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3-Arylindanones and related compounds as antiproliferative agents against colorectal cancer

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Abstract

Diverse benzylidene indanones and their derivatives were synthesized as anticancer agents. Two of the analogues i.e. **7** and **22** exhibited significant antiproliferative activity against several human cancer cell lines. Both the compounds possessed antimitotic activity and induced apoptosis in DLD1 colorectal adenocarcinoma cells through activation of caspase pathways. In cell cycle analysis, both the compounds induced predominantly G2/M phase arrest in DLD1 cells. Molecular docking studies revealed that compound **7** occupies colchicine binding pocket of β-tubulin. Both the compounds were safe in acute oral toxicity in rodents. Both the compounds are further being optimized for better efficacy.

Keywords: cancer; apoptosis; caspase; antitubulin; acute oral toxicity.

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1. INTRODUCTION

Colorectal cancer is a multifaceted disease which is therapeutically challenged. Globally, it is the third most common cancer after lung and breast cancers, which has caused about 1.80 million human deaths in 2018 (WHO Factsheet, 2018). It is more prevalent in developing countries and less pervasive among women than men. It is estimated that there will be 60% of rise in global colorectal cancer burden by 2030 (Farooqi et al., 2019). Most of the colorectal cancers are due to old age, life style factors and only a few cases due to genetic disorders. Some risk factors include diet, smoking, obesity, and lack of physical activity. The common treatments include surgery, radiation, chemotherapy, and target therapy. Chemotherapy is an integral part of treatment in advance stages of the disease which includes 5-fluorouracil, irinotecan, oxaliplatin, and capecitabine etc. (Fig. 1). Available therapies for treatment show

only limited efficacy and there are many complications with relapse in 30-50% of cases (Thomas, 2016), which warrants for the development of new, efficient, and safer chemotherapeutics.

Tubulin is a globular protein that plays an important role in cell growth and development. Alpha-beta tubulin dimer polymerises to microtubules and afterwards depolymerises also (Jordan & Wilson, 2004). Subsequently, microtubule bundles are formed and lead to mitosis in cells during G2/M phase. This polymerisation/depolymerisation process is in dynamic equilibrium. Any disturbance in this process leads to cell cycle arrest. Owing to an important role in mitosis, tubulin has been an important target for cancer drug development. Several successful drugs have been developed and being used as clinical drugs notably paclitaxel, docetaxel, vincristine, vinblatine and many more are as clinical candidates (Fig. 1) (Negi et al., 2015).

Fragment Based Drug Discovery (FBDD) is one of the recent approaches in medicinal chemistry to achieve bioactive compounds [Keseru et al. 2016]. It identifies low molecular weight fragment or motif exhibiting quality interactions with biological targets to elicit a particular pharmacological response. In the present study, a specific motif 3,4,5-trimethoxyphenyl is placed at ring A of indanone pharmacophore. Some of the naturally occurring antitubulin compounds like colchicine, podophyllotoxin, and combretastatin A4 etc possess this fragment which interacts with the β-tubulin at α/β junctions to elicit antitubulin effects (Negi et al., 2015**)**. Further, we have diversified the indanone based anticancer pharmacophore especially for nitrogen moiety. Twenty six diverse 3-arylindanone based compounds were prepared and evaluated for biological activity against a penal of human cancer cell lines. Two of the potential compounds were evaluated for detailed cancer pharmacology. Both the potent compounds were also evaluated for *in-vivo* acute oral toxicity in Swiss-albino mice.

2 EXPERIMENTAL SECTION

2.1. Chemistry

The syntheses of potent compounds **7** and **22** are given in the manuscript, rest of the compounds are given in Supplementary information (S1-S3).

*2.1.1. Synthesis of compound **7***

Indanone **1** (250mg, 0.64 mmol) was taken in dioxane (10mL). To this stirred reaction mixture selenium dioxide (300mg, 2.72 mmol) was added in fractions and reaction mixture was refluxed for 5 h. Dioxane was evaporated and residue was taken in ethyl acetate. It was washed with water, dried over anhydrous sodium sulphate and evaporated. The crude mass was purified through silica gel column to get compounds **6**, **7** and **8**.

*2-Hydroxy, 3-(3´,4´,5´-trimethoxyphenyl)-4,5,6-trimethoxy-ind-2-en-1-one (**7**)*

Yield:23%; m.p.=oil; ^{1}H NMR (300MHz, $CDCl_3$): δ3.32 (s, 3H, OCH_3), 3.80 (s, 6H, 2xOCH_3), 3.88 (s, 3H, OCH_3), 3.96 (s, 6H, 2xOCH_3), 6.65 (s, 2H, 2xCH of 3-phenyl ring), 6.97 (s, 1H, 7-CH); ^{13}C NMR (75MHz, $CDCl_3$): δ56.02, 56.22, 56.48, 60.86, 61.10, 61.45, 105.33, 105.93, 124.71, 127.68, 128.46, 129.10, 138.51, 147.51, 148.36, 152.34, 153.96, 160.40, 192.02; ESI mass for $C_{21}H_{22}O_8$, 401 $[M-H]^+$, 441 $[M+K]^+$, Negative mode: 401 $[M-H]^-$; HRMS (ESI-TOF) m/z [M+H] Calcd for $C_{21}H_{22}O_8$ 401.1236 , found 401.1235.

*3-(3´,4´,5´-Trimethoxyphenyl)-4,5,6-trimethoxy--indan-1,2-dione (**6**)*

Yield:16%; m.p.= 193-195^{o}C ; ^{1}H NMR (300MHz, $CDCl_3$): δ3.61 (s, 3H, OCH_3), 3.75 (s, 9H, 3xOCH_3), 4.05 (s, 3H, OCH_3), 4.08 (s, 3H, OCH_3), 4.71 (s, 1H, 3-CH), 6.33 (s, 2H, 2xCH of 3-phenyl ring), 7.61 (s, 1H, 7-CH); ^{13}C NMR (75MHz, $CDCl_3$): δ49.87, 56.61, 56.82, 61.02, 61.24, 61.64, 102.42, 105.91, 133.16, 133.67, 137.98, 138.20, 150.99, 151.95,

154.01, 155.68, 187.19, 198.44; ESI mass for $C_{21}H_{22}O_8$, 403 $[M+H]^+$, 425 $[M+Na]^+$, 441 $[M+K]^+$, Negative mode: 401 $[M-H]^-$; HRMS (ESI-TOF) m/z [M+H] Calcd for $C_{21}H_{22}O_8$ 403.1392, found 403.1384.

*1,2,3,5,6,7-Hexamethoxy-indeno[2,1b]-9-oxo-benzo[b]furan (**8**)*

Yield:40%; m.p.=oil; ^{1}H NMR (300MHz, $CDCl_3$): δ3.90 (s, 3H, OCH_3), 3.95 (s, 3H, OCH_3), 3.98 (s, 3H, OCH_3), 4.00 (s, 3H, OCH_3), 4.02 (s, 3H, OCH_3), 4.07 (s, 3H, OCH_3), 7.04 (s, 1H, 4-CH), 8.07 (s, 1H, 8-CH); ^{13}C NMR (75MHz, $CDCl_3$): δ56.14, 56.52, 60.88, 61.14, 61.27, 61.85, 105.23, 106.33, 126.62, 129.94, 132.65, 136.51, 141.19, 146.76, 146.91, 148.96, 153.42, 153.77, 156.09, 187.73; ESI mass for $C_{21}H_{20}O_8$, 400 $[M]^+$.

*2.1.2. Synthesis of 3-(4'-N,N-Dimethylaminophenyl)-4,5,6-trimethoxyindan-1-one (**22**)*

It was prepared as per our previously reported method (Saxena et al., 2008).

