±0.16[a] | 70.00±0.16 | 84.61±0.16 | 85.71±0.16 | 87.50±0.16 | 100 |
| 3a | 14.89±0.00 | 23.40±0.16 | 41.07±0.16 | 45.28±0.16 | 56.25±0.28 | 64.28 |
| 3b | 11.48±0.16 | 23.40±0.16 | 41.07±0.16 | 45.28±0.16 | 52.12±0.16 | 59.56 |
| 3c | 11.48±0.16 | 16.80±0.16 | 36.00±0.16 | 40.57±0.16 | 52.12±0.16 | 59.56 |
| 3d | 14.89±0.28 | 16.80±0.16 | 36.00±0.16 | 42.85±0.00 | 52.12±0.16 | 59.56 |
| 4a | 14.89±0.28 | 23.40±0.16 | 41.07±0.16 | 45.28±0.16 | 56.25±0.28 | 64.28 |
| 4b | 32.76±0.28 | 53.40±0.28 | 69.23±0.16 | 73.85±0.16 | 81.25±0.16 | 92.85 |
| 4c | 7.02±0.23 | 40.00±0.20 | 65.38±0.25 | 71.42±0.20 | 68.75±0.23 | 78.57 |
| 4d | 18.51±0.16 | 30.00±0.00 | 46.15±0.00 | 52.42±0.16 | 58.37±0.16 | 66.70 |
| 4e | 7.02±0.23 | 22.60±0.12 | 53.84±0.20 | 67.85±0.43 | 68.75±0.20 | 78.57 |
| 4f | 7.02±0.23 | 17.60±0.14 | 40.46±0.12 | 48.28±0.23 | 57.87±0.23 | 66.13 |
| 5d | 9.75±0.25 | 20.00±0.00 | 42.30±0.32 | 55.42±0.12 | 64.12±0.12 | 73.28 |
| 6d | 14.89±0.00 | 23.40±0.16 | 41.07±0.16 | 47.71±0.16 | 54.25±0.16 | 62.00 |
| 7d | 13.33±0.16 | 13.40±0.16 | 41.07±0.16 | 45.28±0.16 | 52.12±0.16 | 59.56 |
| 8a | 7.87±0.16 | 16.80±0.16 | 36.00±0.16 | 42.85±0.00 | 52.12±0.16 | 59.56 |
| 8b | 1.70±0.23 | 35.00±0.32 | 52.00±0.31 | 59.00±0.42 | 70.37±0.23 | 80.42 |

| | | | | | | |
|---|---|---|---|---|---|---|
| **8c** | 4.16±0.16 | 34.00±0.16 | 53.84±0.16 | 70.00±0.16 | 75.00±0.28 | 85.71 |
| **8d** | 18.51±0.16 | 36.80±0.16 | 56.46±0.33 | 69.14±0.33 | 73.00±0.33 | 83.42 |
| **9a** | 20.20±0.16 | 40.00±0.16 | 59.07±0.16 | 69.14±0.16 | 77.12±0.16 | 88.13 |
| **9b** | 9.79±0.16 | 16.80±0.16 | 41.07±0.16 | 54.85±0.16 | 64.62±0.16 | 73.85 |
| **9c** | 9.79±0.16 | 13.40±0.16 | 41.07±0.16 | 45.28±0.16 | 48.00±0.16 | 54.85 |
| **9d** | 9.79±0.16 | 13.40±0.16 | 41.07±0.16 | 47.71±0.16 | 54.25±0.16 | 62.00 |
| **10a** | 16.66±0.28 | 23.40±0.16 | 41.07±0.16 | 50.00±0.28 | 54.25±0.16 | 62.00 |
| **10b** | 9.79±0.16 | 16.80±0.16 | 41.07±0.16 | 45.28±0.16 | 56.25±0.28 | 64.29 |
| **10c** | 2.91±0.16 | 13.40±0.16 | 28.30±0.16 | 33.42±0.16 | 39.62±0.16 | 45.28 |
| **10d** | 9.79±0.16 | 16.80±0.16 | 36.00±0.16 | 45.28±0.16 | 52.12±0.16 | 59.56 |
| **11a** | 2.91±0.16 | 3.40±0.16 | 20.61±0.16 | 21.42±0.28 | 30.00±0.16 | 34.28 |
| **11b** | 2.91±0.16 | 6.80±0.16 | 26.15±0.16 | 26.28±0.16 | 33.37±0.16 | 38.13 |
| **11c** | 9.79±0.16 | 13.40±0.16 | 20.61±0.16 | 26.28±0.16 | 31.25±0.28 | 35.71 |
| **11d** | 6.25±0.28 | 8.00±0.16 | 18.00±0.16 | 27.14±0.20 | 36.25±0.20 | 41.42 |

[a] Significant difference at $P < 0.01$ vs. control value (student's-t-test).

### 2.2.2. Ulcerogenic effect

The occurrence of gastrointestinal (GI) damage (bleeding and ulceration) is probably among the most prevalent and serious side effects associated with the use of non-steroidal anti-inflammatory drugs (NSAIDs)[23-26]. Observation of the gastrointestinal mucosa for the presence of lesions following oral administration of graded doses (10, 30 and 50 mg/Kg) of the test compounds as well as the reference drug has been taken as an indication for the ulcerogenic effects [27]. Both the frequency of ulceration (expressed as the ratio of ulcerated animals) and the severity of ulceration (expressed as ulcer index) were used for comparison of the test compounds and INM.

The most three active anti-inflammatory compounds (**4b, 8c** and **9a**) was evaluated for their ulcerogenic effects in rats [23, 27]. Results are recorded in **Table 2.** The tested compounds showed superior GI safety profile, since they gave 100% protection in the population of the test animals at oral doses 10, 30 and 50 mg/Kg. INM was found to cause 66.67% and 100% ulceration at 10 and 30 mg/kg, respectively.

