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FULL PAPER

# Exploration of imidazole and imidazopyridine dimers as anticancer agents: Design, synthesis, and structure–activity relationship study

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## Abstract

Dimerization of proteins/receptors plays a critical role in various cellular processes, including cell proliferation and differentiation. Therefore, targeting such dimeric proteins/receptors by dimeric small molecules could be a potential therapeutic approach to treating various diseases, including inflammation-associated diseases like cancer. A novel series of bis-imidazoles (**13–18**) and bis-imidazo[1,2-*a*]pyridines (**19–28**) were designed and synthesized from Schiff base dimers (**1–12**) for their anticancer activities. All the synthesized compounds were screened for anticancer activities against three cancer cell lines, including cervical (HeLa), breast (MDA-MB-231), and renal cancer (ACHN). From structure–activity relationship studies, imidazo[1,2-*a*]pyridines (**19–28**) showed remarkable cytotoxic activities, with compounds **19** and **24** showing the best inhibitory activities against all three cell lines. Especially, both **19** and **24** were very effective against the breast cancer cell line (**19**, $GI_{50}$ = 0.43 μM; **24**, $GI_{50}$ = 0.3 μM), exceeding the activity of the control adriamycin ($GI_{50}$ = 0.51 μM). The in vivo anticancer activity results of compounds **19** and **24** were comparable with those of the animals treated with the standard drug tamoxifen. Therefore, the dimeric imidazo[1,2-*a*]pyridine scaffold could serve as a potential lead for the development of novel anticancer agents.

KEYWORDS

anticancer activity, bis-imidazoles, bis-imidazopyridines, cytotoxicity studies, Schiff base dimers

## 1 | INTRODUCTION

Dimerization of proteins/receptors plays critical roles in various cellular processes, including cell proliferation and differentiation. Dimers exist as homodimers, physical association of two identical proteins or heterodimers, or interaction of two different proteins. In some cases, dimerization is thought to be important for receptor function. For example, the cellular growth factors vascular endothelial growth factor and platelet-derived growth factor are monomers in the cell membrane, but ligand binding induces dimerization of these receptors.[1,2] Structural studies have shown that certain cytokines, such as human growth hormone and erythropoietin are bivalent, and one ligand binds simultaneously to two receptors and creates a 1:2 ligand–receptor complex.[3,4] Upon ligand binding, the estrogen receptor (ER) isoforms ERα and ERβ form homo- or heterodimers.[5–7] Heat shock protein 90, a chaperone protein which is responsible for the maturation of numerous signaling proteins including the proteins required for tumor growth, exists as a homodimer.[8] The presence of such dimeric proteins/receptors as therapeutic targets suggests that an interaction/disruption of these dimers by dimeric small molecules could be a potential therapeutic approach for treating various diseases.

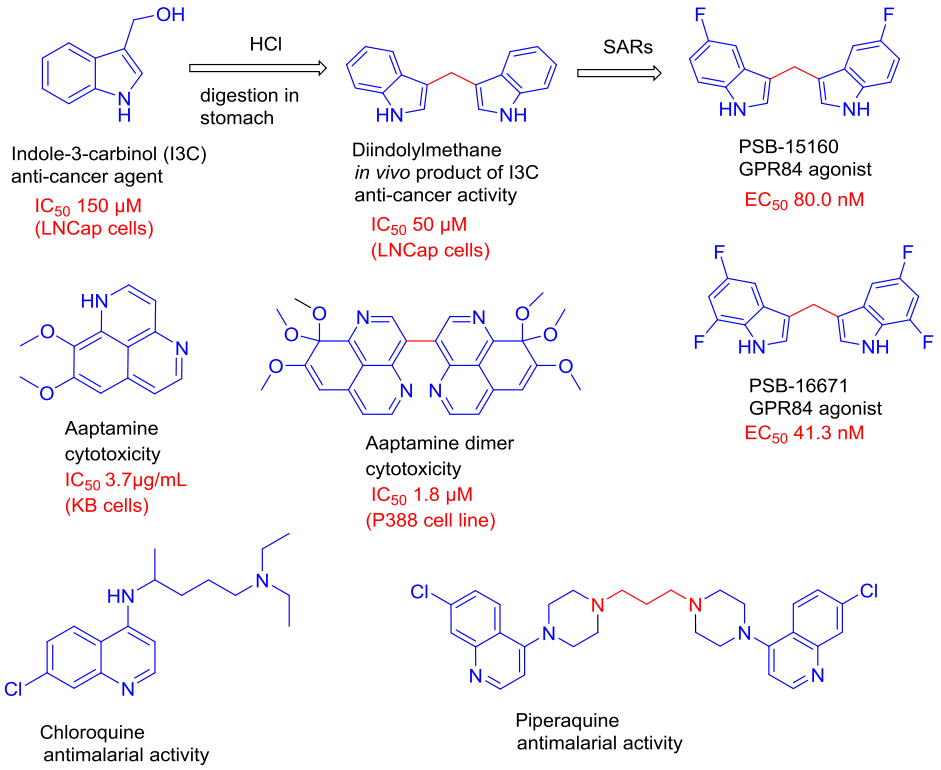



**FIGURE 1** Representative examples of biologically important dimers and their monomers

There are many natural and synthetic dimers with potential biological activities such as anticancer,[9] anti-HIV,[10] antimalarial,[11] antibacterial,[12] as well as opioid antagonist.[13,14] For example, indol-3-carbinol (I3C, Figure 1), which is found in cruciferous vegetables,[15] is a naturally occurring modulator of carcinogenesis that is dependent on the conversion of its dimer, diindolylmethane (DIM)[16,17] in the stomach during digestion. The structure–activity relationship (SAR) of DIM has been extensively studied and many analogs have been developed with the immune stimulatory G protein-coupled receptor 84 (GPR84) agonistic activities, especially the fluoro substituted DIMs (e.g., PSB-15160 and PSB-16671, see Figure 1).[18,19] GPR84 has been proposed as a drug target for acute myeloid leukemia and might have potential for treating other cancers as well. Similarly, numerous synthetic bis-indoles possess cytotoxic activities compared with its monomer.[20] Aaptamine (Figure 1), a naphthyridine alkaloid isolated from marine sponge *Aaptos aaptos*, was found to possess cancer cell growth inhibitory activity,[21] while its dimer bis-aaptamine (Figure 1), isolated from the marine sponge *Aaptos suberitoides* showed potent cytotoxicity against P388 cell lines.[22] Research has been focused on the replacement of chloroquine by its dimeric piperaquine for the treatment of malarial disease caused by *Plasmodium falciparum*.[23]

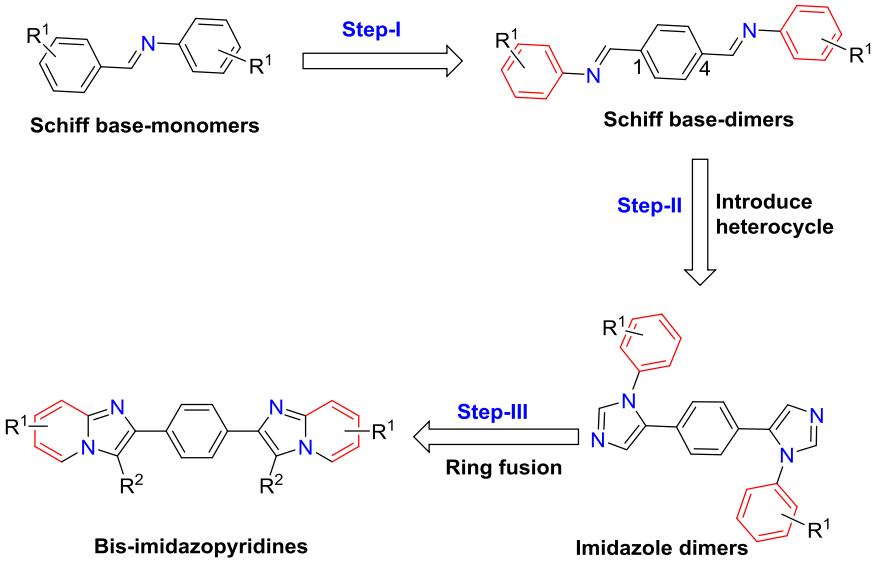
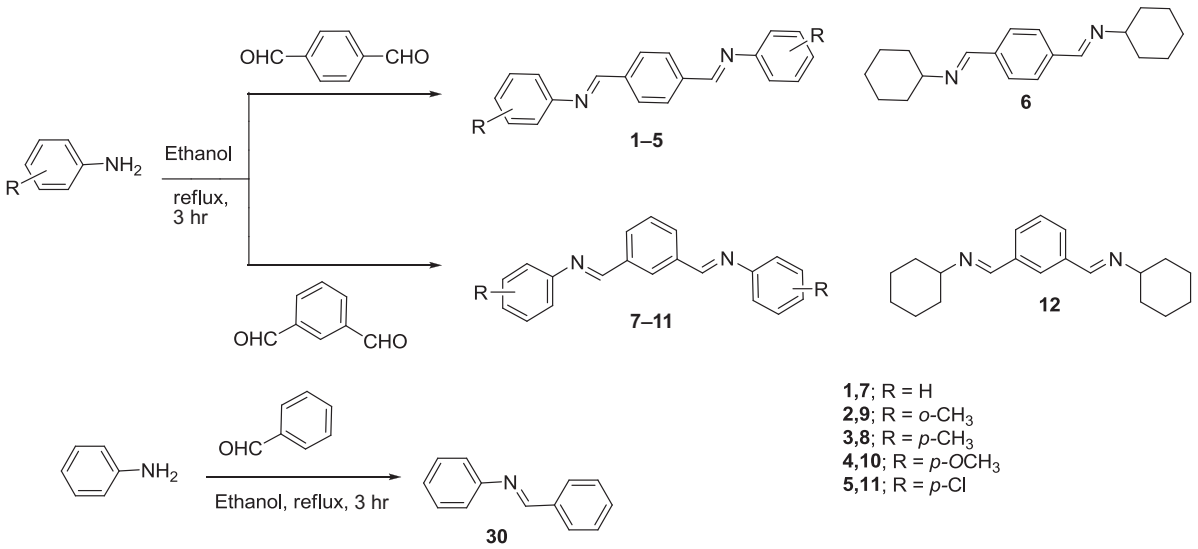



**FIGURE 2** Rational design of bis-imidazoles and bis-imidazopyridines from Schiff base dimers




**SCHEME 1** Preparation of Schiff bases. Reagents and conditions: ethanol, reflux, 3 hr. For the synthesis of **6** and **12**, cyclohexylamine was used

Schiff bases have been shown to exhibit a broad range of biological activities[24,25] and serve as a building block for the synthesis of biologically important heterocycles, such as imidazoles[26] and imidazopyridines.[27] The clinically useful anticancer drug, dacarbazine[28] holds imidazole moiety. Similarly, imidazopyridine drugs such as zolpidem[29] (used in the treatment of insomnia) and alpidem (as an anxiolytic agent),[30] are available in the market. Although a large number of imidazole and imidazopyridine analogs have been studied for various biological activities, their dimeric analogs are not well explored either synthetically and biologically.

Given the biological importance of Schiff bases, an attempt has been made in the present work to synthesize their 1,4-dimeric analogs (Step I, Figure 2). The utility of Van Leusen synthesis was explored to prepare imidazole dimers from the corresponding Schiff base dimers (Step II, Figure 2). In addition to that, the hydrogen bonding property in the imidazole scaffold encouraged us to design the bis-imidazopyridine analogs as a fusion between the phenyl ring and the imidazole ring (Step III, Figure 2). All the prepared dimers of Schiff base, imidazole, and imidazopyridine were biologically evaluated for their anticancer activities. For selected compounds, anticancer activities demonstrated an in vivo model.