Yield:29%; m.p.=89-91^{o}C; ^{1}H NMR (300MHz, $CDCl_3$): δ2.54 (dd, 1H, 2-CH_2, J=17.1Hz and 2.4Hz), 3.10-3.19 (dd, 1H, 2-CH_2, J=11.4Hz and 8.1Hz), 2.98 (s, 6H, N-$(CH_3)_2$), 3.36 (s, 3H, OCH_3), 3.89 (s, 6H, 2xOCH_3), 4.48-4.52 (dd, 1H, 3-CH, J=2.4Hz and 5.4Hz), 6.63 (d, 2H, 2xCH of 3-Phenyl, J=8.7Hz), 6.94-6.97 (d, 2H, 2xCH of 3-Phenyl, J=8.7Hz), 7.07 (s, 1H, 7-CH); ^{13}C NMR (75MHz, $CDCl_3$): δ41.14, 41.18, 47.80, 56.59, 60.54, 61.23, 100.67, 107.78, 113.39, 128.25, 132.49, 132.70, 145.69, 149.80, 150.85, 155.09, 206.35; ESI mass for $C_{27}H_{27}NO_5$, 342 $[M+H]^+$, 364 $[M+Na]^+$; ESI-HRMS for $C_{27}H_{28}NO_5$ calc, 342.1705 ; found 342.1685.

2.2 Biological evaluation

The details of all the biological assays are in Supplementary information (S4-S8).

2.2.1 Cytotoxicity evaluation by Sulphorhodamine assay

Cytotoxic activity of the compounds was assessed against human cancer cell lines *viz.* A549 (lung carcinoma), DU145 (Prostate carcinoma), FaDU (Hypopharyngeal carcinoma), DLD1 (colorectal adenocarcinoma), MCF-7 (Breast adenocarcinoma), and healthy/normal cell line HEK-293 (Human embryonic kidney) by Sulphorhodamine B dye based plate assay as per reported method (Adaramoye et al., 2011). Tamoxifen, doxorubicin, podophyllotoxin and paclitaxel were used as positive controls.

2.2.2 Cell cycle analysis

The effect of potent compounds **7** and **22** on DLD1 cell division cycle was assessed by flow cytometry with PI-stained cellular DNA (Sarkar et al., 2009).

2.2.3 Tubulin polymerisation assay

Tubulin polymerization assay experiment was performed using 'assay kit' procured from Cytoskeleton, Inc. Denver, USA. The procedure was followed as per reported protocol (Lee & Timassheff et al., 1997). The standard antimitotic agents nocodazole, podophyllotoxin (as destabilizers) and paclitaxel (as stabilizer) were used as positive controls.

2.2.4 Apoptosis induction by caspase pathway

Lysate of DLD1 cells was prepared after treating with compounds **7** and **22** and further processed as described in Supplementary information. Doxorubicin was used as positive control.

2.2.5 Molecular docking studies

Docking studies of compound **7** (Destabilizer) was determined at colchicine binding pocket of β-tubulin (Prota et al., 2014), while both the enantiomers compound **22** (R) and **22** (S) (Stabilizers) were assessed at paclitaxel binding pocket of tubulin (Atherton et al., 2017) using docking software AutoDock Vina. Further, preliminary 'Drug likeliness' was also assessed for both the potent compounds **7** and **22** as per 'Lipinski's rule of five' using Discovery Studio 3.5v software (Accelrys, USA).

2.2.6 Acute oral toxicity

Considering significant anti-cancer activity of compounds **7** and **22** in *in-vitro* models, two different acute oral toxicity experiments were carried out in accordance with the Organization for Economic Co-operation and Development (OECD) test guideline No 423 (1987) in Swiss albino mice (Allan et al., 2007) after IAEC (Institutional Animal Ethical Committee) approved protocols *vide* ref. No. CIMAP/IAEC/2016-19/01, dated 09/2/2017.

2.2.7 Statistical analysis

Statistical analysis for cytotoxicity was carried out in Microsoft Excel. The ANOVA followed by Tukey's multiple comparison test was used to assess the statistical significance of vehicle verses treatment groups. Results are presented as the means ± SE. Differences with a P value <0.05 were considered significant. In acute oral toxicity, Turkey's multiple column comparison method was used at 5% significant level (n=6).

3 RESULTS

3.1 Chemical Syntheses

The synthetic strategy was as depicted in scheme 1. Gallic acid was starting substrate, which was modified to indanones **1** as per our reported method (Saxena et al., 2008). Indanone **1** was transformed to various types of indanone derivatives. It underwent Claisen-Schmidt reaction with various aromatic aldehydes in 7% alkali in methanol to give 2-benzylidene indanones **2**-**5** in 79-91% yields. Indanone **1** was oxidised with selenium dioxide in dioxane to give a 1,2-diketo derivative (**6**), keto-enol derivatives **7** and an indolobenzofuran derivatives **8** as a condensed product of **7**. Indanone **1** was treated with dimethylcarbonate in THF using sodium hydride as a base to afford methyl-2-indolylcarboxylate (**9**). Ester **9** was hydrolysed with 5% alkali to get corresponding acid **10**. Indanone **1** was converted to its keto-oxime **11** by treating it with hydroxylamine hydrochloride in ethanol. Oxime **11** underwent Beckmann rearrangement by treating it with *p*-toluene sulphonic acid (*p*-TSA) to afford a lactam **12** (Gawley, 1988). Another indanone series was different than the previous indanones **1**. 3-(4-nitrophenyl)-4,5,6-trimethoxy-indanone-1 (**13**) was prepared in two steps. 3,4,5-Trimethoxyacetophenone was condensed with 4-nitrobenzaldehyde to give a chalcones moiety i.e. 3-(4-nitrophenyl)-1-(3,4,5-trimethoxyphenyl)-2-propen-1-one which was further transformed to indanones **13** by treating with trifluoroacetic acid. Several diverse analogues were prepared onto indanones **13**. It was treated with aromatic benzaldehydes to get 2-benzylidenes (**14**-**18**). Pyridine-2-carboxaldehyde reacted with indanones **13** in an alkaline medium to give 2-pyridinyl-methine indanones (**17**). Oxime of indanones **13** was prepared as described previously for **12**. Similarly, another indanones derivative **22** was prepared and further 2-benzylideneindanones (**23**-**26**) were prepared as described earlier. All the intermediates and final compounds were confirmed by spectroscopy [Supplementary information].

3.2 Purity profile of compounds 7 and 22

The purity of potential anticancer lead compounds **7** and **22** was assessed on Waters UPLC system, using column C-18 (BEH 130Å, 1.7× 50mm, 1.7µm, Waters, USA), PDA (190-400 nm) at λ_{max} (282 nm and 263 nm). Under optimised chromatographic condition, compounds **7** and **22** were eluted at 3.01 min and 1.36 min without any interference of any neighbouring peaks. The purity of the synthesized compounds **7** and **22** were 99% and 98.39%, respectively.

3.3 Biological evaluation

3.3.1 Cytotoxicity evaluation by Sulphorhodamine assay

The cytotoxicity results of indanone derivatives exhibiting significant activity among **2-26** and standard drugs are depicted in Table 1. The preliminary evaluation of compounds was done at 50µM concentration. Only six compounds exhibited significant cytotoxicity while two of the compounds i.e. **7** and **22** showed potential cytotoxicity against most of the cell lines. Compound **7** exhibited cytotoxicity IC_{50} in the range of 2.22-4.80 µM while compound **22** had IC_{50} in the range of 1.23-10.87 µM. Selectivity index (SI) was calculated using healthy cell lines (HEK-293) to know the safety profile *in-vitro*. Compounds **7** and **22** exhibited SI >21 and 97 indicating quite safe (SI≥10), as compared to the clinical drugs possessing very poor SI (tamoxifen, 1.29 and doxorubicin, 0.56).