**Table 2 : Ulcerogenic effects of compounds 4b, 8c, 9a and indomethacin.**

| Compd. No. | Dose mg/kg | Ratio of ulcerated animals | Ulcer index (mean±S.E) |
|---|---|---|---|
| **Indomethacin** | 10 | 4/6 | 1.20±0.20 |
| | 30 | 6/6 | 1.5±0.51 |
| | 50 | Not tested | ------- |
| **4b** | 10 | 0/6 | 0.00 |
| | 30 | 0/6 | |
| | 50 | 0/6 | |
| **8c** | 10 | 0/6 | 0.00 |
| | 30 | 0/6 | |
| | 50 | 0/6 | |
| **9a** | 10 | 0/6 | 0.00 |
| | 30 | 0/6 | |
| | 50 | 0/6 | |

### 2.2.2. Acute toxicity ($LD_{50}$)

The median lethal doses ($LD_{50}$) of compounds (**4b** and **9a**) were also determined in mice according to a reported method [26] and were found non toxic up to 400mg/kg (i.p.), whereas that of INM equal to 13 mg/kg (i.p.).

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## 2.3. Molecular modeling study

Phospholipase $A_2$ enzymes hydrolyze phospholipids at the sn-2 position. The sn-2 position of phospholipids contains unsaturated fatty acids such as arachidonic acid. Arachidonic acid (AA) when released from the sn-2 position of phospholipids can be converted into eicosanoids through the action of a variety of different downstream enzymes [28, 29] . Eicosanoids are a family of compounds (including prostaglandins and leukotrienes) that are produced by many cell types (such as macrophages) from arachidonic acid. There has been considerable pharmaceutical interest in characterizing phospholipase $A_2$ enzymes owing to their role in the production of lipid mediators in inflammation, such as arachidonic acid and its eicosanoid derivatives prostaglandin and leukotriene. The anti-inflammatory response of non-steroidal anti-inflammatory drugs (NSAIDs) has been attributed to their binding to PLA2 [30, 31] and COX enzymes [32, 33].

Indomethacin is a well-known non-selective COX inhibitor [34]. Also, indomethacin has also been reported to inhibit group II PLA2 but its unusual kinetic properties [35, 36] could not be explained. The studies indicated very different binding characteristics when compared with other ligands that bind to PLA2 enzymes at the substrate-binding site [37]. It was reported that, indomethacin interacts effectively with the important residues of active site including, Asp 49 and His 48. O3 atom of the carboxylic group of indomethacin interacts with the catalytically important water molecule OW 18 in a manner similar to other inhibitors [30, 31] and substrate analogues [38-41]. In turn, OW 18 is hydrogen bonded to three amino acids namely; His 48 Nδ1, Asp 49 Oδ1 and Cys 45 O. The carboxylic oxygen atom O3 also forms hydrogen bond with another water molecule OW 41 which in turn is hydrogen bonded to Gly 30 N. In addition it forms a hydrogen bond with one more water molecule OW 158. The carboxylic group oxygen atom O2 of indomethacin forms hydrogen bond with the side chain of Lys 69 at a distance of 2.6A°. In addition, indomethacin forms an array of van der Waals contacts with residues Pro 56, Cys 61 and Pro 68, **Figure 1**.

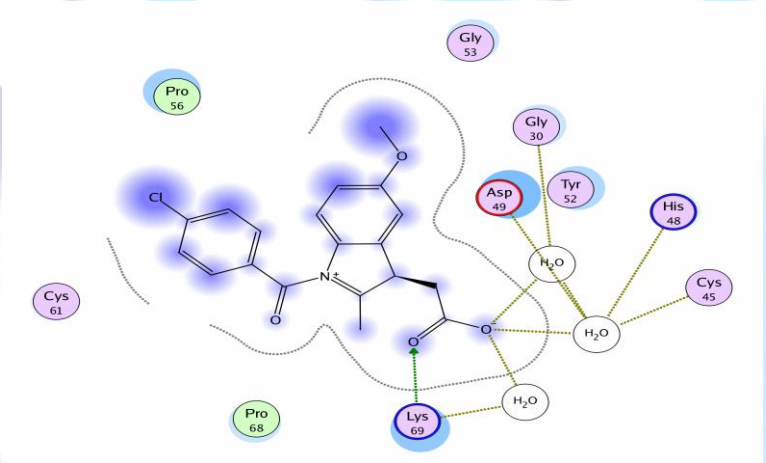


**Figure 1**: Diagrammatic representation of indomethacin interaction to Phospholipase A2.

Because the similarity in the structure, function and pharmacological effects between human secretory phospholipase A2 and snake venom secretory phospholipase A2, snake venom secretory phospholipase A2 has been used for molecular target to evaluate the anti-inflammatory effects of the synthesized compounds .

Docking results for 29 of the synthesized compounds were given in, **Table 3** with the corresponding activities towards carrageenan induced paw edema in rats biological assay. X-ray crystal structure of Phospholipase A2 enzyme was taken from PDB entry 2OTH, having resolution of 1.4 Å.The synthesized compounds have been docked in the Phopholipase A2 enzyme, using the MOE parameters determined above. The binding affinity of the docked molecules was evaluated by (S) kcal/mole, hydrogen bonds in addition to the hydrophobic interactions at the enzyme pocket. The pose of each compound which reveals the highest binding affinity are presented in **Table 3**.