## 2 | RESULTS AND DISCUSSION

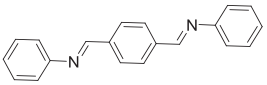
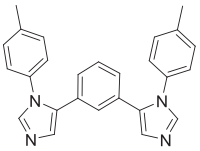
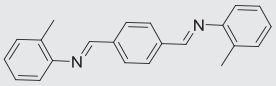
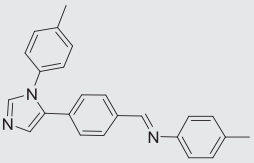
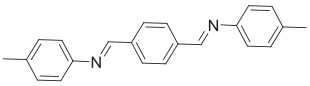
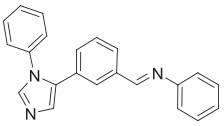
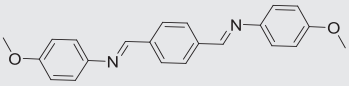
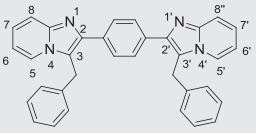
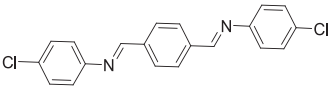
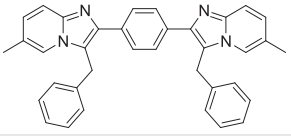
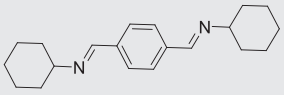
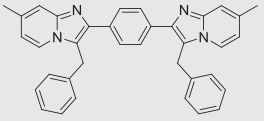
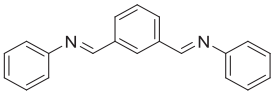
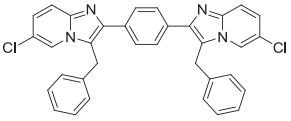
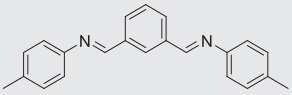
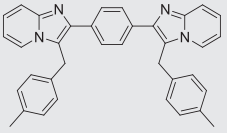
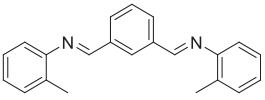
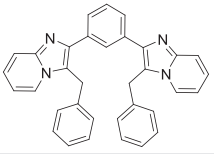
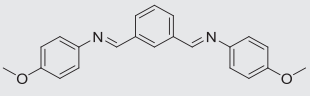
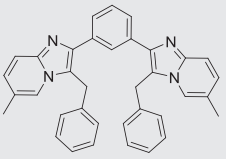
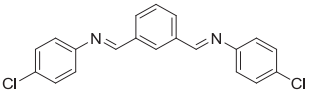
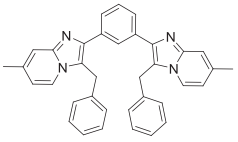
### 2.1 | Chemistry

The first series of 1,4-isomers (**1–6**) of dimeric Schiff bases were prepared from terephthalaldehyde and a variety of amines that diverge the electronic effects of the aromatic ring. The substituents on the phenyl ring of the amine were electron-donating methyl groups (*ortho*- and *para*-substitution) as shown in compounds **2, 3** and *para*-methoxy group as indicated in **4**, electron-withdrawing chloro group at *para*-position as demonstrated in compound **5** along with the parent compound **1** (unsubstituted one). To study the hydrophobic effect, the benzene ring was replaced by the cyclohexyl group as shown in compound **6**. The next series of Schiff bases (**7–12**) were derived from isophthalaldehyde to visualize the effect of biological activity on 1,3-positional isomers (Scheme 1). The substitution pattern was the same as that of the earlier series. Further, to explore the effect of 1,2-positional isomers of the corresponding Schiff bases, we intended to react various amines with phthalaldehyde. However, the reaction did not yield the desired product, probably due to the steric factor. Compound **30** was synthesized from the reaction of aniline and benzaldehyde (equimolar ratio) using the reaction condition of Scheme 1. The chemical structures of the synthesized compounds are summarized in Table 1.

Next, imidazole analogs were synthesized from the corresponding dimeric Schiff base by Van Leusen imidazole synthesis[31] using *p*-toluenesulfonylmethyl isocyanide (TOSMIC) in the presence of $K_2CO_3$. The reaction yielded both the dimeric imidazoles (**13–16**) as well as imidazole–Schiff base hybrid analogs (**17–18**, Scheme 2). The imidazopyridine derivatives (**19–28**, Scheme 3) were synthesized by multicomponent reactions ($A^3$-coupling and 5-exo-dig cyclo isomerization)[27] in which terephthalaldehyde was reacted with 2-aminopyridines and phenylacetylenes in the presence of $CuSO_4$ and D-glucose in ethanol. On the other hand, isophthalaldehyde was used for the preparation of corresponding 1,3-positional isomers of imidazopyridines in the same condition (Scheme 3). However, it was not possible to synthesize 1,2-positional isomers of imidazopyridines (e.g., imidazo[1,2-*a*]pyridine) using phthalaldehyde. For the comparison, the monomer **29** (Scheme 3) was synthesized from the reaction of 2-aminopyridine and benzaldehyde under the same above condition.

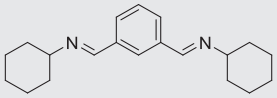
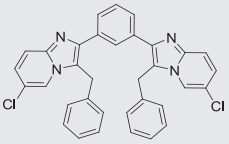
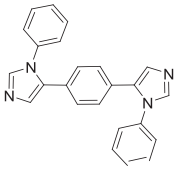
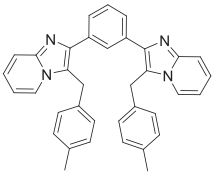
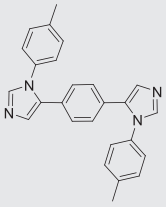
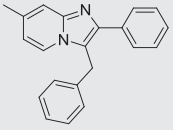
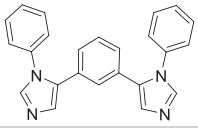
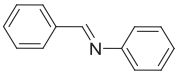
The structures of the synthesized compounds were characterized by $^1H$, $^{13}C$ NMR, HRMS (for selected compounds), and elementary analysis (C, H, and N). Besides, IR spectra were recorded for functional group assignment.

**TABLE 1** Structure of synthesized compounds, melting points, and yields

| Compound | Structure | Melting point (°C) | Yield (%) | Compound | Structure | Melting point (°C) | Yield (%) |
|---|---|---|---|---|---|---|---|
| 1 |  | 150–152 | 85 | 16 |  | 255–257 | 35 |
| 2 |  | 171–173 | 85 | 17 |  | 203–205 | 18 |
| 3 |  | 183–185 | 82 | 18 |  | 198–200 | 15 |
| 4 |  | 192–194 | 78 | 19 |  | 184–186 | 70 |
| 5 |  | 155–157 | 73 | 20 |  | 190–192 | 72 |
| 6 |  | 130–132 | 81 | 21 |  | 204–206 | 73 |
| 7 |  | 112–114 | 75 | 22 |  | 260–262 | 73 |
| 8 |  | 127–129 | 80 | 23 |  | 198–200 | 69 |
| 9 |  | 150–152 | 65 | 24 |  | 170–172 | 73 |
| 10 |  | 137–139 | 75 | 25 |  | 185–187 | 71 |
| 11 |  | 106–108 | 78 | 26 |  | 196–198 | 72 |

(Continues)

**TABLE 1** (Continued)

| Compound | Structure | Melting point (°C) | Yield (%) | Compound | Structure | Melting point (°C) | Yield (%) |
|---|---|---|---|---|---|---|---|
| **12** |  | 115–117 | 78 | **27** |  | 240–242 | 78 |
| **13** |  | 262–264 | 32 | **28** |  | 182–184 | 75 |
| **14** |  | 290–292 | 38 | **29**[28] |  | 150–152 (144–146)[a] | 78 |
| **15** |  | 234–236 | 33 | **30** |  | Viscous oil | 74 |

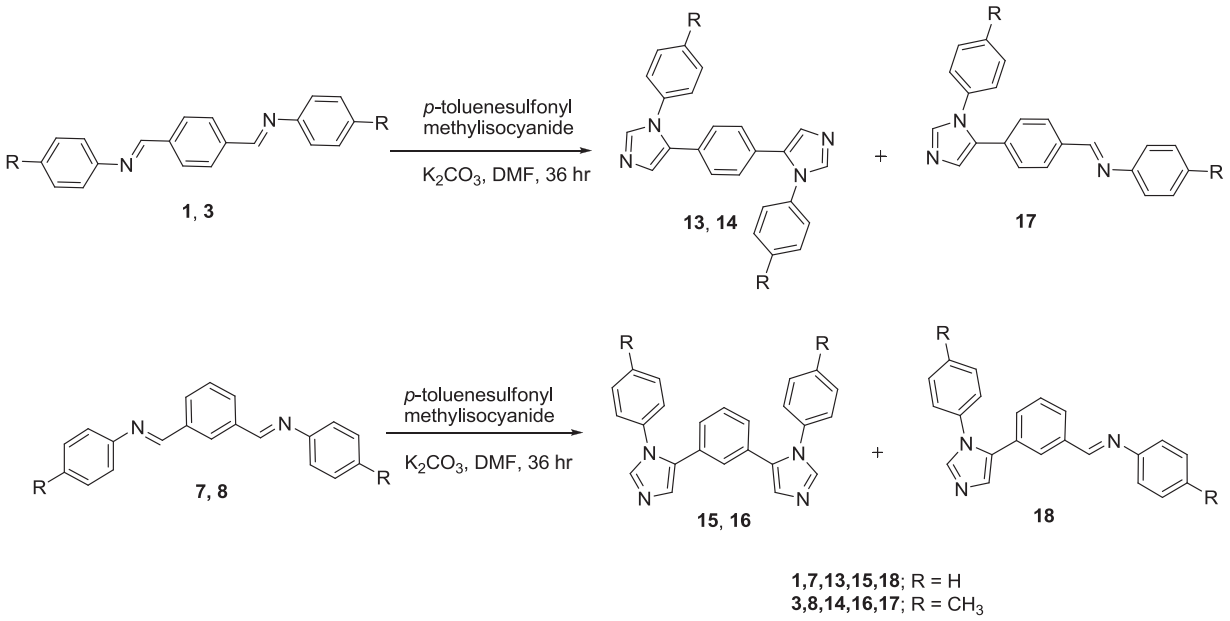
[a]Literature melting point.

## 2.2 | Pharmacology

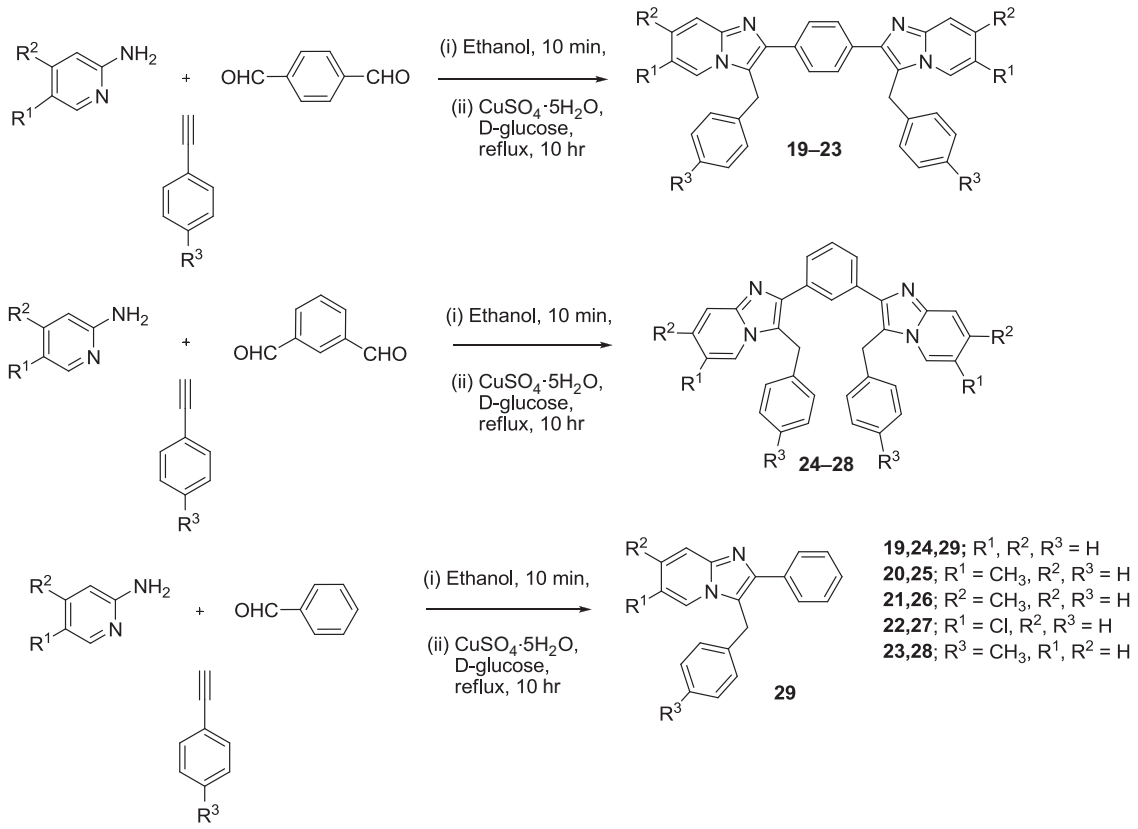
### 2.2.1 | In vitro anticancer activity and SARs

All the synthesized compounds **1–30** were evaluated for their in vitro anticancer activities against three human cancer cell lines (cervical HeLa, breast MDA-MB-231, and renal ACHN) using SRB assay. The concentrations required for 50% growth inhibition ($GI_{50}$) are listed in Table 2. The $GI_{50}$ values are listed in parentheses below along with this sequence of these cell lines written here. The $LC_{50}$ and TGI were also calculated and are mentioned in Table 2.