3.2.2 Cell cycle analysis

Cell cycle has sequence of events which duplicates its genome and ultimately leads to cell division into two daughter cells. There are several regulators at various phases of cell cycle progression (Sherr, 2000). Compounds **7** and **22** both induced G2/M phase arrest in DLD1 colorectal cancer cell lines (Figure 2). This effect was more prominent at 24h but at 48h this

effect was almost negligible. Both the compounds (**7** and **22**) also exerted S phase arrest in DLD1 cells. However, G2/M phase arrest was comparatively more predominated over S phase arrest.

3.2.3 Tubulin polymerisation assay

Compounds **7** and **22** exhibiting potential cytotoxicities were further evaluated for their effect on tubulin polymerisation. As shown in Table 1, compound **7** inhibited tubulin polymerisation strongly (IC_{50}=0.57μM) thus acting as microtubule destabiliser quite comparable to podophyllotoxin (IC_{50}=0.72μM) and nocodazole (IC_{50}=0.62μM). Conversely, compound **22** exhibited stabilizing effect which was much weaker (EC_{50}=1.8μM) than that of paclitaxel (EC_{50}=0.025μM) (Fig. 3). Overall, both the compounds exhibited antimitotic effects.

3.2.4 Apoptosis induction by caspase pathway

In western blot experiments, compounds **7** and **22** cleaved PARP (Poly ADP-ribose polymerase) enzyme at their IC_{50} values (Figure 4). Both the compounds cleaved PARP at their IC_{50} concentrations (2.22μM and 1.23μM) to indicate apoptosis induction through caspase cascade pathway. During apoptosis, PARP is cleaved by the activation of caspase pathway. However, this effect was moderate and only up to 24h of exposure of compounds.

3.2.5 Molecular docking studies

In molecular docking studies, compound **7**, podophyllotoxin, and colchicine all the ligands bound to the colchicine binding pocket (PDB ID: **4O2B**) and shared almost equal interacting amino acids within the region of 4Å radius (Table S7A). There were seven common amino acid residues (LEU β:248, , LYS β:254, LEU β:255, ASN β:258, , ALA B:316, LYS β:352, ALA β:354) to all the three ligands, indicating the occupancy of same pocket. Compound **7**

showed docking energy of -6.1 kcal/mol which was comparable with the standard destabilizers podophyllotoxin (-7.2 kcal/mol) and colchicine (-8.5 kcal/mol) (Fig. 5A).

Another tubulin target PDB ID: **5M5C** a tubulin-paclitaxel bound complex was taken for docking studies of compound **22** and control drug paclitaxel (Table S7B). Both **22** (R) and **22** (S) enantiomers were used for docking studies. There were nine common residual amino acids (VAL β:23, LEU β:230, ALA β:233, PHE β:272, LEU β:275, PRO β:360, ARG β:369, LEU β:371, SER β:374) common in paclitaxel and compounds **22** (R) and **22** (S) indicating that these ligands occupy almost the same binding pocket of tubulin. Compound **22** (R) showed relatively low binding (-6.5 kcal/mol) as compared to isomer **22** (S) (-7.1 kcal/mol) of whereas paclitaxel showed little higher docking energy of -8.2 kcal/mol (Fig. 5B). Both the compounds **7** and **22** did not show any deviation as per Lipinski rule [Supplementary information, Tables S7A, S7B, and S7C].

3.2.6 Acute oral toxicity

Owing to potential antiproliferative efficacy of compounds **7** and **22**, both were evaluated for safety aspects in rodent model by two independent experiments (Table 2 and Figure 6A/B; Table 3 and Figure 7A/B). Four different oral doses (5 mg/kg, 50 mg/kg, 300 mg/kg, and 1000 mg/kg) of compounds **7** and **22** were given to Swiss-albino mice as single oral dose.

There was no morbidity or mortality in experimental animals treated with compound **7** up to the dose level 1000 mg/kg in the acute oral toxicity test. Most of biochemical parameters showed non-significant changes. However, SGOT and SGPT levels showed significant increase in group of animals treated with the compound at 1000 mg/kg as single acute oral dose. When an anticancer compound administered orally, it is quite possible. However, safety index and therapeutic profile of the compound may be important to decide its dose and route of administration to use this compound judiciously (Muller & Milton, 2012). Other

parameters studied showed non-significant changes. It was absolutely safe up to 300 mg/kg dose.

However, compound **22** was safer than **7** up to 1000 mg/kg oral dose. There was no morbidity or mortality in the animals treated with compound **22** up to the dose level 1000 mg/kg in the acute oral toxicity. All the haematological, biochemical parameters and gross pathological analysis showed non-significant changes.

DISCUSSION

Colorectal cancer is one of the most frequent malignancies in the world. Despite the improved management in the chemotherapy, side effects and drug resistance have shown limitations and led to the search for superior treatments. In our studies, both the compounds **7** and **22** exhibited potential cytotoxicities against colorectal adenocarcinoma cells. Hence, detailed pharmacology of these compounds was explored against this cell line.

Tubulin-microtubule dynamic equilibrium is an important aspect in cell growth and development. Antimitotic agents, both microtubule stabilizers like paclitaxel and microtubule destabilizers like podophyllotoxin suppress spindle dynamics inducing mitotic arrest leading to cell death in rapidly dividing cancer cells (Cao et al., 2018; Borisy et al., 2016). Both the compounds (**7** & **22**) showed antimitotic activity. Highly dynamic spindle microtubules are considered remarkable therapeutic targets for the development of cancer chemotherapeutics (Brouhard & Rice, 2018). Further, in molecular docking studies, there were fourteen residual amino acids in the binding pocket of compound **7** common to colchicine binding pocket. It indicates almost same binding location of compound **7** to the β-tubulin. Burns *et al.* (1992) reported that ALA β:316 is an important residue that is directly involved in the binding of trimethoxyphenyl unit of both colchicine and podophyllotoxin (Burns, 1992). It was further elaborated that although trimethoxyphenyl unit is important for binding to β:316, but

hydrophobicity around this residue also plays a role to some extent as it specifies the rate of conformational changes of the ligand. The designed compound **7**, possesses similar arrangements of trimethoxyphenyl unit in ring A and also exhibits similar effects of microtubule destabilization as by colchicine and podophyllotoxin. On the other hand, compound **22** exhibited weak effect of microtubule stabilization. Paclitaxel, a standard microtubule stabilizer, binds to the luminal surface of β-tubulin in microtubules also known as paclitaxel binding site. Nogales *et al.* (1998) found LEU β:275 as a crucial residue in M-loop of binding pocket which is the main interaction point (Nogales et al., 1998). Further, Field *et al.* (2013) revealed that in S9-S10 loop amino acid residues PRO β:360 and ARG β:369 also play important roles (Field et al., 2013). All the three residues were present in the binding pocket of ligands **22**, and paclitaxel, which indicates the occupancy of similar binding pocket by both the ligands. In docking studies with both the enantiomers **22**(R) and **22** (S) independently, **22** (S) showed better binding affinity than the **22**(R) isomer. However, both the enantiomers occupied all the three crucial residues (LEU β:275, PRO β:360, and ARG β:369) to exhibit microtubule stabilization effect.

Oncogenic processes mainly target on cell cycle regulators and abandon their control leading to uncontrolled proliferation of cells which is the hallmark of cancer cells (Hanahan & Weinberg, 2011). Compounds **7** and **22** exhibited G2/M phase arrest in DLD1 cells at 24 h. But, this effect was negligible at 48 h. It might be due to faster metabolism of both the compounds by cancer cells. In general, antimitotic agents induce G2/M phase arrest due to interference in mitotic phase (Negi et al., 2015). Triggering of cell cycle arrest in cancer cells comprises one of the most prevalent strategies to halt or restrict cancer spreading (Hartwell & Weinert, 1989). As a result of cell cycle arrest compound **22** exhibited inductions of apoptosis. But, it was not visible in case of compound **7**. Many compounds induce apoptosis as a result of cell cycle interruption.