Each pose generated by the placement methodology is subjected to scoring in an effort to identify the most favorable poses. London dG (S) scoring method was used in this study which estimates the free energy of binding of the ligand from a given pose. The higher the absolute value of dG, the better affinity between the ligand and the enzyme active site could be predicted. A rough correlation was made between the binding free energy (dG) values of the synthesized compounds and their anti-inflammatory activity. The docking results for the synthesized compounds revealed that most of the compounds bind to Phospholipase A2 enzyme through hydrogen bond to the residue Lys 69. The benzoimidazotriazole ring in those compounds was oriented in the hydrophobic cage of receptor wherein, it is interacted with residues Gly 30, Asp 49, Tyr 52 and Gly 53.

These residues may be involved in flexible alignment of compounds in the hydrophobic cage of enzyme. Docking analysis revealed that the most active compound **4b** interacts with phospholipase A2 enzyme through hydrogen bonding between the nitrogen of the 2-iminothiazoline ring with Lys 69 and hydrophobic interactions with Gly 30, Asp 49, Tyr 52 and Gly 53, **Figure 2**. The final score of the best conformer pose for compound **4b** was

-13.6896 which indicates compound **4b** is active at lowest energy of conformation. There exist a good correlation between percentage activity and energy of conformation which suggests that the most active compound **4b** has lowest score, **Table 3**.

The hydrogen bonding score was found to be 27.5% and interatomic distance was recorded to be 2.93 Å. The C-4 substituted 2-iminothiazoline imparts flexibility to the compound and interacted with the aforementioned residues through bonding and nonbonding interactions. Apparently, the residue Lys 69 may contribute to the binding and stabilization of compound **4b** in the cavity space of phospholipase A2. Furthermore compounds **8b**, **8c** and **8d** interacted with phospholipase A2 enzyme through hydrogen bonding with residue Lys 69 in which hydrogen bond scores were observed to be 24%, 21% and 35% respectively. In compound **8c**, N at position 4 of benzoimidazotriazole ring binds through water molecule to the amino acid residue Gly 30, which may offer proper fit between drug and enzyme, **Figure 2**. The final score for compounds **8b**, **8c** and **8d** was observed to be -9.7217, -11.6357 and -11.0337 respectively which suggests that compound **8c** elicited superior biological response as compared to compounds **8b** and **8d** due to low energy of conformation, as shown in **Table 3**.

Second most active compound **9a** interacts with phospholipase A2 enzyme through hydrogen bonding wherein again the nitrogen atom of the 2-imino group attached to the thiazoline ring forms a hydrogen bond with the essential Lys 69 residue. Also compound **9a** forms different Van der Waals interactions with Gly 30, Trp 31, Asp 49 and Tyr 52, **Figure 2**. The lower activity of some compounds, e.g. compound **3a,** despite having lower final scores, may be attributed to the lower lipophilicity of these compounds, for example although compound **3a** has final score function of -9.0637 compared to compound **9b**, its final score is -8.8890, compound **9b** has higher activity as compound **3a** has Clog P of 2.83 compared to compound **9b** which Clog P of 4.03. In conclusion, results of in vivo anti-inflammatory activity complies with results of the docking study.

**Table 3: Scoring Function database of the tested ligands.**

| Compound no. | S kcal/mole | Activity relative to INM after 4 hrs (%) | Compound no. | S kcal/mole | Activity relative to INM after 4 hrs (%) |
|---|---|---|---|---|---|
| **3a** | -9.0637 | 64.28 | 8c | -11.6357 | 85.71 |
| **3b** | -8.1275 | 59.56 | 8d | -11.0337 | 83.42 |
| **3c** | -8.3334 | 59.56 | 9a | -12.4889 | 88.13 |
| **3d** | -8.1451 | 59.56 | 9b | -8.8890 | 73.85 |
| **4a** | -8.0188 | 64.28 | 9c | -7.8615 | 54.85 |
| **4b** | -13.6896 | 92.85 | 9d | -8.1591 | 62.00 |
| **4c** | -9.0985 | 78.57 | 10a | -8.1837 | 62.00 |
| **4d** | -8.5386 | 66.70 | 10b | -8.6284 | 64.29 |
| **4e** | -9.0762 | 78.57 | 10c | -7.5767 | 45.28 |
| **4f** | -8.6695 | 66.13 | 10d | -7.7030 | 59.56 |
| **5d** | -8.8368 | 73.28 | 11a | -7.2707 | 34.28 |
| **6d** | -7.8916 | 62.00 | 11b | -6.9096 | 38.13 |
| **7d** | -7.6028 | 59.56 | 11c | -6.8610 | 35.71 |
| **8a** | -7.0167 | 59.56 | 11d | -6.9945 | 41.42 |
| **8b** | -9.7217 | 80.42 | INM | - | 100 |