At first, Schiff base dimers **1–12** were tested in all three cell lines (Table 2). Unfortunately, none of them showed any anticancer activity. Next, the corresponding imidazoles were screened. Compound **13** without any substitution on phenyl rings showed




**SCHEME 2** Preparation of imidazole dimers and imidazole–Schiff base hybrids. Reagents and conditions: *p*-Toluenesulfonylmethyl isocyanide, $K_2CO_3$, DMF, 36 hr. DMF, dimethylformamide




**SCHEME 3** Preparation of imidazopyridine dimers. Reagents and conditions: (i) Ethanol, 10 min, (ii) $CuSO_4{\cdot}5H_2O$, D-glucose, reflux, 10 hr

better cell growth inhibition compared with the Schiff base dimers. Methyl substitution at the *para*-position on the phenyl ring (**14**) increased the cell growth inhibitory activity against all three cancer cell lines (HeLa; $GI_{50}$ = 1.86 μM, MDA-MB-231; $GI_{50}$ = 1.16 μM, ACHN; $GI_{50}$ = 3.78 μM), which was comparable to the standard compound adriamycin. The 1,3-positional isomers **15** and **16** had low cytotoxic activities. The imidazole–Schiff base hybrid molecules **17** and **18** showed significant inhibitory activities on cancer cell growth (**17**: $GI_{50}$ = 1.92 μM, $GI_{50}$ = 1.20 μM, $GI_{50}$ = 2.24 μM and **18**: $GI_{50}$ = 3.10 μM, $GI_{50}$ = 1.90 μM, $GI_{50}$ = 3.86 μM, respectively) at all cell lines. In spite of the lower $LC_{50}$ and TGI values for the compounds **14**, **17**, and **18**, it was clearly shown that the drastic improvement of the cytotoxicity of imidazole derivatives was due to the introduction of additional nitrogen in the imidazole ring. Encouraged by this result, imidazo[1,2-*a*]pyridines were screened and all of them showed good to excellent activities. The unsubstituted parent analog **19** showed a remarkable activity ($GI_{50}$ = 0.55 μM, $GI_{50}$ = 0.43 μM, $GI_{50}$ = 0.55 μM), which exceeds the activity of the standard (ariamycin). This result suggests the importance of the bis-imidazopyridine ring. Next, we focused on the effect of various substituents in the ring on the anticancer activity. The introduction of the electron-donating methyl group at the position 5 resulted in a comparable inhibitory activity (**20**: $GI_{50}$ = 1.20 μM, $GI_{50}$ = 0.88 μM, $GI_{50}$ = 1.16 μM) whereas the 6-methyl derivative showed a weak inhibitory activity (**21**: $GI_{50}$ = 2.25 μM, $GI_{50}$ = 2.05 μM, $GI_{50}$ = 1.90 μM) when compared with **19**. The introduction of the electron-withdrawing chloro group resulted in a poor inhibitory activity (**22**: $GI_{50}$ = 5.24 μM, $GI_{50}$ = 4.50 μM, $GI_{50}$ = 7.72 μM) implying that the electron-donating or electron-withdrawing groups in the imidazopyridine ring exercise no positive influence on the anticancer activity. Therefore we did not go for substituent with more electron donating effect such as the methoxy group in the imidazopyridine moiety toward anticancer activity.

Besides, the 1,3-positional isomers also showed good to excellent anticancer activities. The unsubstituted compound **24** ($GI_{50}$ = 0.36 μM, $GI_{50}$ = 0.30 μM, $GI_{50}$ = 0.38 μM) showed the best anticancer activity, and in fact compound **24** was the most potent anticancer agent of the present series. The 5-methyl (**25**: $GI_{50}$ = 0.84 μM, $GI_{50}$ = 0.65 μM, $GI_{50}$ = 0.98 μM) and 6-methyl (**26**: $GI_{50}$ = 0.97 μM, $GI_{50}$ = 0.58 μM, $GI_{50}$ = 0.85 μM) derivatives showed equipotent cell growth inhibitory activity to the compound **24** and the standard adriamycin. The electron-withdrawing chloro group was not suitable on the imidazopyridine ring as it demonstrated a weak activity (**27**: $GI_{50}$ = 4.00 μM, $GI_{50}$ = 1.60 μM, $GI_{50}$ = 1.82 μM). The 4-methyl substitution on the benzylic ring **28** ($GI_{50}$ = 0.73 μM, $GI_{50}$ = 0.59 μM, $GI_{50}$ = 0.62 μM) was also effective for the anticancer activity against all three cell lines. The monomers of the imidazopyridine scaffold **29** and the Schiff base **30** did not prove versatile.

Figure 3 summarizes the SARs of the investigated imidazopyridine dimers for their anticancer activities in all three cancer cell lines. All the above facts clearly emphasize that the dimeric

**TABLE 2** Anticancer activity of the synthesized compounds against three cell lines[a]

| | HeLa | | | MDA-MB-231 | | | ACHN | | |
|---|---|---|---|---|---|---|---|---|---|
| Compound | $LC_{50}$ (μM) | TGI (μM) | $GI_{50}$ (μM) | $LC_{50}$ (μM) | TGI (μM) | $GI_{50}$ (μM) | $LC_{50}$ (μM) | TGI (μM) | $GI_{50}$ (μM) |
| **1** | >10 | >10 | >10 | >10 | >10 | >10 | >10 | >10 | >10 |
| **2** | >10 | >10 | >10 | >10 | >10 | >10 | >10 | >10 | >10 |
| **3** | >10 | 9.47 | >10 | >10 | >10 | >10 | >10 | >10 | >10 |
| **4** | >10 | >10 | >10 | >10 | >10 | >10 | >10 | >10 | >10 |
| **5** | >10 | >10 | >10 | >10 | >10 | >10 | >10 | >10 | >10 |
| **6** | >10 | >10 | >10 | >10 | >10 | >10 | >10 | >10 | >10 |
| **7** | >10 | 9.79 | 8.23 | >10 | >10 | >10 | >10 | >10 | >10 |
| **8** | >10 | 9.67 | >10 | >10 | >10 | 8.90 | >10 | >10 | >10 |
| **9** | >10 | >10 | 9.12 | >10 | 8.45 | >10 | >10 | >10 | 7.50 |
| **10** | >10 | >10 | 8.23 | >10 | >10 | >10 | >10 | >10 | >10 |
| **11** | >10 | >10 | >10 | >10 | >10 | >10 | >10 | >10 | >10 |
| **12** | >10 | >10 | >10 | >10 | >10 | >10 | >10 | >10 | >10 |
| **13** | >10 | 9.76 | 4.23 | >10 | >10 | 5.14 | >10 | >10 | 8.24 |
| **14** | >10 | 9.76 | 1.86 | >10 | >10 | 1.16 | >10 | >10 | 3.78 |
| **15** | >10 | >10 | >10 | >10 | >10 | 6.88 | >10 | >10 | 9.88 |
| **16** | >10 | 9.76 | 6.85 | >10 | >10 | 4.26 | >10 | >10 | 7.15 |
| **17** | >10 | >10 | 1.92 | >10 | >10 | 1.20 | >10 | >10 | 2.24 |
| **18** | >10 | >10 | 3.10 | >10 | >10 | 1.90 | >10 | >10 | 3.86 |
| **19** | **>10** | **>10** | **0.55** | **>10** | **>10** | **0.43** | **>10** | **>10** | **0.55** |
| **20** | >10 | >10 | 1.20 | >10 | >10 | 0.88 | >10 | >10 | 1.16 |
| **21** | >10 | >10 | 2.25 | >10 | >10 | 2.05 | >10 | >10 | 1.90 |
| **22** | >10 | 3.73 | 5.24 | >10 | >10 | 4.50 | >10 | >10 | 7.72 |
| **23** | >10 | 9.76 | 0.96 | >10 | >10 | 1.30 | >10 | >10 | 1.32 |
| **24** | **>10** | **>10** | **0.36** | **>10** | **>10** | **0.30** | **>10** | **>10** | **0.38** |
| **25** | >10 | >10 | 0.84 | >10 | >10 | 0.65 | >10 | >10 | 0.98 |
| **26** | >10 | >10 | 0.97 | >10 | >10 | 0.58 | >10 | >10 | 0.85 |
| **27** | >10 | 9.74 | 4.00 | >10 | >10 | 1.60 | >10 | >10 | 1.82 |
| **28** | >10 | 9.76 | 0.73 | >10 | >10 | 0.59 | >10 | >10 | 0.62 |
| **29** | >10 | >10 | >10 | >10 | >10 | >10 | >10 | >10 | 8.24 |
| **30** | >10 | >10 | >10 | >10 | >10 | >10 | >10 | >10 | >10 |
| Adriamycin[b] | >10 | >10 | 0.52 | >10 | >10 | 0.51 | >10 | >10 | 0.58 |

Abbreviations: $GI_{50}$, concentration of drug causing 50% inhibition of cell growth; $LC_{50}$, concentration of drug causing 50% cell kill; TGI, concentration of drug causing total inhibition of cell growth.
Bold values indicate the activity best compounds.
[a]Inhibitory activity was expressed in micromolar.
[b]Positive control.

imidazopyridines were more important for the anticancer activity when compared with the monomer as well as to the Schiff base dimers and imidazole dimers. In the dimer series of imidazopyridines, the 1,3-positional isomer was found to be more effective than the 1,4-positional isomer. In both 1,4 and 1,3 isomers, the unsubstituted parent compounds **19** (1,4 isomer) and **24** (1,3 isomer) were the two best compounds among all compounds tested in the present study. Especially, both **19** and **24** were very effective against the breast cancer cell line (MDA-MB-231) among all three cell lines selected (see Figure 4).

ERs (ERα, ERβ) are prominent in breast cancer cells and therefore current breast cancer drugs like tamoxifene, rolaxifene, toremifene act as either agonist or antagonist by binding to this receptor.[32,33] As mentioned earlier, the ERs form homo-or heterodimers upon ligand binding. Based on these facts and to account for the mechanistic pathway of the imidazopyridines dimers, particularly toward breast cancer cell line, we believe that the two effective positional isomers **19** and **24** bind to the ERs and upon binding, the receptors could be dimerized. However, the mechanistic studies are under process and the results will be explored elsewhere.