Apoptosis is a controlled and energy dependent cellular process which leads to programmed cell death (Susan, 2007). During apoptosis PARP is cleaved by caspases and inactivated (Chiarugi & Moskowitz, 2002). PARP cleavage in DLD1 cells by both the compounds clearly indicates activation of caspase pathway to trigger apoptosis. However, this effect was moderate in both the cases and only up to 24h. There were similar indications in cell cycle analysis where both the compounds exhibited prominent effects only up to 24h. It indicates that the antiproliferative effects of both the compounds are early on DLD1 cells. Both apoptotic signalling pathways induce cell death by activating protease enzymes i.e. caspase. Caspases 8, 9, and 10 are initiators while caspases 3, 6, and 7 are effector caspases (Degterev et al., 2003). Cancer cells circumvent apoptosis and continue proliferating cells. Induction of apoptosis by drug entities is preferred in cancer drug discovery (Olsson & Zhivotovsky, 2014).

There was no deviation in any of the parameters of compounds **7** and **22** specified in Lipinski rule, which indicates good druggability of both the compounds. Drug candidates complying rule of five tend to have lower attrition during clinical trials and are more successful in reaching to market (Leeson & Springthorpe, 2007).

Assessment and optimization of safety of drug candidates is an important aspect in drug discovery and development to recognise the molecules with balanced safety-efficacy profile *via* therapeutic window. Acute oral toxicity is used to assess the adverse effects of investigational drug within a short time after single oral dose or multiple doses given within 24h (IUPAC Gold Book, 2004).

Both the compounds were safe and well tolerated by experimental animals in acute oral toxicity. However, experiments with prolonged exposure of drug candidate will be required during future development through sub-acute, chronic and sub-chronic toxicity experiments. Nowadays, safety has become most important concern and a key criterion for successful new drugs due to regulatory, societal and legal concerns (Ray, 2009).

4. CONCLUSION

In summary, diverse analogues of 1-indanone were prepared as possible anticancer agents. Two of the analogues exhibited potential antiproliferative activity against colorectal adenocarcinoma. These analogues showed antimitotic effects and induced G2/M phase arrest in cell cycle analysis. Both the potential lead compounds moderately induced apoptosis in DLD1 cells through induction of caspase pathway. Both the lead compounds showed good druggability under Lipinski rule and were found to be safe in rodent models. Thus, both the indanone based lead compounds **7** and **22** are potential anticancer agents. The optimization of these lead compounds is underway to get more efficacious compounds from this series.

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CONFLICT OF INTEREST

Authors declare no conflict of interest.

REFERENCES

Adaramoye O. A., Sarkar J., Singh N., *et al.* (2011). Antiproliferative action of *Xylopia aethiopica* fruit extract on human cervical cancer cells. *Phytotherapy Research,* 25, 1558-63.

Allan J. J., Damodaran A., Deshmukh N. S., Goudar K. S., Amit A. (2007). Safety evaluation of a standardized phytochemical composition extracted from *Bacopa monnieri* in Sprague--Dawley rats. *Food Chemical Toxicol*ogy, 45,1928-37.

Atherton J., Jiang K., Stangier M. M., Luo Y., Hua S., Houben K., Van Hooff J. J., Joseph A. P., Scarabelli G., Grant B. J., Roberts A. J. (2017). A structural model for microtubule minus-end recognition and protection by CAMSAP proteins. *Nature Structural Molecular Biol*ogy, 24, 931-43.

Borisy G, Heald R, Howard J, Janke C, Musacchio A, Nogales E. (2016). Microtubules: Fifty years on from the discovery of tubulin. *Nature*, 17, 322-28.

Brouhard G. J., Rice L. M. (2018). Microtubule dynamics: an interplay of biochemistry and mechanics. *Nature Rev Mol Cell Biology*; 19: 451-463.

Burns R. G. (1992). Analysis of the colchicine-binding site of beta-tubulin. *FEBS J,* 297,205-8.

Cancer Key Facts, World Health Organisation, 12 September 2018.

Cao Y. N., Zheng L. L., Wang D., Liang X. X., Gao F., Zhao X. L. (2018). Recent advances in microtubule stabilizing agents. *European J. Medicinal Chemistry*; 143: 806-28.

Chiarugi A., Moskowitz M. A. (2002). Cell biology. PARP-1- a perpetrator of apoptotic cell death? *Science,* 297,200-201.

Degterev A., Boyce M., Yuan J. (2003). A decade of caspases. *Oncogene*, 22, 8543-67.

Farooqi A. A., De La Roche M., Djamgoz M. B. A., Siddik Z. H. (2019). Overview of the oncogenic signaling pathways in colorectal cancer: Mechanistic insights. *Seminars in Cancer Biology*; doi: 10.1016/j.semcancer.2019.01.001 .

Field J. J., Diaz J. F., Miller J. H. (2013). The binding sites of microtubule-stabilizing agents. *Chemistry and Biology*, 20: 301-315.

Gawley R. E. (1988) Beckmann Rearrangement. *Organic Reactions*, 35, 1-420.

Hanahan D., Weinberg R. A. (2011). Hallmarks of cancer: the next generation. *Cell*, 144, 646-74.

Hartwell L. H., Weinert T. A. (1989). Checkpoints: controls that ensure the order of cell cycle events. *Science*, 246, 629-34.

IUPAC Gold Book. *PAC* (2004), *76* 1033 (IUPAC Recommendations **2003**).

Jordan M. A., Wilson L. (2004). Microtubules as a target for anticancer drugs. *Nature Reviews Cancer*; 4, 253-65.

Keseru G. M., Erlanson D. A., Ferenczy G. G., Hann M. M., Murray C.W., Pickett S. D. (2016). Design and principles for fragment libraries: maximizing the value of learnings from pharma fragment based drug discovery (FBDD) programs for use in academia. *J. Medicinal Chemistry*, 59, 8189-8206.

Lee J. C., Timassheff S. N. (1997). *In vitro* reconstitution of calf brain microtubules: effects of solution variables. *Biochemistry*,16,1754-64.

Leeson P. D., Springthorpe B. (2007). The influence of drug-like concepts on decision-making in medicinal chemistry. *Nature Reviews Drug Discovery*, 6, 881-90.

Muller P. Y., Milton M. N. (2012). The determination and interpretation of the therapeutic index in drug development. *Nature Reviews Drug Discovery*, 11:751-61.

Negi A. S., Gautam Y., Alam S., Chanda D., Luqman S., Sarkar J., Khan F., Konwar R. (2015). Natural antitubulin agents: importance of 3,4,5-trimethoxyphenyl fragment. *Bioorganic Medicinal Chemistry,* 23, 373-89.

Nogales E., Wolf S. G., Downing K. H. (1998). Structure of the alpha beta tubulin dimer by electron crystallography. *Nature,* 391,199-203.

Olsson M., Zhivotovsky B. (2014). Caspases and cancer. *Cell Death Differentiation,* 18,1441-49.

Prota A. E., Danel F., Bachmann F., Bargsten K., Buey R. M., Pohlmann J., Reinelt S., Lane H., Steinmetz M. O. (2014). The novel microtubule-destabilizing drug BAL27862 binds to the colchicine site of tubulin with distinct effects on microtubule organization. *J. Molecular Biology,* 426, 1848-60.

Ray A. (2009). Beyond Debacle and Debate: Developing Solutions in Drug Safety. *Nature Reviews Drug Discovery*, 8,775-79.

Sarkar J., Singh N., Meena S., Sinha S. (2009). Staurosporine induces apoptosis in human papillomavirus positive oral cancer cells at G2/M phase by disrupting mitochondrial membrane potential and modulation of cell cytoskeleton. *Oral Oncology,* 45, 974-79.