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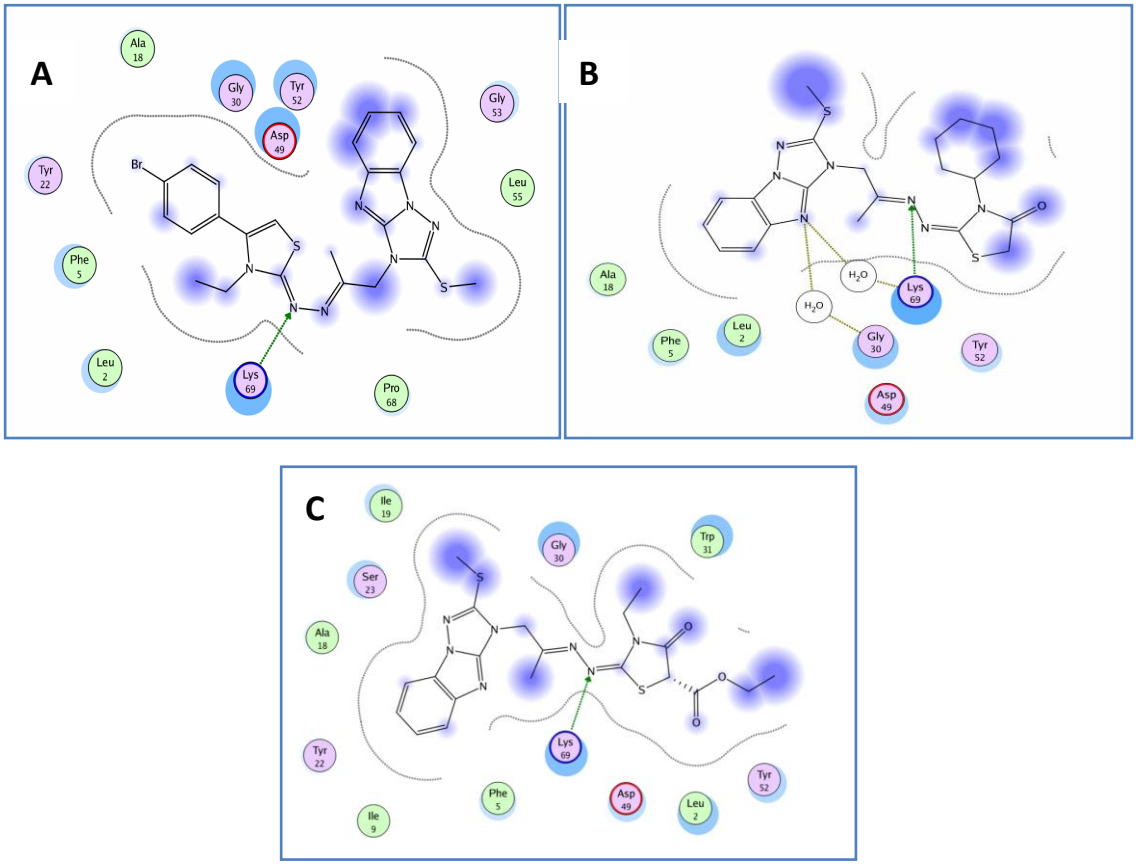


**Figure 2**: Docked complexes of compounds **4b**, **8c** and **9a** into PLA-2 active site.
**A:** 2D structure of compound (**4b**) docked into PLA-2 active site **B:** 2D structure of compound (**8c**) docked into PLA-2 active site
**C:** 2D structure of compound (**9a**) docked into PLA-2 active site

Melting points were determined on an electrothermal melting point apparatus [Stuart Scientific, model SMP3, Staffordshire, UK], and were uncorrected. Pre-coated silica gel plates (kieselgel 0.25 mm, 60G F254, Merck, Darmstadt, Germany) were used for TLC monitoring of reactions. The developing solvent systems of Hexane:ethylacetate (4:6 and 6:4 v/v) were used and the spots were detected at 254 nm wavelength using ultraviolet lamp (Spectroline, model CM-10, Seattle, USA). IR spectra (KBr discs) were recorded on a Shimadzu IR-470 spectrometer (Shimadzu, Kyoto, Japan) at Faculty of Pharmacy, Assiut University, Assiut. $^{1}$H-NMR Spectra were scanned on Varian EM-360 L NMR spectrometer (60 MHz, Varian, CA, USA) at Faculty of Pharmacy, Assiut University, Assiut. Chemical shifts were expressed in δ-value (ppm) relative to TMS as an internal standard, using $CDCl_3$, unless otherwise specified, as a solvent, and deuterium oxide was used for the detection of exchangeable protons.

Mass spectra were recorded with Gas Chromatography Mass, Quadruple-2010 Plus (Shimadzu, Kyoto, Japan) at the unit of Microanalysis, Faculty of Science, Cairo University. Elemental microanalyses were performed on a Vario elemental analyzer III (Vario, Hanau, Germany) at the unit of Microanalysis, Faculty of Science, Cairo University. Most of the required chemicals were of the commercial grade: ethyl-2-chloroacetoacetate, diethyl-2-bromomalonate (Merck, Germany) acetic anhydride, sodium acetate and chloroacetone (obtained from the local market). Thiosemicarbazone derivatives **(3a-d)** were prepared according to our previous work[22].

## 3.1. Chemistry

### General method for synthesis of 3-[(3-Alkyl(aryl)-5-ethoxycarbonyl-4-oxo-thiazolidin-2-ylidene)hydrazonopropylidenyl]-2-(methylthio)-3H-[1,2,4]triazolo[1,5-a]benzimidazole, compounds (9a-d):

To a mixture of 1-[2-(methylthio)-3H-[1,2,4]triazolo[1,5-a]benzimidazol-3-yl]acetone $N^4$-alkyl(aryl) thiosemicarbazones **(3a-d)** (1mmole), diethyl-2-bromomalonate (0.85 mL, 5 mmole), and anhydrous sodium acetate (1.64 g, 20 mmole) in absolute ethanol (80 mL) was refluxed for 20-24 h with continuous stirring. The solvent was evaporated, and the obtained solid was triturated with cold water followed by cold diethyl ether, dried, and recrystallized from aqueous ethanol.