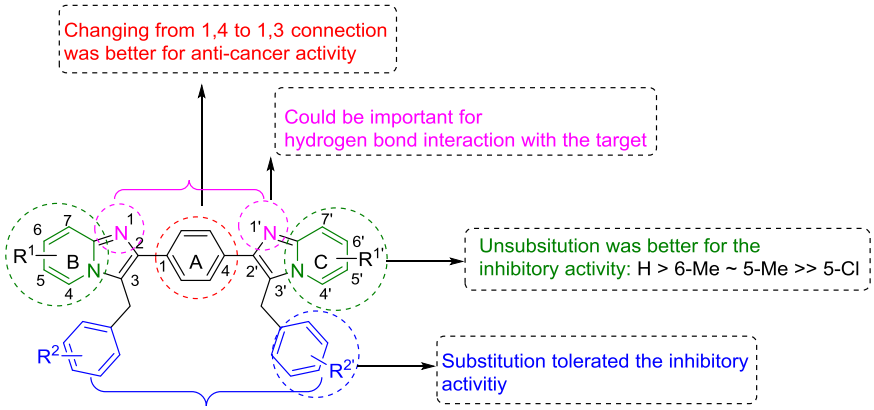



**FIGURE 3** Structure–activity relationships of imidazopyridine dimers as anticancer agents ($R^1 = R^{1'}$, and $R^2 = R^{2'}$ as these are symmetrical dimers)

### 2.2.2 | In vivo anticancer activity

Selected compounds such as **19** and **24** were tested for in vivo anticancer activities. In the experiment design, the animals were divided into five groups each consisting of six rats. Group I: Control rats received normal pelleted diet. Group II: Mammary carcinoma was induced in overnight fasted rats by a single dose of 7,12-dimethylbenz[*a*]anthracene (DMBA) in olive oil (25 mg/kg body weight) by gastric intubation.[34] Group III: Mammary carcinoma rats treated with compound **19** (50 mg/kg body weight) for 14 continuous days by gastric intubation. Group IV: Mammary carcinoma rats treated with compound **24** (50 mg/kg body weight) for 14 continuous days by gastric intubation. Group V: Mammary carcinoma rats treated with (standard drug) tamoxifen (20 mg/kg body weight) for 14 continuous days by gastric intubation.

The values outside the normal range of the hematological parameters are diagnostic for cancer.[34] In Table 3, the results of the hematological assay of the whole blood sample of the rats of five groups are denoted. The contents of hemoglobin, packed cell volume (PCV), white blood cells (WBC), red blood cells (RBC), and platelets are found to be elevated in the breast cancer-induced rats (Group II) compared with the control rats (Group I). When compound **19** (Group III) and **24** (Group IV) were treated, the contents of hemoglobin, PCV, WBC, RBC, and platelets were found to be comparable to the standard drug, tamoxifen (Group V).

Serum cancer markers carcinoembryonic antigen (CEA) and cancer antigen 15-3 (CA 15-3) are used to monitor response to cancer treatment, particularly breast cancer treatment and disease recurrence.[35] In Table 4, CEA and CA are elevated in cancer-induced rats (Group II) whereas the antigens were reduced due to the effect of compound **19** (Group III) and compound **24** (Group IV), which was also comparable to the drug tamoxifen (Group V).

Liver marker enzymes act as significant indicators of malignant disorders.[36] The concentrations of liver marker enzymes in the serum, such as aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), and acid phosphatase (ACP) in the experimental groups are shown in Table 5. A marked upsurge in the levels of serum marker enzymes was found in cancer-induced rats (Group II) compared with the control group (Group I)

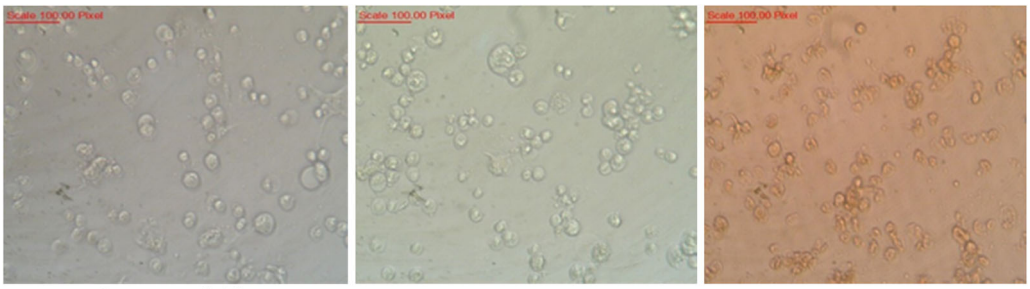



**FIGURE 4** Cytotoxic activity of the compounds **19** and **24** against MDA-MB-231 cell line (adriamycin used as a positive control)

**TABLE 3** Hematological parameters of breast cancer-induced rats

| Groups | Hb (g/dl) | PCV (%) | WBC (thousands/mm$^3$) | RBC (millions/mm$^3$) | Platelets (lakhs/mm$^3$) |
|---|---|---|---|---|---|
| Group I | 14.33 ± 0.32 | 43 ± 0.96 | 7.16 ± 0.10 | 6.08 ± 0.09 | 8.30 ± 0.10 |
| Group II | 8.86 ± 0.25 | 26.60 ± 0.75 | 10.18 ± 0.19 | 5.11 ± 0.11 | 4.48 ± 0.24 |
| Group III | 13.51 ± 0.20 | 40.55 ± 0.61 | 9.25 ± 0.10 | 5.25 ± 0.16 | 6.18 ± 0.09 |
| Group IV | 13.61 ± 0.21 | 40.85 ± 0.64 | 8.85 ± 0.21 | 5.40 ± 0.08 | 6.58 ± 0.25 |
| Group V | 13.91 ± 0.28 | 41.75 ± 0.85 | 8.08 ± 0.21 | 6.38 ± 0.14 | 7.13 ± 0.12 |

*Note:* Values are expressed as mean ± SD for six animals.
Abbreviations: Hb, hemoglobin; PCV, packed cell volume; RBC, red blood cells; SD, standard deviation; WBC, white blood cells.

**TABLE 4** Analysis of serum cancer markers of the animals of five groups

| Groups | CEA (ng/ml) | CA 15-3 (ng/ml) |
|---|---|---|
| Group I | 0.42 ± 0.04 | 0.56 ± 0.05 |
| Group II | 4.2 ± 0.08 | 8.1 ± 0.20 |
| Group III | 2.45 ± 0.15 | 3.75 ± 0.22 |
| Group IV | 2.21 ± 0.07 | 3.08 ± 0.21 |
| Group V | 1.7 ± 0.12 | 2.75 ± 0.13 |

*Note:* Values are expressed as mean ± SD for six animals.
Abbreviations: CA 15-3, cancer antigen 15-3; CEA, carcinoembryonic antigen; SD, standard deviation.

rats. However, these levels were substantially decreased in the animals treated with compound **19** (Group III) and compound **24** (Group IV). The results were comparable with that of the animals treated with the standard drug tamoxifen (Group V).

In Figure 5, no histopathological alterations were observed in the mammary gland of the control group (Group I). The histopathological features of the tumor developed in rats (Group II) injected by DMBA were shown to possess anaplastic activity. However, these levels were substantially decreased and the histopathological structures were restored in the animals treated with compound **19** (Group III) and compound **24** (Group IV). The in vivo anticancer activity results of compounds **19** and **24** results were comparable with that of the animals treated with the standard drug tamoxifen (Group V).

The histopathology of the liver is shown in Figure 6. No histopathological alterations were observed in the liver of control group (Group I). The alterations in the histopathological features of the liver of rats injected by DMBA are shown in Group II. However, the histopathological structures were restored in the animals treated with compound **19** (Group III) and compound **24** (Group IV).

The in vivo anticancer activity results of compounds **19** and **24** results were comparable with that of the animals treated with the standard drug tamoxifen (Group V).

## 3 | CONCLUSION

A novel series of dimers of imidazole (**13–18**) and imidazo[1,2-*a*]pyridine (**19–28**) were designed and synthesized from Schiff base dimers (**1–12**). To study the SARs of each class, all the synthesized compounds were screened for anticancer activities against HeLa, MDA-MB-231, and ACHN cell lines. The bis-imidazoles **14**, imidazole–Schiff base hybrid analog **17** and imidazopyridines (**19–28**) showed good to excellent anticancer activities against three cell lines. Especially, imidazopyridines were more promising as anticancer agents. The substituents in the imidazopyridine ring, namely, 1,4-bis(3-benzyl-6-methylimidazo[1,2-*a*]-pyridin-2-yl)benzene (**20**), 1,4-bis(3-benzyl-7-methylimidazo[1,2-*a*]pyridin-2-yl)benzene (**21**), 1,4-bis(3-benzyl-6-chloroimidazo[1,2-*a*]pyridin-2-yl)benzene (**22**), 1,4-bis(3-(4-methylbenzyl)imidazo[1,2-*a*]pyridin-2-yl)-benzene (**23**), 1,3-bis(3-benzyl-6-methylimidazo[1,2-*a*]pyridin-2-yl)benzene (**25**), 1,3-bis(3-benzyl-7-methylimidazo[1,2-*a*]pyridin-2-yl)benzene (**26**), 1,3-bis(3-benzyl-6-chloroimidazo[1,2-*a*]pyridin-2-yl)benzene (**27**), and 1,3-bis(3-(4-methylbenzyl)imidazo[1,2-*a*]pyridin-2-yl)benzene (**28**), demonstrated good activity; however, the unsubstituted analogs, 1,4-bis-(3-benzylimidazo[1,2-*a*]pyridin-2-yl)benzene (**19**) and 1,3-bis(3-benzylimidazo[1,2-*a*]pyridin-2-yl)benzene (**24**), showed the best result and, in fact, they were the two best compounds among all series in the present study. In vivo studies were proved that these two compounds (**19** and **24**) were effective in treating the cancers. Although the molecular targets for these new class of compounds as anticancer agents were not possible to explore in the present study, we believe that this novel class of compounds could target ERs, as they are potential target in treating the breast cancer. However, the mechanistic studies for identifying molecular target for these new inhibitors are under progress and it will be explored in the future. In summary, 1,4- and 1,3-positional isomers of dimeric imidazo[1,2-*a*]pyridine could serve as potent leads for the development of anticancer agents.

## 4 | EXPERIMENTAL

### 4.1 | Chemistry

#### 4.1.1 | General

Melting points were determined on Mettler FP 51 apparatus (Mettler Instruments, Switzerland) and are uncorrected. They are expressed in degree centigrade (°C). The IR spectra (in KBr pellets) were recorded on a Shimadzu FT-IR 157 spectrophotometer (Shimadzu). $^{1}H$ and $^{13}C$ NMR spectra (see the Supporting Information) were recorded using $CDCl_3$ as solvent and TMS as an internal standard on a Bruker

**TABLE 5** Analysis of serum liver enzymes of the animals of five groups

| Groups | AST (μmoles of pyruvate liberated/l) | ALT (μmoles of pyruvate liberated/l) | ALP (μmoles of phenol liberated/l) | ACP (μmoles of phenol liberated/l) |
|---|---|---|---|---|
| Group I | 45.63 ± 1.98 | 50.63 ± 1.54 | 144.78 ± 1.38 | 78.18 ± 1.91 |
| Group II | 96.30 ± 2.28 | 104.56 ± 1.78 | 196.13 ± 1.80 | 56.05 ± 1.37 |
| Group III | 59.21 ± 1.84 | 67.26 ± 1.85 | 163.05 ± 2.79 | 65.53 ± 0.79 |
| Group IV | 56.08 ± 1.16 | 65.2 ± 0.70 | 156.83 ± 1.36 | 66.56 ± 1.13 |
| Group V | 51.60 ± 0.95 | 61.88 ± 0.94 | 152.35 ± 0.83 | 68.13 ± 0.63 |

*Note:* Values are expressed as mean ± SD for six animals.
Abbreviations: ACP, acid phosphatase; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; SD, standard deviation.