Saxena H. O., Faridi U., Srivastava S., Kumar J. K., Darokar M. P., Luqman S., Chanotiya C. S., Krishna V., Negi A. S., Khanuja S. P. S. (2008). Gallic acid based indanone derivatives as anticancer agents. *Bioorganic Medicinal Chemistry Letters*, 18, 3914-18.

Sherr C. J. (2000). The Pezcoller lecture: cancer cell cycles revisited. *Cancer Research,* 60, 3689-95.

Susan E. (2007). Apoptosis: a review of programmed cell death. *Toxicology Pathology,* 35, 495-516.

Thomas S. N.(2016) *Science Translational Medicine*, 8, 352.

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Supplementary information

^{1}H NMR, ^{13}C NMR and Mass analysis data of compounds, detailed biological protocols and scanned spectra are available online version.

Figure legends:

Figure 1. Chemical structure of some clinical drugs for colorectal cancer, microtubule stabilizers (Paclitaxel, docetaxel), destabilizers (Colchicine, podophyllotoxin, combretastatin A4), and indanone core.

Figure 2: Cell cycle analysis of compounds **7** and **22** in DLD1 colorectal cancer cells.

Figure 3. Effects of compound **7** and compound **22** on tubulin kinetics (Positive controls, PAC=Paclitaxel as microtubule stabilizer, PDT=podophyllotoxin as microtubule destabilizer)

Figure 4. Cleavage of PARP (Poly ADP-ribose polymerase) by compounds **7** and **22** at their IC_{50}(s)

Figure 5A: 2D docking poses of compound **7**, colchicine, and paclitaxel with PDB ID: **4O2B** (Tubulin-colchicine complex).

Figure 5B: 2D docking poses of isomeric compounds **22** (R), **22** (S) and paclitaxel with PDB ID: **5M5C** (Microtubule-paclitaxel bound complex)

Figure 6 A. Effect of **7** as a single acute oral dose at 5, 50, 300 and 1000 mg/kg on absolute and relative organ weights in Swiss albino mice (Mean±SE; n=6).

Figure 6B. Effect of **7** as a single acute oral dose at 5, 50, 300 and 1000 mg/kg body weight on differential leucocytes counts in Swiss albino mice (Mean±SE; n=6).

Figure 7A. Effect of **22** as a single acute oral dose at 5, 50, 300 and 1000 mg/kg on absolute and relative organ weights in Swiss albino mice (Mean±SE; n=6).

Figure 7B. Effect of **22** as a single acute oral dose at 5, 50, 300 and 1000 mg/kg body weight on differential leucocytes counts in Swiss albino mice (Mean±SE; n=6).

Table 1: *In-vitro* Cytotoxicity of indanones and related derivatives against various human cancer cells by sulphorhodamine assay (IC_{50} in µM).

S. no.	Cytotoxicity IC_{50} in µM					Cytotoxicity CC_{50} in µM	Selectivity Index (SI)**	Antitubulin	
	A549	DU145	FaDu	DLD1	MCF-7	HEK-93	IC_{50}(DLD1)/ CC_{50}	IC_{50} (Inhibition)	EC_{50} (Stabilization)
7	4.80	2.89	2.40	**2.22**	3.63	47.15	**21.24**	**0.57**	----
15	>40	>40	>40	7.02	>40	ND	---	---	---
17	>40	>40	>40	>40	>40	ND	---	---	---
18	>40	>40	>40	>40	>40	ND	---	---	---
22	2.23	2.47	10.87	**1.23**	>40	119.44	**97.11**	---	**1.8**
25	20.73±1.99	24.40±1.94	>40	24.91±1.18	>40	ND	---	---	---
Tamoxifen	15.68	19.82	8.29	16.34	8.61	21.0	1.29	---	---
Doxorubicin	0.63	0.30	<1.25	2.19±0.34	5.83±0.32	3.90	0.56	---	---
Nocodazole	---	---	---	---	---	---	---	0.62±	---
Podophyllotoxin	<1.25	<1.25		0.104±0.013	<1.25	---	---	0.74±0.12	---
Paclitaxel	ND	ND	ND	ND	1.02±1.7	>25	---	---	0.025

* IC_{50}>40µM was considered as inactive; **SI=IC_{50}/CC_{50}; IC_{50} against DLD1 cells, CC_{50} against HEK-93 cells

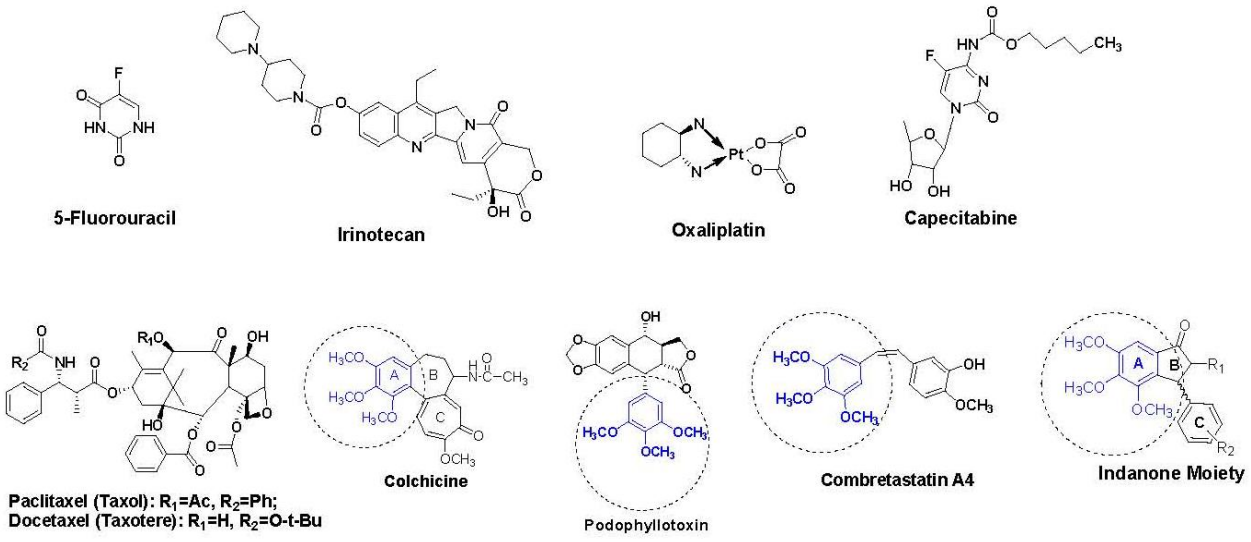
Table 2. Effect of compound **7** as a single acute oral dose at 5, 50, 300, and 1000 mg/kg on body weight, haematological and serum biochemical parameters in Swiss albino mice (Mean±SE; n=6; *significant compared to control, P<0.05).