**3-[(3-Ethyl-5-ethoxycarbonyl-4-oxo-thiazolidin-2-ylidene)hydrazonopropylidenyl]-2-(methylthio)-3H-[1,2,4]triazolo[1,5-a]benzimidazole (9a):**

M.p. 133-135°C, yield 80%, $R_f$: 0.30 (n-hexane : ethyl acetate 6:4); IR (KBr) ύ ($cm^{-1}$): 1729 (C=O), 1619, 1576, 1484 (C=N/C=C).$^1$H-NMR ($CDCl_3$): (ppm) 1.00-1.50 (two overlapping triplets, 6H, $NCH_2C\underline{H_3}$ &$OCH_2C\underline{H_3}$), 2.05 (s, 3H, S-$CH_3$), 2.70 (s, 3H, N=C-$CH_3$), 3.80 (s, 1H, CH of thiazolidinone), 3.70-4.60 (two overlapping quartets, 4H, N-$C\underline{H_2}$-$CH_3$&O-$C\underline{H_2}$-$CH_3$), 5.05 (s, 2H, N=C-$CH_2$), 7.20-7.80 (m, 4H, Ar-H). EI-MS m/z: 474.00 (M+, 28.57%), 95 (100). Anal. Calcd. (%) for $C_{20}H_{23}N_7O_3S_2$: C, 50.72; H, 4.90; N, 20.70. Found: C, 50.65; H, 4.76; N, 20.52.

**3-[(3-Isopropyl-5-ethoxycarbonyl-4-oxo-thiazolidin-2-ylidene) hydrazonopropylidenyl]-2-(methylthio)-3H-[1,2,4]-triazolo[1,5-a]benzimidazole (9b):**

M.p. 144-146°C, yield 88%, $R_f$: 0.41 (n-hexane : ethyl acetate 6:4); IR (KBr) ύ ($cm^{-1}$): 1720 (C=O), 1626, 1584, 1474 (C=N/C=C).$^1$H-NMR ($CDCl_3$): (ppm) 0.90-1.60 (m, 9H, $CH(C\underline{H_3})_2$&O-$CH_2$-$C\underline{H_3}$), 2.10 (s, 3H, S-$CH_3$), 2.70 (s, 3H, N=C-$CH_3$), 3.60 (s, 1H, CH of thiazolidinone), 3.80-4.50 (m, 3H, O-$C\underline{H_2}$-$CH_3$, -$C\underline{H}(CH_3)_2$), 5.10 (s, 2H, N=C-$CH_2$), 7.20-7.90 (m, 4H, Ar-H). Anal. Calcd. (%) for $C_{21}H_{25}N_7O_3S_2$: C, 51.73; H, 5.17; N, 20.11. Found: C, 51.59; H, 4.95; N, 19.98.

**3-[(3-Cyclohexyl-5-ethoxycarbonyl-4-oxo-thiazolidin-2-ylidene) hydrazonopropylidenyl]-2-(methylthio)-3H-[1,2,4]-triazolo[1,5-a]benzimidazole (9c):**

M.p. 166-168°C, yield 85%, $R_f$: 0.47 (n-hexane : ethyl acetate 6:4); IR (KBr) ύ ($cm^{-1}$): 1705 (C=O), 1618, 1555, 1482 (C=N/C=C).$^1$H-NMR (DMSO-$d_6$): (ppm) 0.90-1.90 (m, 13H, O-$CH_2$-$C\underline{H_3}$, $(CH_2)_5$ of cyclohexyl), 2.05 (s, 3H, S-$CH_3$), 2.70 (s, 3H, N=C-$CH_3$), 3.65(s, 1H, CH of thiazolidinone), 3.80-4.70 (m, 3H, NC$\underline{H}$ of cyclohexyl, O-$C\underline{H_2}$-$CH_3$), 5.05 (s, 2H, N=C-$CH_2$), 7.10-7.90 (m, 4H, Ar-H). Anal. Calcd. (%) for $C_{24}H_{29}N_7O_3S_2$: C, 54.63; H, 5.54; N, 18.58. Found: C, 54.45; H, 5.33; N, 18.46. Anal. Calcd. for: $C_{14}H_{12}N_2O_4$ (272.26): C, 61.76; H, 4.44; N, 10.29%. Found: C, 61.80; H, 4.52; N, 10.35%.

**3-[(3-Phenyl-5-ethoxycarbonyl-4-oxo-thiazolidin-2-ylidene) hydrazonopropylidenyl]-2-(methylthio)-3H-[1,2,4]triazolo[1,5-a]benzimidazole (9d):**

M.p. 155-157°C, yield 83%, $R_f$: 0.44 (n-hexane : ethyl acetate 6:4), crystallized from ethyl acetate; IR (KBr) ύ ($cm^{-1}$): 1731 (C=O), 1621, 1574, 1483 (C=N/C=C).$^1$H-NMR ($CDCl_3$): (ppm) 1.3 (t, 3H, O-$CH_2$-$C\underline{H_3}$), 1.85 (S, 3H, S-$CH_3$), 2.65 (s, 3H, N=C-$CH_3$), 3.80 (s, 1H, CH of thiazolidinone), 4.30 (q, 2H, O-$C\underline{H_2}$-$CH_3$), 4.95 (s, 2H, N=C-$CH_2$), 7.00-7.90 (m, 9H, Ar-H). Anal. Calcd. (%) for $C_{25}H_{25}N_7O_2S_2$: C, 55.26; H, 4.44; N, 18.80. Found: C, 55.11; H, 4.34; N, 18.66.

## General method for synthesis of 3-[(5-Ethoxycarbonyl-4-methyl-3-substituted-thiazolin-2-ylidene) hydrazonopropylidenyl]-2-(methylthio)-3H-[1,2,4]triazolo-[1,5-a]benzimidazole, compounds (10a-d):

To a mixture of 1-[2-(methylthio)-3H-[1,2,4]triazolo[1,5-a]benzimidazol-3-yl]acetone $N^4$-alkyl(aryl) thiosemicarbazones **(3a-d)** (1mmol), ethyl-2-chloroacetoacetate (0.21 mL, 1.5 mmole), and anhydrous sodium acetate (0.246 g, 3 mmole) in absolute ethanol (30 mL), glacial acetic acid (0.5 mL) was added as a catalyst. The reaction mixture was heated under reflux for 22-24 h with continuous stirring, partially concentrated under reduced pressure, and then allowed to attain room temperature; crushed ice water was poured on it. The precipitated solid was collected by filtration, washed thoroughly with water, dried, and recrystallized from aqueous ethanol.