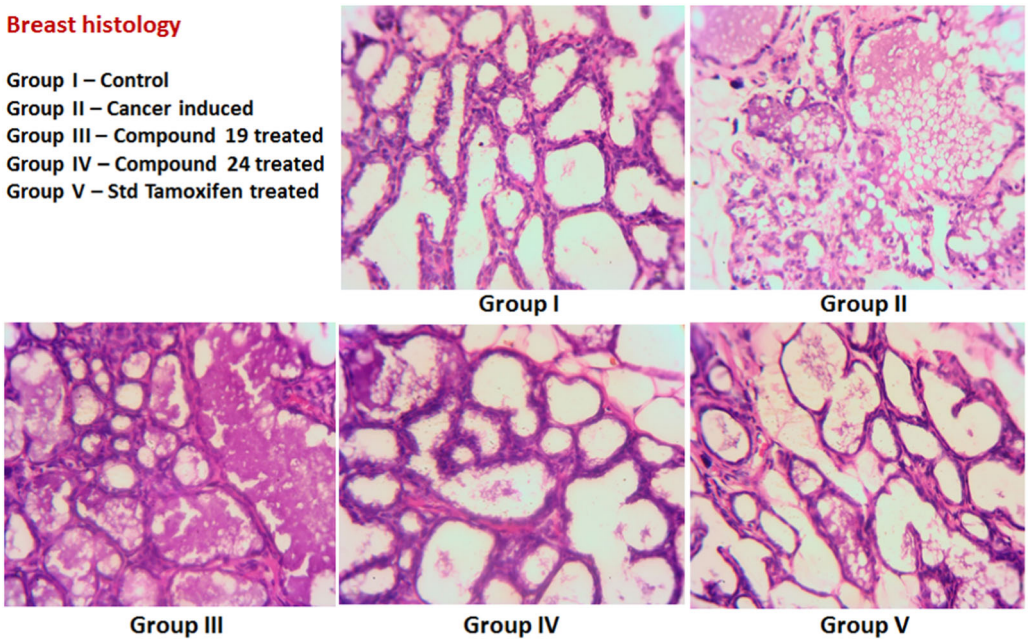



**FIGURE 5** Representative photomicrographs of breast tissues of Group I to Group V

300 MHz/400 MHz NMR spectrometer (Bruker). Chemical shift values are given in $\delta$ (ppm scale). Mass spectra were recorded on a Bruker Maxis HRMS (Bruker) by ESI techniques and Auto Spec EI Shimadzu QP 2010 PLUS GC-MS mass spectrometer (Shimadzu). Microanalyses were performed on a Vario EL III model CHNS analyzer (Vario, Germany) at the Department of Chemistry, Bharathiar University. The purity of the compounds was checked by thin-layer chromatography (TLC) on silica gel plates using petroleum ether and ethyl acetate.

The InChI codes of the investigated compounds together with some biological activity data are provided as Supporting Information.

### 4.1.2 | General procedure for the preparation of Schiff bases 1–12 and 30

To a solution of corresponding dialdehyde (10.0 mmol) in ethanol (10 ml), corresponding amines (20.0 mmol) were added and refluxed for 6 hr. The obtained precipitate was collected by filtration and

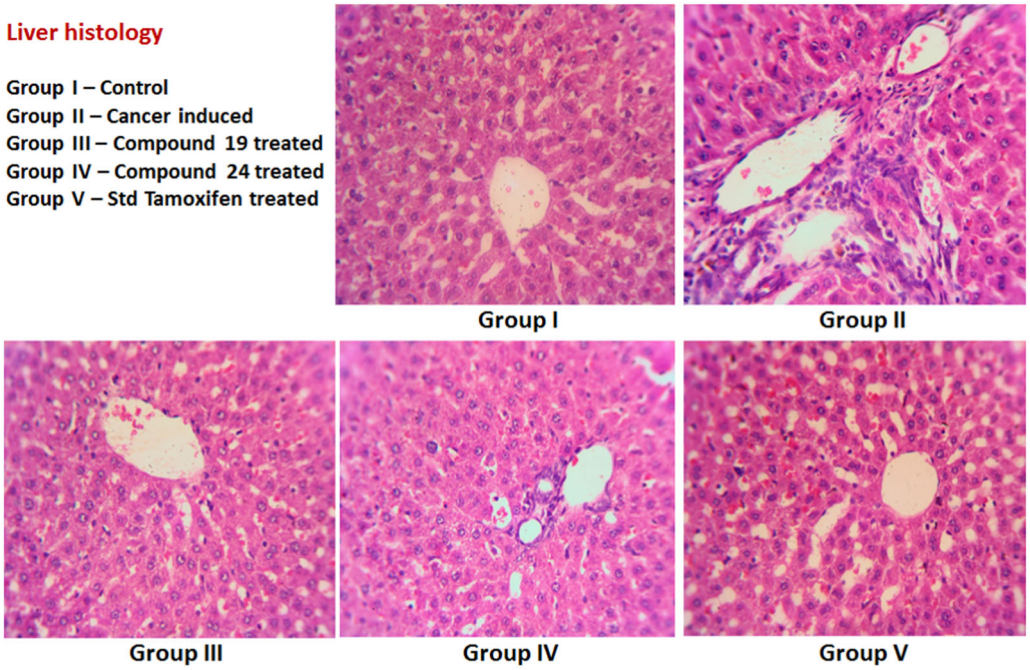



**FIGURE 6** Representative photomicrographs of liver tissues of Group I to Group V

recrystallized from ethanol. The compound **30** was synthesized from the reaction of aniline and benzaldehyde (equimolar ratio).

*(N,N′E,N,N′E)-N,N′-(1,4-Phenylenebis(methan-1-yl-1-ylidene))dianiline (**1**)*

White solid. IR (KBr) $\nu_{max}$ ($cm^{-1}$): 3,019, 2,872, 1,611, 1,042, 838. $^1H$ NMR ($CDCl_3$) $\delta$ (ppm): 7.27–7.29 (m, 3H), 7.42 (t, 2 H, $J$ = 6.80 Hz), 8.02 (s, 2H), 8.52 (s, 1H, CH═N). $^{13}C$ NMR ($CDCl_3$) $\delta$ (ppm): 120.94, 129.18, 129.23, 130.04, 138.85, 151.68, 159.44. EI-MS, *m/z* (%): 285 (10), 282 (100), 207 (38), 181 (35), 130 (20), 94 (42), 72 (55), 54 (45). Anal. calcd. for $C_{20}H_{16}N_2$ (284): C, 84.48, H, 5.67, N, 9.85. Found: C, 84.69, H, 5.07, N, 10.24%.

*(N,N′E,N,N′E)-N,N′-(1,4-Phenylenebis(methan-1-yl-1-ylidene))bis(2-methylaniline) (**2**)*

White solid. IR (KBr) $\nu_{max}$ ($cm^{-1}$): 3,020, 2,874, 1,609, 1,041, 836. $^1H$ NMR ($CDCl_3$) $\delta$ (ppm): 2.39 (s, 3H), 6.98 (d, $J$ = 7.80 Hz, 1H), 7.13–7.25 (m, 3H), 8.03 (s, 2H), 8.43 (s, 1H, CH═N). $^{13}C$ NMR ($CDCl_3$) $\delta$ (ppm): 17.87, 117.49, 126.03, 126.75, 129.04, 130.36, 132.20, 138.80, 150.81, 158.53. Anal. calcd. for $C_{20}H_{20}N_2$ (312): C, 84.58, H, 6.45, N, 8.97. Found: C, 84.47, H, 6.58, N, 8.95%.

*(N,N′E,N,N′E)-N,N′-(1,4-Phenylenebis(methan-1-yl-1-ylidene))bis(4-methylaniline) (**3**)*

Yellowish solid. IR (KBr) $\nu_{max}$ ($cm^{-1}$): 3,015, 2,875, 1,607, 1,075, 829. $^1H$ NMR ($CDCl_3$) $\delta$ (ppm): 2.39 (s, 3H), 7.18 (d, 2H, $J$ = 6.80 Hz), 7.22 (d, 2H, $J$ = 8.00 Hz), 8.00 (s, 2H), 8.53 (s, 1H, CH═N). $^{13}C$ NMR ($CDCl_3$) $\delta$ (ppm): 21.05, 120.91, 129.04, 129.84, 136.30, 138.85, 149.29, 158.62. Anal. calcd. for $C_{22}H_{20}N_2$ (312): C, 84.58, H, 6.45, N, 8.97. Found: C, 84.50, H, 6.46, N, 9.04%.

*(N,N′E,N,N′E)-N,N′-(1,4-Phenylenebis(methan-1-yl-1-ylidene))bis(4-methoxyaniline) (**4**)*

Brown solid. IR (KBr) $\nu_{max}$ ($cm^{-1}$): 3,016, 2,871, 1,600, 1,035, 830. $^1H$ NMR ($CDCl_3$) $\delta$ (ppm): 3.85 (s, 3H), 6.95 (d, $J$ = 7.80 Hz, 2H), 7.30 (d, $J$ = 7.40 Hz, 2H), 8.00 (s, 2H), 8.54 (s, 1H, CH═N). $^{13}C$ NMR ($CDCl_3$) $\delta$ (ppm): 55.52, 114.46, 122.29, 129.20, 130.78, 137.00, 157.53, 158.58. Anal. calcd. for $C_{22}H_{20}N_2O_2$ (344): C, 76.72, H, 5.85, N, 8.13. Found: C, 76.76, H, 5.83, N, 8.09%.

*(N,N′E,N,N′E)-N,N′-(1,4-Phenylenebis(methan-1-yl-1-ylidene))bis(4-chloroaniline) (**5**)*

Yellow solid. IR (KBr) $\nu_{max}$ ($cm^{-1}$): 3,018, 2,875, 1,604, 1,035, 829. $^1H$ NMR ($CDCl_3$) $\delta$ (ppm): 7.19 (d, 2H, $J$ = 8.80 Hz), 7.38 (d, 2H, $J$ = 8.50 Hz), 8.01 (s, 2H), 8.49 (s, 1H, CH═N). $^{13}C$ NMR ($CDCl_3$) $\delta$ (ppm): 122.28, 129.24, 129.35, 130.05, 138.58, 150.11, 159.82. Anal. calcd. for $C_{20}H_{14}Cl_2N_2$ (353): C, 68.00, H, 3.99, N, 7.93. Found: C, 68.04, H, 3.94, N, 7.97%.

*(N,N′E,N,N′E)-N,N′-(1,4-Phenylenebis(methan-1-yl-1-ylidene))dicyclohexanamine (**6**)*

White solid. IR (KBr) $\nu_{max}$ ($cm^{-1}$): 3,021, 2,855, 1,621, 1,065, 920, 840. $^1H$ NMR ($CDCl_3$) $\delta$ (ppm): 1.24–1.45 (m, 3H), 1.53–1.87 (m, 7H), 3.16–3.25 (m, 1H), 7.75 (s, 2H), 8.32 (s, 1H, CH═N). $^{13}C$ NMR ($CDCl_3$) $\delta$ (ppm): 24.80, 25.63, 34.33, 70.14, 128.23, 138.28, 158.17. Anal. calcd. for $C_{20}H_{28}N_2$ (296): C, 81.03, H, 9.52, N, 9.45. Found: C, 81.10, H, 9.49, N, 9.41%.

*(N,N′E,N,N′E)-N,N′-(1,3-Phenylenebis(methan-1-yl-1-ylidene))dianiline) (**7**)*

Yellow liquid. IR (KBr) $\nu_{max}$ ($cm^{-1}$): 3,019, 2,850, 1,618, 1,067, 842. $^1H$ NMR ($CDCl_3$) $\delta$ (ppm): 7.25–7.28 (m, 3H), 7.38–7.43 (m, 2H), 7.55 (m, 1H), 8.00 (d, 1H, Hz, $J$ = 7.52 Hz), 8.38 (s, 1H), 8.53 (s, 1H, CH═N). $^{13}C$ NMR ($CDCl_3$) $\delta$ (ppm): 120.85, 129.24, 129.35, 129.80, 130.85, 136.25, 137.10, 149.12, 158.72. Anal. calcd. for $C_{20}H_{16}N_2$ (284): C, 84.48, H, 5.67, N, 9.85. Found: C, 84.40, H, 5.69, N, 9.91%.

*(N,N′E,N,N′E)-N,N′-(1,3-Phenylenebis(methan-1-yl-1-ylidene))bis(4-methylaniline) (**8**)*

White solid. IR (KBr) $\nu_{max}$ ($cm^{-1}$): 3,017, 2,880, 1,615, 1,081, 837. $^1H$ NMR ($CDCl_3$) $\delta$ (ppm): 2.38 (s, 3H), 7.17 (d, 2H, $J$ = 8.50 Hz), 7.21 (d, 2H, $J$ = 8.00 Hz), 7.57 (m, 1H), 8.03 (d, $J$ = 7.50 Hz, 1H), 8.39 (s, 1H), 8.55 (s, 1H, CH═N). $^{13}C$ NMR ($CDCl_3$) $\delta$ (ppm): 21.03, 120.86, 129.2, 129.84, 131.02, 134.12, 136.16, 136.93, 149.09, 158.73. Anal. calcd. for $C_{22}H_{20}N_2$ (312): C, 84.58, H, 6.45, N, 8.97. Found: C, 84.59, H, 6.46, N, 8.95%.