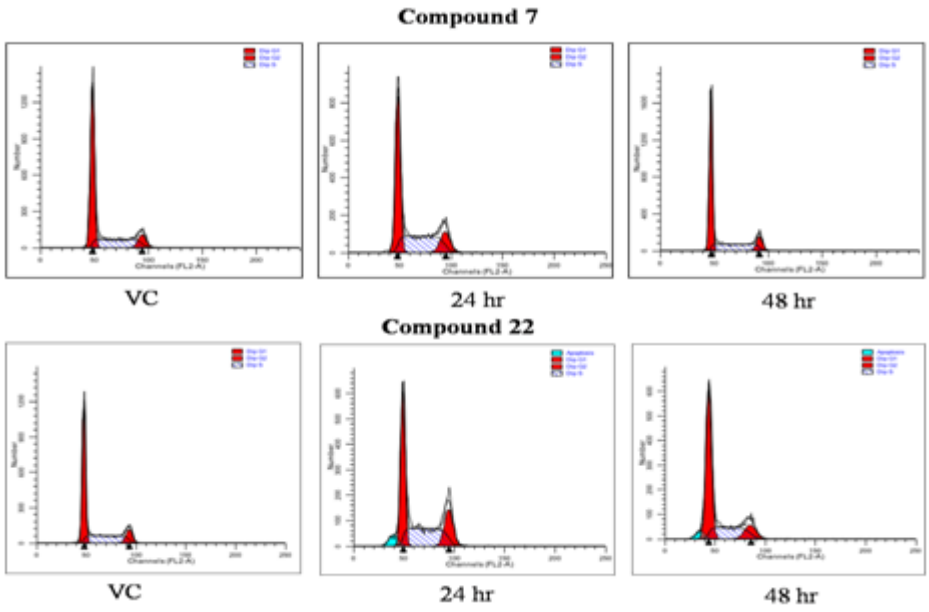
Parameters		Dose of compound 7 at mg/kg body weight as single oral dose				
		Control	5 mg/kg	50 mg/kg	300 mg/kg	1000 mg/kg
Body weight	**Body weight (g)**	33.51±1.04	31.03±2.20	29.30±1.01	28.81±0.60	29.38±1.21
Haematological parameters	**Haemoglobin (g/dL)**	13.96±0.21	14.66±0.41	15.55±0.61	14.30±0.57	12.81±0.68
	RBC (million/mm^3)	9.36±0.50	9.07±0.68	9.79±0.75	8.86±0.46	8.57±0.41
	WBC(thousands/mm^3)	6.56±0.12	7.56±1.56	10.33±1.84	9.05±1.68	10.39±0.60
Liver Function Test	**ALP (U/L)**	44.71±6.44	46.14±2.48	47.82±5.71	57.47±6.46	49.39±6.71
	SGOT (U/L)	45.16±3.59	43.24±19.75	53.67±9.07	53.03±3.81	**103.91±15.33***
	SGPT (U/L)	26.64±1.90	27.49±1.59	22.49±1.37	23.40±2.10	**49.73±3.92***
	Albumin(g/dL)	2.66±0.10	2.73±0.15	2.56±0.15	2.89±0.07	2.42±0.10
	Protein(mg/ml)	2.82±0.08	3.24±0.03	2.79±0.07	2.64±0.04	2.91±0.12
	Total Bilirubin(mg/dL)	0.51±0.02	0.56±0.02	0.55±0.04	0.54±0.07	0.50±0.02
Kidney Function Test	**Creatinine (mg/dL)**	0.76±0.10	0.42±0.03	1.03±0.21	0.86±0.20	0.78±0.21
Lipid Profile	**Cholesterol (mg/dL)**	177.91±18.68	193.29±18.65	171.03±15.64	150.90±8.79	162.08±23.25
	Triglycerides (mg/dL)	141.33±22.51	114.67±11.58	113.21±21.38	123.63±16.75	95.41±2.82

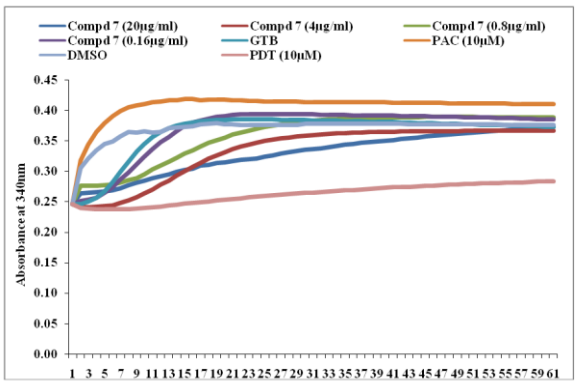
Table 3. Effect of compound **22** as a single acute oral dose at 5, 50, 300, and 1000 mg/kg on body weight, haematological and serum biochemical parameters in Swiss albino mice (Mean±SE; n=6).

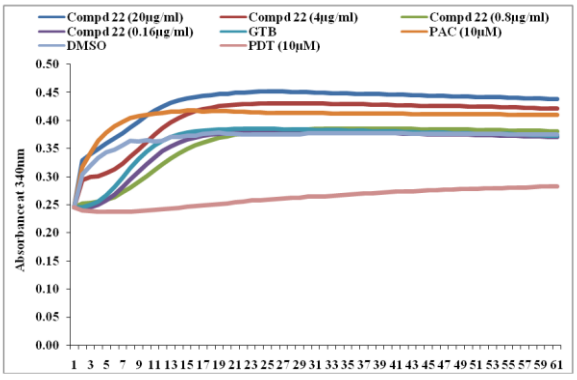
Parameters		Dose of compound 22 at mg/kg body weight as single oral dose				
		Control	5 mg/kg	50 mg/kg	300 mg/kg	1000 mg/kg
Body weight	Body weight (gm)	28.48±1.00	27.17±0.64	31.47±0.88	24.10±0.43	26.05±0.53
Haematological parameters	Haemoglobin (gm/dL)	10.84±0.50	9.96±0.71	10.91±0.59	9.66±0.63	12.89±0.83
	RBC (million/mm^3)	9.85±0.57	8.90±0.59	7.63±0.41	7.51±1.43	8.98±0.90
	WBC(thousands/mm^3)	5.80±0.58	7.80±1.06	9.79±0.86	10.28±0.83	8.09±0.77
Liver Function Test	ALP (U/L)	98.03±15.84	100.76±10.57	105.63±10.50	91.52±7.60	90.89±6.99
	SGOT (U/L)	48.55±4.94	30.83±1.59	36.99±4.15	48.22±6.07	41.47±4.78
	SGPT (U/L)	23.06±1.33	20.27±3.14	24.42±1.77	22.34±1.19	21.57±2.85
	Albumin(g/dL)	1.86±0.16	1.81±0.16	2.81±0.05	1.26±0.16	2.47±0.10
	Serum Protein(mg/ml)	2.71±0.13	2.86±0.12	2.81±0.17	3.08±0.21	2.69±0.12
	Bilirubin(mg/dL)	0.41±0.02	0.46±0.05	0.43±0.06	0.57±0.07	0.39±0.03
Kidney Function Test	Creatinine (mg/dL)	0.60±0.06	0.54±0.05	0.87±0.20	0.78±0.18	0.49±0.02
Lipid profile	Cholesterol (mg/dL)	108.63±7.46	130.07±8.73	177.98±17.68	169.27±19.21	214.71±25.45
	Triglycerides (mg/dL)	80.67±9.76	85.85±11.08	104.70±16.38	96.33±12.49	90.09±13.77