**3-[(5-Ethoxycarbonyl-4-methyl-3-ethyl-thiazolin-2-ylidene) hydrazonopropylidenyl]-2-(methylthio)-3H-[1,2,4]triazolo[1,5-a]benzimidazole (10a):**

M.p. 147-149°C, yield 84%, $R_f$: 0.44 (n-hexane : ethyl acetate 6:4); IR (KBr) ύ ($cm^{-1}$): 1683 (C=O), 1625, 1590, 1474 (C=N/C=C).$^1$H-NMR ($CDCl_3$): (ppm) 1.10-1.50 (m, 6H, $NCH_2C\underline{H_3}$&$OCH_2C\underline{H_3}$), 2.05 (s, 3H, S-$CH_3$), 2.55 (s, 3H, C4-$CH_3$ of thiazoline), 2.70 (s, 3H, N=C-$CH_3$), 4.00 (q, 2H, N-$C\underline{H_2}$-$CH_3$), 4.35 (q, 2H, O-$C\underline{H_2}$-$CH_3$), 5.10 (s, 2H, N=C-$CH_2$), 7.20-8.00 (m, 4H, Ar-H). Anal. Calcd. (%) for $C_{21}H_{25}N_7O_2S_2$: C, 53.48; H, 5.34; N, 20.79. Found: C, 53.24; H, 5.18; N, 20.57.

**3-[(5-Ethoxycarbonyl-4-methyl-3-isopropyl-thiazolin-2-ylidene) hydrazonopropylidenyl]-2-(methylthio)-3H-[1,2,4]-triazolo[1,5-a]benzimidazole (10b):**

M.p. 150-152°C, yield 88%, $R_f$: 0.35 (n-hexane : ethyl acetate 6:4); IR (KBr) ύ ($cm^{-1}$): 1682 (C=O), 1624, 1590, 1495 (C=N/C=C).$^1$H-NMR ($CDCl_3$): (ppm) 1.30 (t, 3H, O-$CH_2C\underline{H_3}$), 1.50 (d, 6H, -$CH(C\underline{H_3})_2$), 2.05 (s, 3H, S-$CH_3$), 2.55 (s, 3H, C4- $CH_3$ of thiazoline), 2.60 (s, 3H, N=C-$CH_3$), 4.00-4.70 (m, 3H, -$C\underline{H}(CH_3)_2$, O-$C\underline{H_2}$-$CH_3$), 5.00 (s, 2H, N=C-$CH_2$), 7.10-7.70 (m, 4H, Ar-H). Anal. Calcd. (%) for $C_{22}H_{27}N_7O_2S_2$: C, 54.41; H, 5.60; N, 20.19. Found: C, 54.23; H, 5.40; N, 19.90.

**3-[(5-Ethoxycarbonyl-4-methyl-3-cyclohexyl-thiazolin-2-ylidene) hydrazonopropyliden-yl]-2-(methylthio)-3H-[1,2,4]triazolo[1,5-a]benzimidazole (10c):**

M.p. 122-124°C, yield 90%, $R_f$: 0.50 (n-hexane : ethyl acetate 6:4),; IR (KBr) ύ ($cm^{-1}$): 1699 (C=O), 1635, 1560, 1487 (C=N/C=C).$^1$H-NMR ($CDCl_3$): (ppm) 1.00-1.90 (m, 13H, -$CH_2$-$C\underline{H_3}$, $(CH_2)_5$ of cyclohexyl), 2.00 (s, 3H, S-$CH_3$), 2.55 (s, 3H, C4- $CH_3$ of thiazoline), 2.70 (s, 3H, N=C-$CH_3$), 3.70-4.20 (m, 1H, NCH of cyclohexyl), 4.25 (q, 2H, O-$C\underline{H_2}$-$CH_3$), 5.05 (s, 2H, N=C-$CH_2$), 7.10-7.90 (m, 4H, Ar-H). EI-MS m/z: 525 (M+, 92.31), 226 (100). Anal. Calcd. (%) for $C_{25}H_{31}N_7O_2S_2$: C, 57.12; H, 5.94; N, 18.65. Found: C, 56.90; H, 5.84; N, 18.44.

**3-[(5-Ethoxycarbonyl-4-methyl-3-phenyl-thiazolin-2-ylidene) hydrazonopropylidenyl]-2-(methylthio)-3H-[1,2,4]triazolo[1,5-a]benzimidazole (10d):**

M.p. 130-132°C, yield 83%, $R_f$: 0.48 (n-hexane : ethyl acetate 6:4); IR (KBr) ύ ($cm^{-1}$): 1698 (C=O), 1626, 1545, 1495 (C=N/C=C).$^1$H-NMR ($CDCl_3$): (ppm) 1.45 (t, 3H, -$CH_2$-C$\underline{H_3}$), 1.85 (s, 3H, S-$CH_3$), 2.35 (s, 3H, C4-$CH_3$ of thiazoline), 2.70 (s, 3H, N=C-$CH_3$), 4.35 (q, 2H, O-C$\underline{H_2}$-$CH_3$), 5.00 (s, 2H, N=C-$CH_2$), 7.00-7.90 (m, 9H, Ar-H). Anal. Calcd. (%) for $C_{25}H_{25}N_7O_2S_2$: C, 57.78; H, 4.85; N, 18.87. Found: C, 57.67; H, 4.68; N, 18.70.