*(N,N′E,N,N′E)-N,N′-(1,3-Phenylenebis(methan-1-yl-1-ylidene))bis(2-methylaniline) (**9**)*

Yellow liquid. IR (KBr) $\nu_{max}$ ($cm^{-1}$): 3,019, 2,884, 1,614, 1,079, 835. $^1H$ NMR ($CDCl_3$) $\delta$ (ppm): 2.39 (s, 3H), 6.98 (d, $J$ = 6.80 Hz, 1H), 7.08–7.26 (m, 3H), 7.60 (m, 1H), 8.08 (d, $J$ = 7.20 Hz, 1H), 8.41 (s, 1H), 8.46 (s, 1H, CH═N). $^{13}C$ NMR ($CDCl_3$) $\delta$ (ppm): 17.87, 117.60, 125.90, 126.76, 129.51, 130.34, 131.02, 131.66, 134.11, 137.07, 150.84, 158.69. Anal. calcd. for $C_{20}H_{20}N_2$ (312): C, 84.58, H, 6.45, N, 8.97. Found: C, 84.53, H, 6.43, N, 9.04%.

*(N,N′E,N,N′E)-N,N′-(1,3-Phenylenebis(methan-1-yl-1-ylidene))bis(4-methoxyaniline) (**10**)*

Brown solid. IR (KBr) $\nu_{max}$ ($cm^{-1}$): 3,018, 2,875, 1,618, 1,076, 841. $^1H$ NMR ($CDCl_3$) $\delta$ (ppm): 3.87 (s, 3H), 6.99 (d, 2H, $J$ = 6.80 Hz), 7.33 (d, 2H, $J$ = 8.80 Hz), 7.59 (m, 1H), 8.03 (d, $J$ = 7.00 Hz, 1H), 8.40 (s, 1H), 8.59 (s, 1H, CH═N). Anal. calcd. for $C_{22}H_{20}N_2O_2$ (344): C, 76.72, H, 5.85, N, 8.13. Found: C, 76.10, H, 5.49, N, 8.41%.

*(N,N′E,N,N′E)-N,N′-(1,3-Phenylenebis(methan-1-yl-1-ylidene))bis(4-chloroaniline) (**11**)*

Yellow solid. IR (KBr) $\nu_{max}$ ($cm^{-1}$): 3,024, 2,879, 1,621, 1,033, 851. $^1H$ NMR ($CDCl_3$) $\delta$ (ppm): 7.18 (d, 2H, $J$ = 9.00 Hz), 7.37 (d, 2H, $J$ = 9.00 Hz), 7.60 (m, 1H), 8.04 (d, 1H, $J$ = 7.50 Hz, 2H), 8.41 (s, 1H), 8.52 (s, 1H, CH═N). $^{13}C$ NMR ($CDCl_3$) $\delta$ (ppm): 122.23, 129.35, 130.21, 131.58, 131.87, 134.19, 136.63, 150.09, 159.71. Anal. calcd. for $C_{20}H_{14}Cl_2N_2$ (353): C, 68.00, H, 3.99, N, 7.93. Found: C, 68.10, H, 3.49, N, 7.91%.

*(N,N′E,N,N′E)-N,N′-(1,3-Phenylenebis(methan-1-yl-1-ylidene))dicyclohexanamine (**12**)*

White solid. IR (KBr) $\nu_{max}$ ($cm^{-1}$): 3,022, 2,875, 1,619, 1,032, 848. $^1H$ NMR ($CDCl_3$) $\delta$ (ppm): 1.24–1.87 (m, 10H), 3.18–3.25 (m, 1H), 7.43 (m, 1H), 7.81 (d, $J$ = 8.40 Hz, 1H), 8.06 (s, 1H), 8.35 (s, 1H, CH═N). $^{13}C$ NMR ($CDCl_3$) $\delta$ (ppm): 24.81, 25.64, 34.36, 69.98, 127.90, 128.75, 129.71, 137.00, 158.20. Anal. calcd. for $C_{20}H_{28}N_2$ (296): C, 81.03, H, 9.52, N, 9.45. Found: C, 81.09, H, 9.50, N, 9.41%.

*(E)-N-Benzylideneaniline (**30**)*

Viscous oil. $^1H$ NMR ($CDCl_3$) $\delta$ (ppm): 7.18–7.21 (m, 3H), 7.28–7.53 (m, 5H), 7.82 (d, $J$ = 7.40 Hz, 2H), 8.42 (s, 1H, CH═N). Anal. calcd. for $C_{13}H_{11}N$ (181.23): C, 86.15, H, 6.12, N, 7.73. Found: C, 86.22, H, 6.08, N, 7.70%.

### 4.1.3 | General procedure for the synthesis of imidazoles 13–16 and the imidazole–Schiff base hybrids 17 and 18

To a solution of the corresponding Schiff base (2.0 mmol) in dimethylformamide, TOSMIC (4.0 mmol) and $K_2CO_3$ (6.0 mmol) were added. The solution was stirred at ambient temperature for 36 hr. The formation of products was monitored by TLC. Water was added to the reaction mixture and extracted using ethyl acetate. The organic layer was dried over anhydrous sodium sulfate and concentrated. The obtained crude product was purified by column chromatography to get the imidazole dimers **13–16** and imidazole–Schiff base hybrid compounds **17** and **18**. The yields of the imidazole dimers **13–16** were 30–40%. The yields of the imidazole–Schiff base hybrid compounds **17** and **18** were 10–20%. The reaction was not completed even after 36 hr and the unreacted starting material was recovered.

*1,4-Bis(1-phenyl-1H-imidazol-5-yl)benzene (**13**)*

White solid. IR (KBr) $\nu_{max}$ ($cm^{-1}$): 2,994, 2,943, 1,639, 1,461, 1,186, 1,074. $^1H$ NMR ($CDCl_3$) $\delta$ (ppm): 7.03 (s, 1H), 7.20–7.25 (m, 3H), 7.27 (s, 2H), 7.28–7.30 (m, 2H), 7.68 (s, 1H). Anal. calcd. for $C_{24}H_{18}N_4$ (362): C, 79.54, H, 5.01, N, 15.46. Found: C, 79.59, H, 5.05, N, 14.32%.

*1,4-Bis(1-p-tolyl-1H-imidazol-5-yl)benzene (**14**)*

White solid. IR (KBr) $\nu_{max}$ ($cm^{-1}$): 2,995, 2,946, 1,645, 1,462, 1,182, 1,076. $^1H$ NMR ($CDCl_3$) $\delta$ (ppm): 2.40 (s, 3H), 7.02 (s, 1H), 7.05 (d, $J$ = 8.00 Hz, 2H), 7.18 (d, $J$ = 8.00 Hz, 2H), 7.26 (s, 2H), 7.66 (s, 1H). $^{13}C$ NMR ($CDCl_3$) $\delta$ (ppm): 21.11, 125.44, 127.49, 127.90, 128.47, 130.92, 132.47, 134.04, 138.28, 139.19. HRMS (ESI): $m/z$ $[M+H]^+$ calcd. for $C_{26}H_{22}N_4$ 391.1843, found: 391.1922. Anal. calcd. for $C_{26}H_{22}N_4$ (390): C, 79.97, H, 5.68, N, 14.35. Found: C, 80.07, H, 5.55, N, 14.38%.

*1,3-Bis(1-phenyl-1H-imidazol-5-yl)benzene (**15**)*

White solid. IR (KBr) $\nu_{max}$ ($cm^{-1}$): 2,993, 2,942, 1,647, 1,460, 1,186, 1,074. $^1H$ NMR ($CDCl_3$) $\delta$ (ppm): 7.19–7.23 (m, 3H), 7.21 (s, 1H), 7.28–7.31 (m, 2H), 7.29 (m, 1H), 7.67 (d, $J$ = 7.40 Hz, 1H), 7.70 (s, 1H), 7.91 (s, 1H). $^{13}C$ NMR ($CDCl_3$) $\delta$ (ppm): 125.40, 127.41, 127.87, 128.11, 128.38, 128.94, 130.06, 130.86, 132.19, 138.30, 139.24. Anal. calcd. for $C_{24}H_{18}N_4$ (362): C, 79.54, H, 5.0, N, 15.46. Found: C,79.57, H, 5.05, N, 15.38%.

*1,3-Bis(1-p-tolyl-1H-imidazol-5-yl)benzene (**16**)*

White solid. IR (KBr) $\nu_{max}$ ($cm^{-1}$): 2,989, 2,944, 1,642, 1,465, 1,189, 1,076. $^1H$ NMR ($CDCl_3$) $\delta$ (ppm): 2.38 (s, 3H), 7.07 (d, $J$ = 7.40 Hz, 2H), 7.20 (s, 1H), 7.21 (d, $J$ = 7.80 Hz, 4H), 7.28 (m, 1H), 7.65 (d, $J$ = 7.20 Hz, 1H), 7.69 (s, 1H), 7.90 (s, 1H). $^{13}C$ NMR ($CDCl_3$) $\delta$ (ppm): 21.09, 125.40, 127.45, 127.92, 128.03, 128.50, 129.31, 130.90, 132.49, 134.10, 138.29, 139.20. Anal. calcd. for $C_{26}H_{22}N_4$ (390): C, 79.97, H, 5.68, N, 14.35. Found: C, 80.03, H, 5.65, N, 14.32%.

*(E)-4-Methyl-N-(4-(1-p-tolyl-1H-imidazol-5-yl)benzylidene)aniline (**17**)*

Light brown solid. IR (KBr) $\nu_{max}$ ($cm^{-1}$): 2,933, 1,641, 1,615, 1,435, 1,194, 1,075, 956, 821. $^1H$ NMR ($CDCl_3$) $\delta$ (ppm): 2.37 (s, 3H), 2.40 (s, 3H), 7.09–7.13 (m, 4H), 7.18–7.26 (m, 6H), 7.36 (s, 1H), 7.70 (s, 1H), 7.78 (d, $J$ = 7.8 Hz, 2H), 8.43 (s, 1H, CH═N). Anal. calcd. for $C_{24}H_{21}N_3$ (351): C, 82.02, H, 6.02, N, 11.96. Found: C, 82.04, H, 6.05, N, 11.90%.

*(E)-N-(3-(1-Phenyl-1H-imidazol-5-yl)benzylidene)aniline (**18**)*

Light brown solid. IR (KBr) $\nu_{max}$ ($cm^{-1}$): 2,932, 1,639, 1,620, 1,437, 1,191, 1,076, 958, 826. $^1H$ NMR ($CDCl_3$) $\delta$ (ppm): 7.17–7.20 (m, 3H), 7.28–7.34 (m, 7H), 7.34 (s, 1H), 7.38 (m, 1H), 7.68 (s, 1H), 7.79 (d, $J$ = 8.00 Hz, 2H), 8.18 (s, 1H), 8.42 (s, 1H, CH═N). Anal. calcd. for $C_{22}H_{17}N_3$ (323): C, 82.02, H, 6.02, N, 11.96. Found: C, 82.04, H, 6.0 4, N, 11.92%.

### 4.1.4 | General procedure for the synthesis of imidazopyridines 19–29

A mixture of 2-aminopyridine (1 mmol) and the corresponding dialdehyde (1 mmol) in absolute ethanol (5 ml) was stirred for 15 min. To this reaction mixture phenylacetylene (1.5 mmol) was added followed by the addition of $CuSO_4{\cdot}5H_2O$ (15 mol%) and D-glucose (30 mol%). Again ethanol (5 ml) was added to the reaction mixture and refluxed at 100°C for 10 hr. After the completion of reaction as indicated by TLC, the resultant mixture was directly adsorbed on neutral alumina (without workup) and the product was purified by column chromatography. The monomer **29** was synthesized from the reaction of 2-aminopyridine and benzaldehyde under the same above condition.