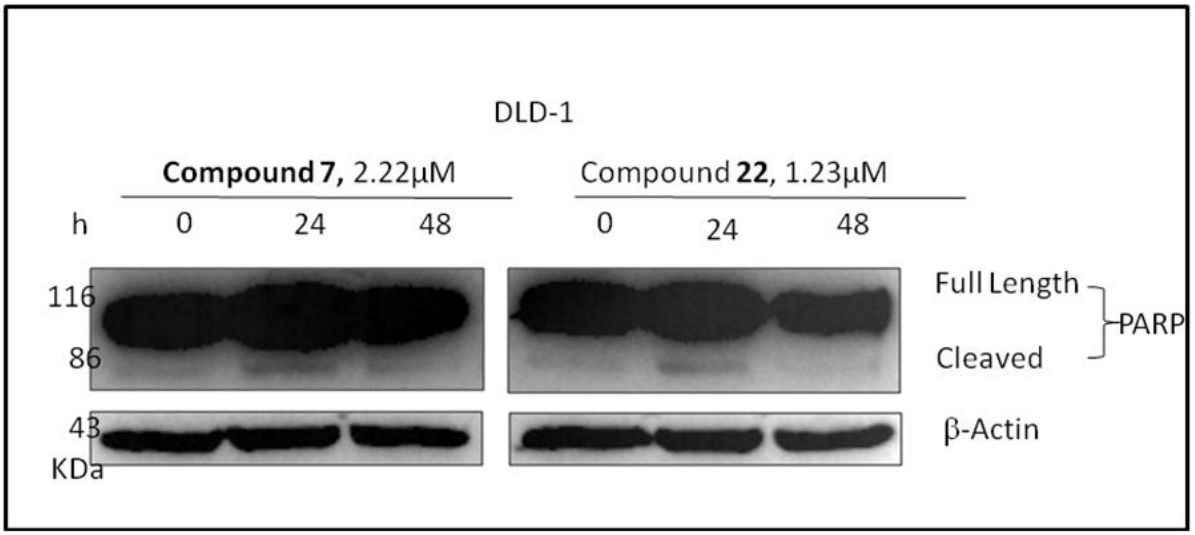
Figure 1

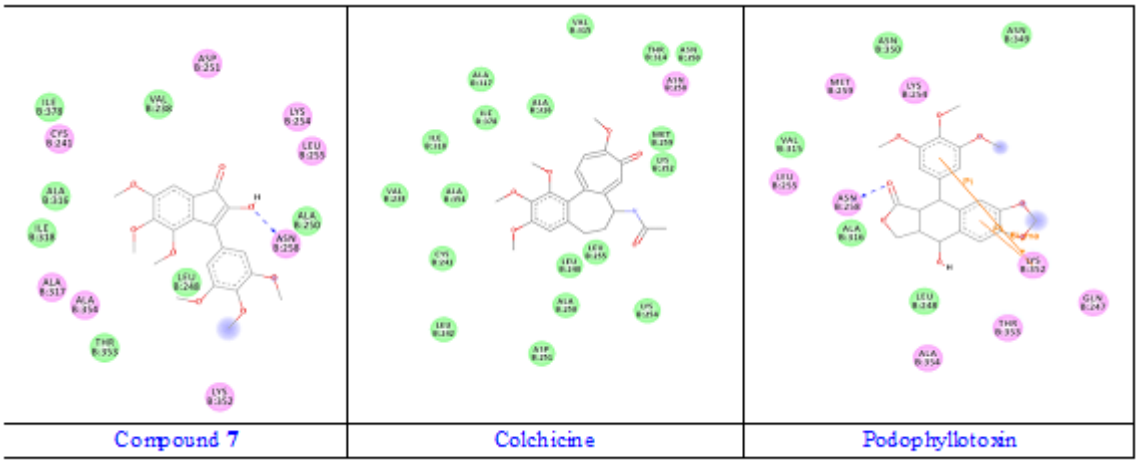


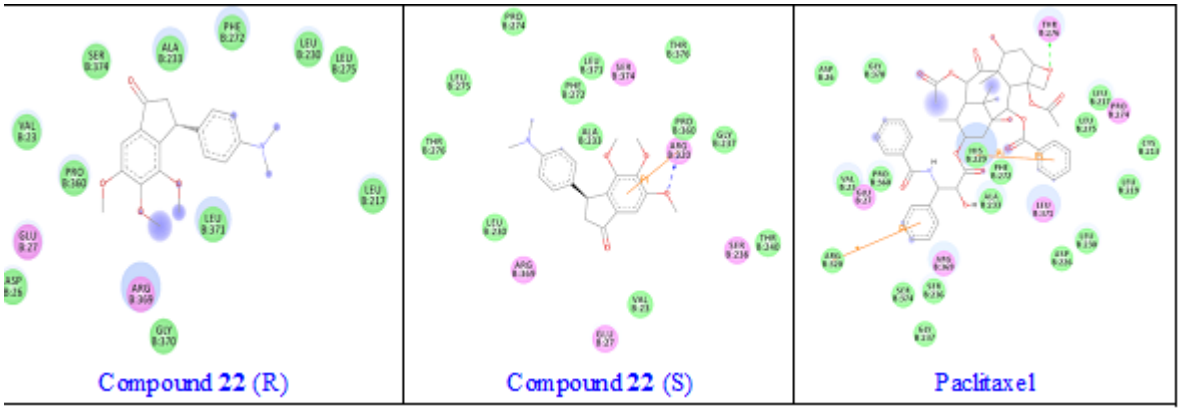


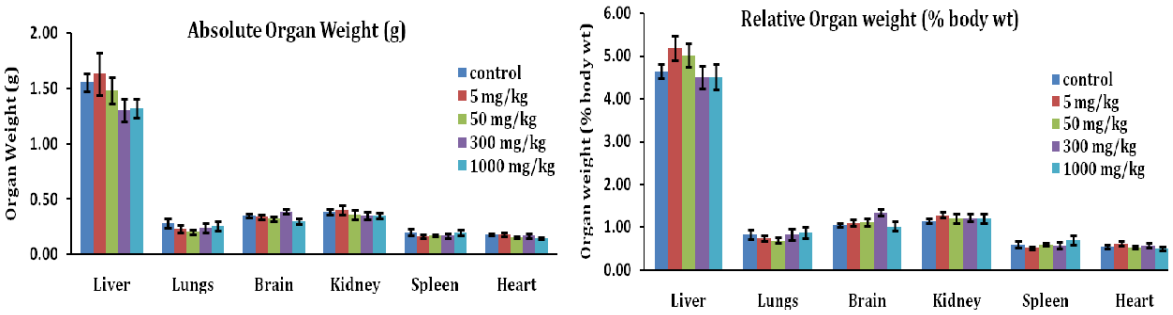


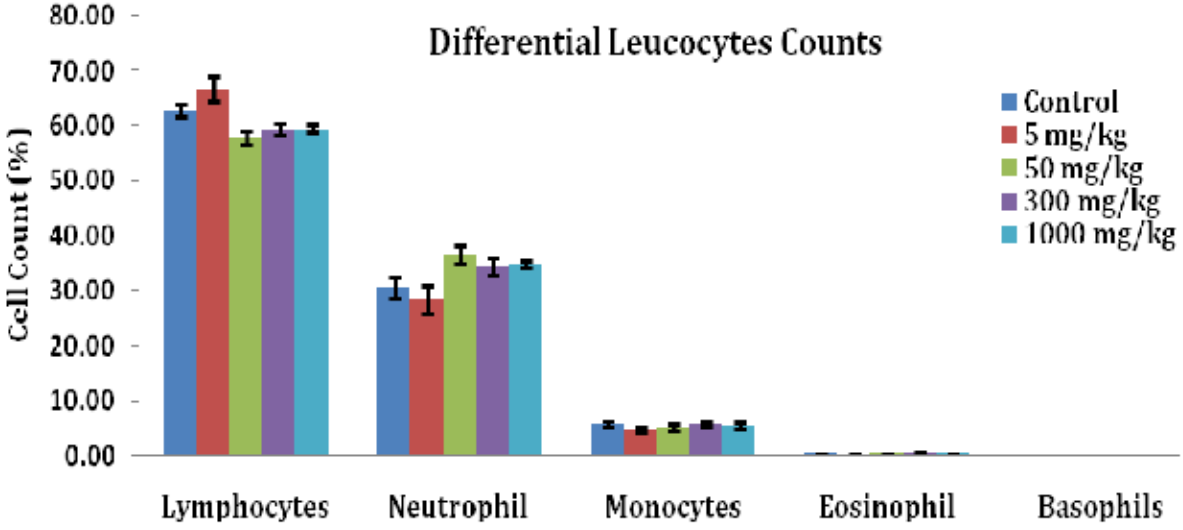


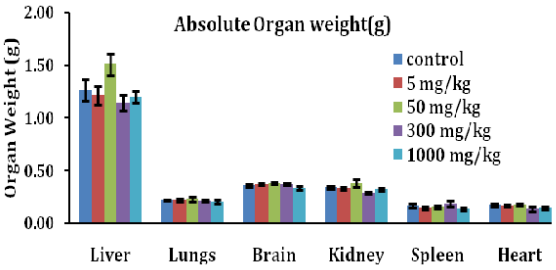


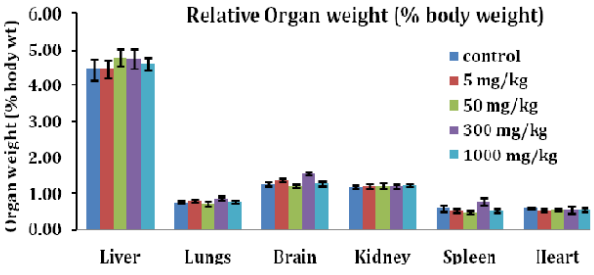


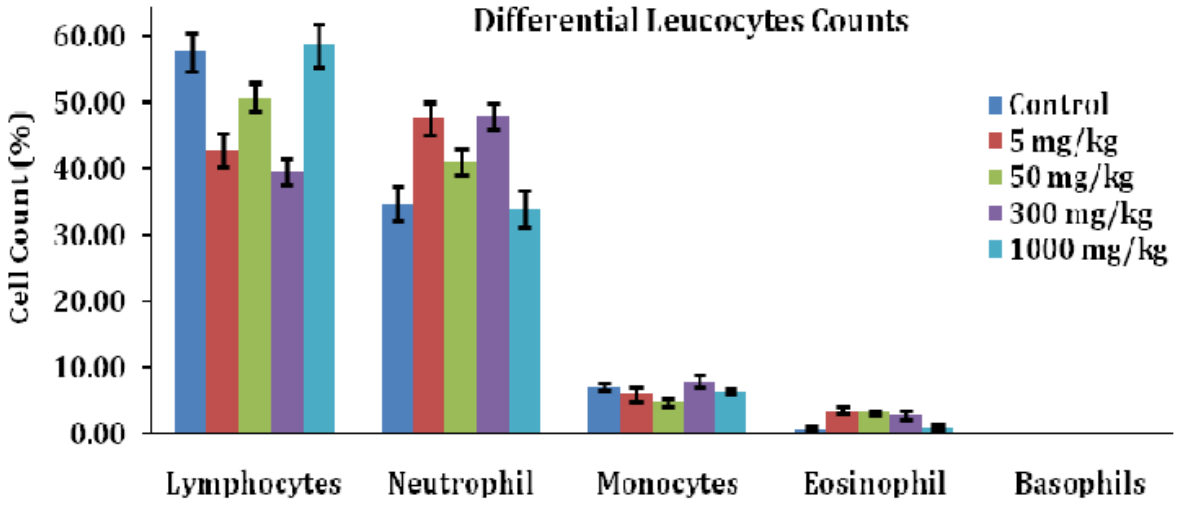




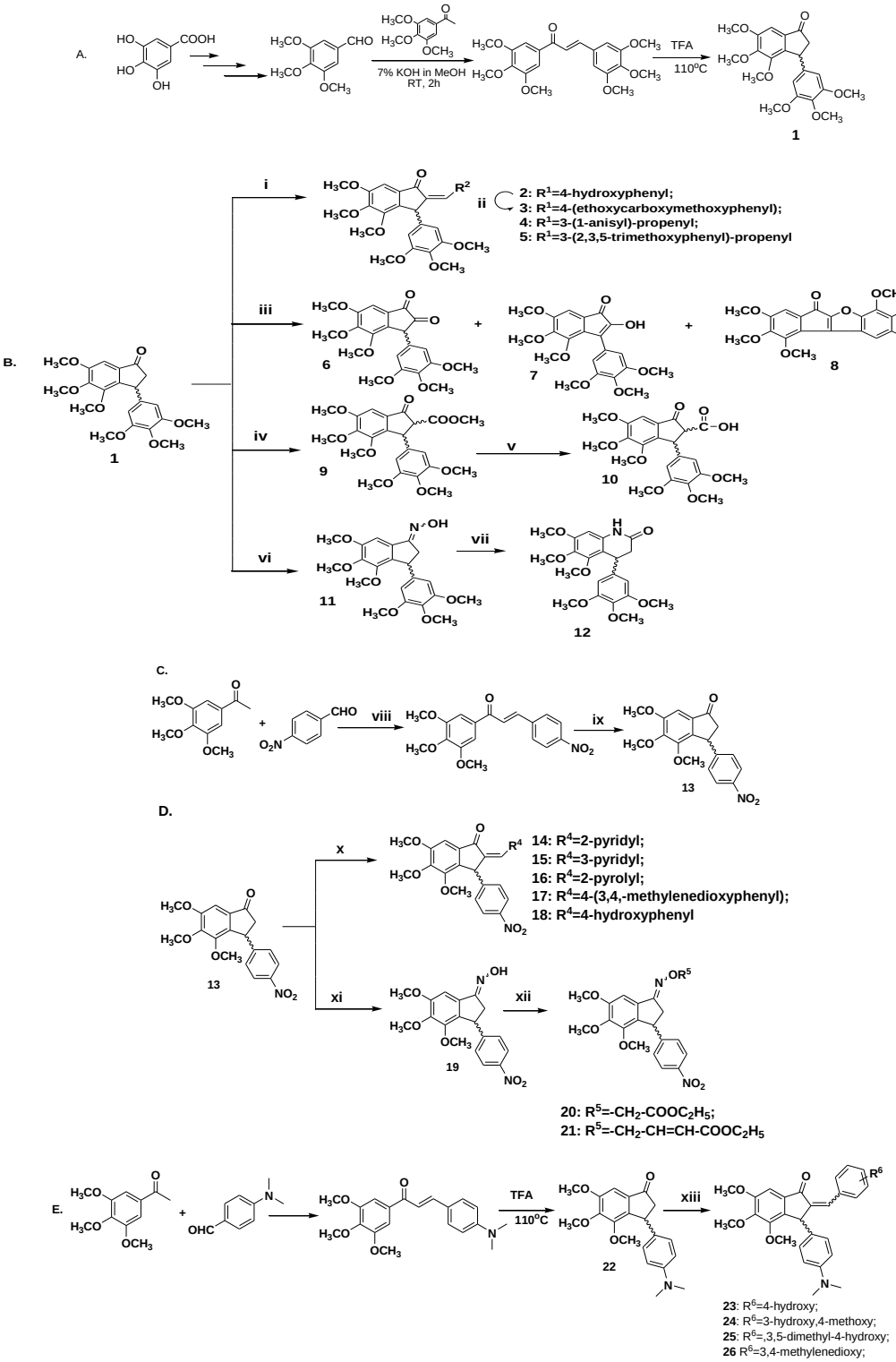








Scheme 1



Reagents and conditions: i) 3% MeOH-KOH, RT, 3-5h, 64-91%; ii) Anhyd. K_2CO_3, dry acetone, for **3** and **20** ethylbromoacetate/ for **21** ethylbromocrotonate, reflux, 2-3h, 73-84%; iii) Dioxane, SeO_2, reflux, 5h, **6**:16%, **7**: 23% and **8**: 40%; iv) Me_2CO_3, NaH, dry toluene, reflux, 6h, 91%; v) 5% KOH, MeOH:H_2O (9:1), 50°C, 1h, 87%; vi) EtOH, $NH_2OH.HCl$, reflux, 3-5h, **11**: 72% and **19**: 79%; vii) p-TSA, dry THF, reflux, 3h, 39%; viii) 7% KOH, MeOH, RT, 2h, 93%; ix) TFA, 110°C, 6h, 41%; x) Piperidine, dry THF, RT, 54-63% for **17** & **18**.