## General method for synthesis of 3-[3-Acetyl-5-(N-substituted acetamido)-2-methyl-2,3-dihydro-[1,3,4]-thiadiazol-2-yl]methyl-2-(methylthio)-3H-[1,2,4]triazolo[1,5-a]benzimidazole, compounds (11a-d):

A solution of 1-[2-(methylthio)-3H-[1,2,4]triazolo[1,5-a]benzimidazol-3-yl]acetone $N^4$-alkyl(aryl) thiosemicarbazones **(3a-d)** (1mmole) in acetic anhydride (12 mL, 120 mmole) was heated under reflux for 10-15 h with continuous stirring and then allowed to attain room temperature. The reaction mixture was slowly added to 400 mL of ice-cooled water and then stirred at room temperature for 1 h. The separated product was collected by filtration, washed thoroughly with water, dried, and recrystallized from appropriate solvent.

**3-[3-Acetyl-5-(N-ethyl acetamido)-2-methyl-2,3-dihydro-[1,3,4]-thiadiazol-2-yl]-methyl-2-(methylthio)-3H-[1,2,4]triazolo[1,5-a]benzimidazole (11a):**

M.p. 130-134°C, yield 44%, $R_f$: 0.44 (n-hexane : ethyl acetate 4:6), crystallized from ethanol; IR (KBr) ύ ($cm^{-1}$): 1664 (C=O), 1611, 1571, 1482 (C=N/C=C).$^1$H-NMR ($CDCl_3$): (ppm) 0.95 (t, 3H, N-$CH_2$C$\underline{H_3}$), 2.05 (s, 3H, S-$CH_3$), 2.10, 2.30 (two s, each 3H, two $COCH_3$), 2.65 (s, 3H, C2-$CH_3$ of thiadiazoline), 3.40 (q, 2H, N-C$\underline{H_2}$-$CH_3$), 4.35, 5.25 (pair of doublets, 2H, N-$CH_2$-C), 7.00-8.00 (m, 4H, Ar-H). EI-MS m/z: 446.00 ($M^+$, 100). Anal. Calcd. (%) for $C_{19}H_{23}N_7O_2S_2$: C, 51.22; H, 5.20; N, 22.01. Found: C, 51.20; H, 5.00; N, 21.90.

**3-[3-Acetyl-5-(N-isopropyl acetamido)-2-methyl-2,3-dihydro-[1,3,4]-thiadiazol-2-yl]-methyl-2-(methylthio)-3H-[1,2,4]triazolo[1,5-a]benzimidazole (11b):**

M.p. 120-122°C, yield 45%, $R_f$: 0.48 (n-hexane : ethyl acetate 4:6), crystallized from ethyl acetate; IR (KBr) ύ ($cm^{-1}$): 1659 (C=O), 1615, 1564, 1486 (C=N/C=C).$^1$H-NMR ($CDCl_3$): (ppm) 0.90 (d, 6H, -CH(C$\underline{H_3}$)$_2$), 1.75 (s, 3H, S-$CH_3$), 2.30 (s, 6H, two $COCH_3$), 2.65 (s, 3H, C2-$CH_3$ of thiadiazoline), 4.10-4.50 (m, 1H, -C$\underline{H}$(CH$_3$)$_2$), 4.40, 5.50 (pair of doublets, 2H, N-$CH_2$-C), 7.10-7.80 (m, 4H, Ar-H). Anal. Calcd. (%) for $C_{20}H_{25}N_7O_2S_2$: C, 52.27; H, 5.48; N, 21.33. Found: C, 52.10; H, 5.33; N, 21.25.

**3-[3-Acetyl-5-(N-cyclohexyl acetamido)-2-methyl-2,3-dihydro-[1,3,4]-thiadiazol-2-yl]-methyl-2-(methylthio)-3H-[1,2,4]triazolo[1,5-a]benzimidazole (11c):**

M.p. 111-113°C, yield 50%, $R_f$: 0.50 (n-hexane : ethyl acetate 4:6), crystallized from ethyl acetate; IR (KBr) ύ ($cm^{-1}$): 1669 (C=O), 1620, 1560, 1480 (C=N/C=C).$^1$H-NMR ($CDCl_3$): (ppm) 0.50-1.70 (m, 10H, $(CH_2)_5$ of cyclohexyl), 1.80 (s, 3H, S-$CH_3$), 2.35 (s, 6H, two $COCH_3$), 2.70 (s, 3H, C2-$CH_3$ of thiadiazoline),3.50-4.10 (m, 1H, NCH of cyclohexyl), 4.35, 5.60 (pair of doublets, 2H, N-$CH_2$-C), 7.20-7.80 (m, 4H, Ar-H). Anal. Calcd. (%) for $C_{23}H_{29}N_7O_2S_2$: C, 55.29; H, 5.85; N, 19.62. Found: C, 54.98; H, 5.77; N, 19.55.

**3-[3-Acetyl-5-(N-phenylacetamido)-2-methyl-2,3-dihydro-[1,3,4]-thiadiazol-2-yl]-methyl-2-(methylthio)-3H-[1,2,4]triazolo[1,5-a]benzimidazole (11d):**

M.p. 142-144 °C, yield 55%, $R_f$: 0.47 (n-hexane : ethyl acetate 4:6), crystallized from ethanol; IR (KBr) ύ ($cm^{-1}$): 1662 (C=O), 1620, 1500, 1480 (C=N/C=C).$^1$H-NMR ($CDCl_3$): (ppm) 1.85 (s, 6H, $COCH_3$&S-$CH_3$), 2.25 (s, 3H, -N(Ph)$COCH_3$), 2.65 (s, 3H, C2-$CH_3$ of thiadiazoline), 4.40, 5.20 (pair of doublets, 2H, N-$CH_2$-C), 6.70-8.00 (m, 9H, Ar-H). Anal. Calcd. (%) for $C_{23}H_{23}N_7O_2S_2$: C, 55.96; H, 4.70; N, 19.86. Found: C, 55.70; H, 4.55; N, 19.67.