*1,4-Bis(3-benzylimidazo[1,2-a]pyridin-2-yl)benzene (**19**)*

White solid. IR (KBr) $\nu_{max}$ ($cm^{-1}$): 3,027, 2,926, 1,602, 1,499, 1,342, 1,049, 800. $^1H$ NMR ($CDCl_3$) $\delta$ (ppm): 4.54 (s, 2H), 6.74 (t, $J$ = 7.40 Hz, 1H), 7.15–7.27 (m, 5H), 7.30–7.33 (m, 2H), 7.73 (d, $J$ = 7.40 Hz, 1H), 7.89 (s, 2H). $^{13}C$ NMR ($CDCl_3$) $\delta$ (ppm): 29.82, 112.59, 117.39, 118.05, 123.49, 124.78, 126.71, 127.04, 127.75, 128.54, 129.14, 130.37, 136.56, 144.67. Anal. calcd. for $C_{34}H_{26}N_4$ (490): C, 83.24, H, 5.34, N, 11.42. Found: C, 83.27, H, 5.32, N, 11.41%.

*1,4-Bis(3-benzyl-6-methylimidazo[1,2-a]pyridin-2-yl)benzene (**20**)*
White solid. IR (KBr) $\nu_{max}$ ($cm^{-1}$): 3,025, 2,924, 1,609, 1,497, 1,345, 1,047, 802. $^1H$ NMR ($CDCl_3$) $\delta$ (ppm): 2.26 (s, 3H), 4.49 (s, 2H), 7.10 (d, $J$ = 7.20 Hz, 1H), 7.14–7.16 (m, 2H), 7.24–7.34 (m, 3H), 7.51 (s, 1H), 7.68 (d, $J$ = 8.20 Hz, 1H), 7.85 (s, 2H). $^{13}C$ NMR ($CDCl_3$) $\delta$ (ppm): 18.35, 29.77, 116.61, 117.80, 121.07, 127.01, 127.74, 127.75, 128.41, 128.42, 129.13, 129.14, 136.67, 145.31, 149.88. HRMS (ESI): $m/z$ $[M+H]^+$ calcd. for $C_{36}H_{30}N_4$: 519.2468, found: 519.2548. Anal. calcd. for $C_{36}H_{30}N_4$ (518): C, 83.37, H, 5.83, N, 10.80. Found: C, 83.34, H, 5.89, N, 10.77%.

*1,4-Bis(3-benzyl-7-methylimidazo[1,2-a]pyridin-2-yl)benzene (**21**)*
White solid. IR (KBr) $\nu_{max}$ ($cm^{-1}$): 3,022, 2,927, 1,615, 1,498, 1,342, 1,044, 807. $^1H$ NMR (DMSO-$d_6$) $\delta$ (ppm): 2.41 (s, 3H), 4.50 (s, 2H), 6.60 (d, $J$ = 7.20 Hz, 1H), 7.14–7.16 (m, 2H), 7.25–7.32 (m, 3H), 7.53 (s, 1H), 7.61 (d, $J$ = 8.00 Hz, 1H), 7.87 (s, 2H). $^{13}C$ NMR (DMSO-$d_6$) $\delta$ (ppm): $^{13}C$ NMR (151 MHz, DMSO) $\delta$ 192.84, 141.73, 140.82, 140.58, 137.77, 137.49, 135.82, 134.98, 133.87, 132.61, 130.10, 128.99, 126.83, 123.89, 119.78, 118.11, 115.29, 114.83, 28.93, 20.93, 15.36. Anal. calcd. for $C_{36}H_{30}N_4$ (518): C, 83.37, H, 5.83, N, 10.80. Found: C, 83.35, H, 5.87, N, 10.78%.

*1,4-Bis(3-benzyl-6-chloroimidazo[1,2-a]pyridin-2-yl)benzene (**22**)*
White solid. IR (KBr) $\nu_{max}$ ($cm^{-1}$): 3,025, 2,922, 1,617, 1,494, 1,339, 1,042, 801. $^1H$ NMR ($CDCl_3$) $\delta$ (ppm): 4.51 (s, 4H), 7.13–7.15 (m, 4H), 7.28–7.33 (m, 3H), 7.41–7.44 (m, 5H), 7.61 (d, $J$ = 8.0 Hz, 2H), 7.74 (d, $J$ = 8.4 Hz, 2H), 8.01 (s, 2H). $^{13}C$ NMR ($CDCl_3$) $\delta$ (ppm): 29.83, 114.13, 119.21, 122.27, 123.50, 124.75, 126.64, 127.12, 127.98, 128.54, 129.15, 130.37, 136.57. Anal. calcd. for $C_{34}H_{24}Cl_2N_4$ (559): C, 72.99, H, 4.32, N, 10.01. Found: C, 72.95, H, 4.30, N, 10.08%.

*1,4-Bis(3-(4-methylbenzyl)imidazo[1,2-a]pyridin-2-yl)benzene (**23**)*
White solid. IR (KBr) $\nu_{max}$ ($cm^{-1}$): 3,020, 2,919, 1,615, 1,484, 1,340, 1,045, 803. $^1H$ NMR ($CDCl_3$) $\delta$ (ppm): 2.33 (s, 3H), 4.49 (s, 2H), 6.86 (t, $J$ = 7.40 Hz, 1H), 7.02 (d, $J$ = 7.80 Hz, 2H), 7.13 (d, $J$ = 8.00 Hz, 2H), 7.28–7.35 (m, 2H), 7.79 (d, $J$ = 7.20 Hz, 1H), 7.90 (s, 2H). $^{13}C$ NMR ($CDCl_3$) $\delta$ (ppm): 21.10, 29.44, 112.69, 117.44, 123.84, 124.93, 127.93, 128.07, 128.35, 129.29, 129.87, 129.95, 133.48, 136.65, 144.59. Anal. calcd. for $C_{36}H_{30}N_4$ (518): C, 83.37, H, 5.83, N, 10.80. Found: C, 83.35. H, 5.87, N, 10.78%.

*1,3-Bis(3-benzylimidazo[1,2-a]pyridin-2-yl)benzene (**24**)*
White solid. IR (KBr) $\nu_{max}$ ($cm^{-1}$): 3,028, 2,919, 1,599, 1,494, 1,343, 1,036, 852. $^1H$ NMR ($CDCl_3$) $\delta$ (ppm): 4.42 (s, 2H), 6.68 (t, $J$ = 8.20 Hz, 1H), 7.07–7.09 (m, 2H), 7.16 (d, $J$ = 7.80 Hz, 1H), 7.20–7.28 (m, 3H), 7.55 (t, $J$ = 8.00 Hz, 1H), 7.65–7.67 (m, 2H), 7.85 (d, $J$ = 7.80 Hz, 1H), 8.18 (s, 1H). $^{13}C$ NMR ($CDCl_3$) $\delta$ (ppm): 29.88, 112.33, 117.72, 118.09, 123.81, 124.36, 127.02, 127.99, 128.18, 128.19, 129.15, 129.39, 134.99, 136.89, 144.10, 144.96. Anal. calcd. for $C_{34}H_{26}N_4$ (490): C, 83.24, H, 5.34, N, 11.42. Found: C, 83.25, H, 5.37, N, 11.38%.

*1,3-Bis(3-benzyl-6-methylimidazo[1,2-a]pyridin-2-yl)benzene (**25**)*
White solid. IR (KBr) $\nu_{max}$ ($cm^{-1}$): 3,027, 2,920, 1,596, 1,498, 1,339, 1,041, 855. $^1H$ NMR ($CDCl_3$) $\delta$ (ppm): 2.32 (s, 3H), 4.43 (s, 2H), 7.05–7.08 (m, 2H), 7.14 (d, $J$ = 7.40 Hz, 1H), 7.19–7.25 (m, 3H), 7.53 (t, $J$ = 8.00 Hz, 1H), 7.58 (s, 1H), 7.61–7.65 (m, 2H), 8.17 (s, 1H). $^{13}C$ NMR (101 MHz, $CDCl_3$) $\delta$ 143.81, 143.63, 136.85, 134.88, 128.96, 128.84, 127.71, 127.64, 127.53, 127.23, 126.67, 121.67, 120.94, 117.49, 116.75, 77.33, 29.67, 18.32. Anal. calcd. for $C_{36}H_{30}N_4$ (518): C, 83.37, H, 5.83, N, 10.80. Found: C, 83.34, H, 5.86, N, 10.80%.

*1,3-Bis(3-benzyl-7-methylimidazo[1,2-a]pyridin-2-yl)benzene (**26**)*
White solid. IR (KBr) $\nu_{max}$ ($cm^{-1}$): 3,026, 2,924, 1,595, 1,495, 1,333, 1,040, 852. $^1H$ NMR (DMSO-$d_6$) $\delta$ (ppm): 2.35 (s, 3H), 4.45 (s, 2H), 6.59 (d, $J$ = 7.40 Hz, 1H), 7.08–7.10 (m, 2H), 7.20–7.29 (m, 3H), 7.41 (t, $J$ = 7.80 Hz, 1H), 7.57 (s, 1H), 7.63 (d, $J$ = 6.80 Hz, 1H), 7.67 (d, $J$ = 7.80 Hz, 1H), 8.03 (s, 1H). $^{13}C$ NMR (DMSO-$d_6$) $\delta$ (ppm): $^{13}C$ NMR (151 MHz, DMSO) $\delta$ 160.00, 147.18, 144.44, 141.98, 137.58, 135.23, 135.06, 128.98, 128.89, 127.71, 126.97, 126.68, 126.68, 126.44, 123.65, 118.01, 115.25, 114.78, 20.86. Anal. calcd. for $C_{36}H_{30}N_4$ (518): C, 83.37, H, 5.83, N, 10.80. Found: C, 83.33, H, 5.91, N, 10.76%.

*1,3-Bis(3-benzyl-6-chloroimidazo[1,2-a]pyridin-2-yl)benzene (**27**)*
White solid. IR (KBr) $\nu_{max}$ ($cm^{-1}$): 3,027, 2,931, 1,594, 1,492, 1,335, 1,042, 851. $^1H$ NMR (DMSO-$d_6$) $\delta$ (ppm): 4.40 (s, 2H), 7.07–7.09 (m, 2H), 7.17 (d, $J$ = 7.80 Hz, 1H), 7.24–7.32 (m, 3H), 7.55 (t, $J$ = 7.40 Hz, 1H), 7.67 (d, $J$ = 7.80 Hz, 1H), 7.72 (s, 1H), 7.84 (d, $J$ = 7.8 Hz, 1H), 8.17 (s, 1H). $^{13}C$ NMR (151 MHz, DMSO-$d_6$) $\delta$ 144.08, 142.33, 137.44, 135.14, 128.94, 127.75, 126.75, 124.65, 124.43, 118.68, 117.04, 112.35, 40.11, 39.97, 39.84, 39.70, 39.56, 39.42, 39.28, 28.85. HRMS (ESI): $m/z$ $[M+H]^+$ calcd for $C_{34}H_{24}Cl_2N_4$: 559.1375, found: 559.1451. Anal. calcd. for $C_{34}H_{24}Cl_2N_4$ (559): C, 72.99, H, 4.32, N, 10.01. Found: C, 72.86, H, 4.35, N, 10.06%.

*1,3-Bis(3-(4-methylbenzyl)imidazo[1,2-a]pyridin-2-yl)benzene (**28**)*
White solid. IR (KBr) $\nu_{max}$ ($cm^{-1}$): 3,024, 2,932, 1,590, 1,491, 1,337, 1,045, 853. $^1H$ NMR (DMSO-$d_6$) $\delta$ (ppm): 2.32 (s, 3H), 4.49 (s, 2H), 6.90 (t, $J$ = 7.20 Hz, 1H), 6.99 (d, $J$ = 7.80 Hz, 2H), 7.08 (d, $J$ = 8.00 Hz, 2H), 7.37–7.43 (m, 2H), 7.63 (t, $J$ = 7.80 Hz, 1H), 7.79 (d, $J$ = 7.20 Hz, 1H), 7.94 (d, $J$ = 7.20 Hz, 1H), 8.25 (s, 1H). $^{13}C$ NMR (DMSO-$d_6$) $\delta$ (ppm): 21.35, 29.66, 112.70, 117.51, 118.42, 123.85, 127.14, 128.01, 128.21, 128.22, 129.15, 129.35, 132.03, 135.13, 136.22, 144.05, 145.11. Anal. calcd. for $C_{36}H_{30}N_4$ (518): C, 83.37, H, 5.83, N, 10.80. Found: C, 83.32, H, 5.85, N, 10.83%.

*3-Benzyl-7-methyl-2-phenylimidazo[1,2-a]pyridine (**29**)*
White solid. $^1H$ NMR ($CDCl_3$) $\delta$ (ppm): 2.26 (s, 3H), 4.48 (s, 2H), 7.11 (dd, $J$ = 1.80, 8.40 Hz, 1H), 7.16 (d, $J$ = 7.4 Hz, 2H), 7.27–7.38 (m, 4H), 7.43–7.45 (m, 2H), 7.51 (s, 1H), 7.69 (d, $J$ = 8.80 Hz, 1H), 7.79 (dd, $J$ = 2.0, 7.40 Hz, 2H). Anal. calcd. for $C_{21}H_{18}N_2$ (298.38): C, 84.53, H, 6.08, N, 9.39. Found: C, 84.52, H, 6.10, N, 9.38%.

## 4.2 | Pharmacology

### 4.2.1 | Anticancer activity (in vitro)

The anticancer screening was performed by SRB assay.[34,37–39] The cell lines were grown in RPMI 1640 medium containing 10% fetal bovine serum and 2 mM L-glutamine. For present screening experiment, cells were inoculated into 96-well microtiter plates in 90 µl at 5,000 cells per well. After cell inoculation, the microtiter plates were incubated at 37°C, 5% $CO_2$, 95% air, and 100% relative humidity for 24 hr before addition of experimental drugs. Experimental drugs were solubilized in appropriate solvent to prepare stock of $10^{-2}$ concentration. At the time of experiment, four 10-fold serial dilutions were made using complete medium. Aliquots of 10 µl of these different drug dilutions were added to the appropriate microtiter wells already containing 90 µl of medium, resulting in the required final drug concentrations.

After compound addition, plates were incubated at standard conditions for 48 hr and assay was terminated by the addition of cold TCA. Cells were fixed in situ by the gentle addition of 50 µl of cold 30% (w/v) TCA (final concentration, 10% TCA) and incubated for 60 min at 4°C. The supernatant was discarded; the plates were washed five times with tap water and air dried. Sulforhodamine B (SRB) solution (50 µl) at 0.4% (w/v) in 1% acetic acid was added to each of the wells, and plates were incubated for 20 min at room temperature. After staining, unbound dye was recovered and the residual dye was removed by washing five times with 1% acetic acid. The plates were air dried. Bound stain was subsequently eluted with 10 mM Trizma base, and the absorbance was read on an enzyme-linked immunosorbent assay (ELISA) plate reader at a wavelength of 540 nm, with 690 nm reference wavelength.

Percentage of growth was calculated on a plate-by-plate basis for test wells relative to control wells. Percentage of growth was expressed as the ratio of average absorbance of the test well to the average absorbance of the control wells × 100. Using the six absorbance measurements (time zero [$T_z$], control growth [$C$], and test growth in the presence of drug at the four concentration levels [$T_i$]), the percentage growth was calculated at each of the drug concentration levels. The dose response parameters were calculated for each test article. Growth inhibition of 50% ($GI_{50}$) was calculated from $[(T_i - T_z)/(C - T_z)] \times 100 = 50$, which is the drug concentration resulting in a 50% reduction in the net protein increase (as measured by SRB staining) in control cells during the drug incubation. The drug concentration resulting in total growth inhibition (TGI) was calculated from $T_i = T_z$. The $LC_{50}$ (concentration of drug resulting in a 50% reduction in the measured protein at the end of the drug treatment as compared with that at the beginning) indicating a net loss of cells following treatment is calculated from $[(T_i - T_z)/T_z] \times 100 = -50$. Values were calculated for each of these three parameters if the level of activity was reached; however, if the effect was not reached or was exceeded, the values for that parameter were expressed as greater or less than the maximum or minimum concentration tested.

### 4.2.2 | Anticancer activity (in vivo)

*Collection of samples*

In the experiment design, the animals were divided into five groups each consisting of six rats. After the experimental regimen (16 weeks), the animals were killed by cervical dislocation under mild chloroform anesthesia. Blood was collected in EDTA and centrifuge tubes by an incision made in the jugular veins and serum was separated by centrifugation at 2,000 rpm for 20 min and utilized for various biochemical assays. The liver and mammary tissue were excised immediately and thoroughly washed with ice-cold physiological saline and blotted dry. A part of the tissues such as liver and breast were removed and fixed in 10% formalin for histopathological study.

*Hematological parameters*

The hematological parameters such as hemoglobin, PCV, WBC, RBC, and platelets, were assayed. The whole blood sample was analyzed for the changes in the blood cells using SYSMEX Xs—800 i automatic hematology analyzer (Bio-Rad Laboratories, Inc.).

### 4.2.3 | Estimation of aspartate transaminase[40]

*Principle*

The enzyme catalysis the following reaction

$$\text{L-Aspartate} + \alpha\text{-oxoglutarate} \xrightarrow{\text{AST}} \text{oxaloacetate} + \text{L-glutamate}.$$

The oxaloacetate is measured by the reaction with 2,4-dinitrophenyl hydrazine giving a brown-colored hydrazone after the addition of sodium hydroxide (NaOH). The color developed is read at 520 nm.

*Reagents*

(a) Phosphate buffer, 0.1 M, pH 7.5, (b) substrate: 146 mg ketoglutarate and 13.3 g aspartic acid in 1 N NaOH. Adjusted the pH to 7.4 and made up to 1,000 ml with phosphate buffer, (c) standard pyruvate, 2 mML: 22 mg sodium pyruvate/100 ml phosphate buffer, (d) dinitrophenyl hydrazine reagent, 1 mmol/l in 1 M HCl, and (e) 0.4 N NaOH.

*Procedure*

To 0.2 ml of sample, added 1.0 ml of the buffered substrate and incubated for 60 min at 37°C. To the control tubes, enzyme was added after arresting the reaction with 1.0 ml of DNPH and the tubes were kept at room temperature for 20 min. Then 10 ml of 0.4 N NaOH was added. A set of standard pyruvate in the concentration 0.4–2.0 µM was also treated in a similar manner. The color developed was read at 520 nm. The enzyme activity was expressed as µmol of pyruvate liberated/l in serum and µmol of pyruvate liberated/min/mg protein in tissues.

### 4.2.4 | Estimation of alanine transaminase[40]

*Principle*

The enzyme catalyses the following reaction

$$\text{L-Alanine} + \alpha\text{-oxoglutarate} \xrightarrow{\text{ALT}} \text{pyruvate} + \text{L-glutamate}.$$

The oxaloacetate is measured by the reaction with 2,4-dinitrophenyl hydrazine giving a brown-colored hydrazone after the addition of NaOH. The color developed is read at 520 nm.

*Reagents*

(a) Phosphate buffer 0.1 M, pH 7.5, (b) substrate: 146 mg ketoglutarate and 17.8 g L-alanine in 1 N NaOH. Adjusted the pH to 7.4 and made up to 1,000 ml with phosphate buffer, (c) standard pyruvate, 2 mM: 22 mg sodium pyruvate/100 ml phosphate buffer, (d) dinitrophenyl hydrazine reagent, 1 mmol/l in 1 M HCl, and (e) 0.4 N NaOH.

*Procedure*

To 0.2 ml of sample, added 1.0 ml of the buffered substrate and incubated for 30 min at 37°C. To the control tubes, enzyme was added after arresting the reaction with 1.0 ml of DNPH and the tube was kept at room temperature for 20 min. Then 10 ml of 0.4 N NaOH was added. A set of standard pyruvate in the concentration 0.4–2.0 µM was also treated in a similar manner. The color developed was read at 520 nm. The enzyme activity was expressed as µmol of pyruvate liberated/l in serum and µmoles of pyruvate liberated/min/mg protein in tissues.

### 4.2.5 | Estimation of alkaline phosphatase[41]

*Principle*

The method used was that of King and Armstrong in which disodium phenyl phosphate is hydrolyzed with the liberation of phenol and inorganic phosphate. The liberated phenol is measured at 700 nm with Folin-Ciocalteu reagent.

*Reagents*

(a) Sodium carbonate/sodium bicarbonate buffer, 100 mM, (b) disodium phenyl phosphate, 100 mM, (c) buffered substrate: mix equal volume of above two solutions (pH 10), (d) Folin-Ciocalteu reagent: mixed 1.0 ml of reagent with 2.0 ml of water, (e) sodium carbonate solution, 15%, (f) standard: 1.0 g crystalline phenol/l in 0.1 M HCl, and (g) working standard: added 100 ml dilute phenol reagent to 5.0 ml of stock standard and diluted to 500 ml with water. This contained 10 µg phenol/ml.

*Procedure*

Pipetted out 4.0 ml of the buffered substrate into a test tube and incubated at 37°C for 5 min. Added 0.2 ml of serum and incubated for 15 min. Removed and immediately added 1.8 ml of diluted phenol reagent. A control was run simultaneously with 4.0 ml buffered substrate and 0.2 ml sample to which 1.8 ml phenol reagent was added immediately. Mixed well and centrifuged. To 4.0 ml of the supernatant added 2.0 ml of sodium carbonate. Standards were also run. Incubated all the tubes at 37°C for 15 min, the color developed were read at 700 nm. The enzyme activity was expressed as µmol of phenol liberated/l in serum and µmol of phenol liberated/min/mg protein.

### 4.2.6 | Estimation of ACP[42]

*Principle*

The method used was that of King and Armstrong in which disodium phenyl phosphate is hydrolyzed with the liberation of phenol and inorganic phosphate. The liberated phenol is measured at 700 nm with Folin-Ciocalteu reagent.

*Reagents*

(i) Citrate buffer: 0.1 M, pH 5. A: citric acid (21.01 g in 1,000 ml), B: sodium citrate (29.41 g in 1,000 ml), 20.5 ml of A and 29.5 ml of B, diluted to a total of 100 ml. (ii) Disodium phenyl phosphate, 100 mM: dissolved 2.18 g in water, heated to boil, cooled and made to a liter. Added 1.0 ml of chloroform and stored in the refrigerator. (iii) Buffer-substrate: prepared by mixing equal volume of the above two solutions. This has a pH of 5.0. (iv) Folin-Ciocalteu reagent: mixed 1.0 ml of reagent with 2.0 ml of water. (v) Sodium carbonate solution, 15%: dissolved 15 g of anhydrous sodium carbonate in 100 ml of water. (vi) Standard phenol solution, 1 g/l: dissolved 19 g pure crystalline phenol in 100 mM HCl and made to a liter with the acid. (vii) Working standard solution: diluted 10 ml of stock standard to 100 ml with water. This contains 100 µg phenol/ml.

*Procedure*

Pipetted out 4.0 ml of the buffered substrate into a test tube and incubated at 37°C for 5 min. Added 0.2 ml of sample and incubated further for exact 60 min, removed and immediately added 1.8 ml of diluted phenol reagent. At the same time a control was set up containing 4.0 ml buffered substrate and 0.2 ml of sample to which 1.8 ml of phenol reagent was added immediately. Mixed well and centrifuged. To 4.0 ml of the supernatant added 2.0 ml of sodium carbonate. Took 4.0 ml of working standard solution and for blank taken 3.2 ml water and 0.8 ml of phenol reagent. Then added 2.0 ml of sodium carbonate. Incubated all the tubes at 37°C for 15 min. Read the color developed at 700 nm.

The activity of serum ACP was expressed in µmol of phenol liberated/l. The activity in tissue homogenate was expressed as µmol of phenol liberated/min/mg protein.

### 4.2.7 | ELISA of CEA and CA 15-3[43]

Quantitative estimation of tumor markers namely, CEA and CA 15-3 was carried out by solid phase ELISA using the UBI MAGIWELL (USA) enzyme immunoassay kit. The desired numbers of coated wells were secured in the holder. Ten microliters of standards, controls, or serum samples were then dispensed into appropriate wells. One well was

saved for the blank, to which standards or enzyme conjugate should not be added. Fifty microliters of antibody solution was dispensed into each well expect the blank well. The wells were then incubated for 1 hr at room temperature. After incubation mixture the wells were rinsed with working washing buffer (50 ml) five times. Then, 100 µl of solution A (a phosphate buffer solution containing hydrogen peroxide) and 100 µl solution B (tetramethylbenzidine solution) was added into each well including the blank well. This was then incubated for 30 min at room temperature, after which, the enzyme reaction was stopped by the addition of 50 µl of stop reagent and the intensity of the color was measured with microreader at 450 nm.

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## CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

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## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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