## 3.2. Biological screening

### 3.2.1. Anti-inflammatory activity

Male adult albino rats and mice were obtained from the animal house, Faculty of Medicine, Assiut University. Indomethacin (INM) (Liometacin® vial, Nile Company, Egypt), carrageenan (Sigma, USA), sodium carboxymethylcellulose (NaCMC) (El Nasr Pharm. Company, Egypt) and normal saline (Almottahedoon Pharma Company, Egypt) were obtained from the local market. Animals were housed in separate cages, 3 animals each, in temperature-controlled rooms at 25˚C. Animals were allowed free access to food and water and maintained at a 12 h light/dark cycle. Work was conducted in accordance with the internationally accepted principles for laboratory animals' use and care as found in the European Community Guidelines [42].

The anti-inflammatory activity of the synthesized compounds (**3a-d**, **4a-f**, **5d**, **6d**, **7d**, **8-11; a-d)** was determined according to paw induced edema method in comparison to indomethacin as a reference drug. The test is based on the pedal inflammation in rat paws induced by subplantar injection of carrageenan suspension (0.2 mL of 1% solution in normal saline) into the right hind paw of the rats.

Male adult albino rats (120-150 g) were divided into groups, each of four animals, they were fed ad libitum with rodent's chow and allowed free access to drinking water. The thickness of rat paw was measured by a Vernier caliper (SMIEC, China) before and 1h after carrageenan injection to detect the carrageenan induced inflammation. Each test compound at a dose of 0.02 mmole/Kg (in 1% sodium carboxymethylcellulose (NaCMC) solution in normal saline) was injected i.p. to different groups of rats. Control group received a vehicle (1% NaCMC solution in normal saline), while reference group received indomethacin i.p. at 0.02 mmole/Kg.

The difference between the thicknesses of the two paws was taken as a measure of edema. The measurement was carried out at 0.5, 1.0, 2.0, 3.0 and 4.0h after injection of the test compounds, reference drug, and control. Results are listed in **Table 1.** The percentages of edema inhibition were calculated according to the following equation:

$$\% \text{ Edema inhibition } = \frac{(V_R - V_L)\text{ control} - (V_R - V_L)\text{ treated}}{(V_R - V_L)\text{ control}} \text{ x } 100$$

Where, $V_R$: Average right paw thickness, $V_L$: Average left paw thickness.

### 3.2.2. Ulcerogenic effect

The ulcerogenecity of the most active compounds (**4b, 8c** and **9a**), regarding their anti-inflammatory activities was carried out according to a reported method [23] on adult male albino rats. Male albino rats were fasted for 24 h. The test compounds and indomethacin were administered orally at doses of 10, 30, and 50 mg/Kg to groups of rats each of 6 animals. After 6 h, the animals were sacrificed, the stomachs were removed and washed with saline, Stomachs of each group and gastric lesions on the mucosa were examined grossly by naked eye or under a binocular magnifier. "Ulcer" was defined as at least one lesion that was 0.5 mm or more in length. All lesions of more than 0.1 mm in length were summed to obtain the ulcer index. The results were listed in **Table 2**.

### 3.2.3. Acute toxicity ($LD_{50}$)

The median lethal dose ($LD_{50}$) of the most active and relatively safe compounds (**4b** and **9a**) was determined in mice[26]. Groups of male adult albino mice, each of four animals (25-30 g), were injected i.p. with graded doses of the test compounds. The percentage mortality in each group of animals was determined 72 h latter to injection.

## 4. Molecular docking:

The docking studies were carried out on Dell Precision™ T3600 Workstation [Intel Xeon E5-1660 3.3GHz, 16GB 1600MHz DDR3, ECC RDIMM 1TB (7200RPM), 1GB NVIDIA Quadro 2000, Windows 7 Professional (64 Bit)]. Molecular Operating Environment (MOE) package version 2011.10 [43] was used for performing docking studies. Docking procedures were performed using Phospholipase A2 as receptor downloading its structure from the Protein Data Bank (PDB) PDB code 2OTH at 2.9 °A resolution.

The original 2OTH PDB file contained crystallized beside the receptor chain and the bound indomethacin- nimesulide, acetonitrile and the solvation system. The receptor (first chain) and the bound indomethacin were kept beside only the waters in the active site, and the nimesulide and acetonitrile beside the rest of the solvation chain were removed. All the 12 Ligands were built in ChemBioDraw Ultra 12.0, and saved in the mol format for further preparation in the MOE. In MOE, the ligands were extracted from their .mol files, hydrogens were added, and energy minimization was performed using the MMFF94 force field [44]. Steepest descent algorithm was used for minimization, followed by conjugate gradient method, until it reached an RMS (root mean square) gradient of 0.00001 kcal/mol/Å.

A database of the ligands was generated for further docking studies in the target receptor. The standard protocol of the procedure in MOE 2011 was applied in this work. The Alpha Triangle placement which derives poses by random superposition of ligand atom triplets alpha sphere dummies in the receptor site is to determine the poses. The London dG scoring function estimates the free energy of binding of the ligand from a given pose. Refinement of the results using MMFF94 force field, and rescoring of the refined results using The London dG scoring function was applied. The output database dock file, **Table 3**, was created with different poses for each ligand and arranged according to the final score function (S), which is the score of the last stage that was not set to none.

The database browser was used for the visual inspection of different poses for each ligand and the best poses were chosen. **Figure 2** shows interaction of some Ligands with PLA2.

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