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Original article

Design and synthesis of spiro derivatives of parthenin as novel anti-cancer agents

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ABSTRACT

Several novel spiro derivatives of parthenin (**1**) have been synthesized by the dipolar cycloaddition using various dipoles *viz*; benzonitrile oxides, nitrones and azides with exocyclic double bond of C ring (α-methylene-γ-butyrolactone). Majority of the compounds exhibited improved anti-cancer activity compared to the parthenin, when screened for their *in vitro* cytotoxicity against three human cancer cell lines *viz.*, SW-620, DU-145 and PC-3. *In vivo* screening of select analog revealed improved anti-cancer activity with low mammalian toxicity as compared to parthenin. The results of the cytotoxicity pattern of these derivatives reveals the SAR of these sesquiterpinoid lactones and possible role of α,β-unsaturated ketone of parthenin in inhibiting NF-kB. A mechanistic correlation of anti-cancer activity along with *in vivo* and western blotting experiments has been described.

1. Introduction

In recent years, the anti-cancer property of various sesquiterpenes has attracted a great deal of interest and extensive research work has been carried out to characterize the anti-cancer activity, the molecular mechanisms and the potential chemo-preventive and chemo-therapeutic application of sesquiterpenoids [1]. Parthenin (**1**), a sesquiterpene lactone (SL) isolated from *Parthenium hysterophorus L* [2], has found interest due to its medicinal properties *viz*., anti-cancer, antibacterial, antiamoebic, anti-inflammatory, lipid peroxidation inhibition, and trypanocidal activity [3–12]. Cytotoxicity, as many other biological activities of sesquiterpene lactones, is known to be mediated by the presence of potential alkylant structure elements such as an α-methylene-γ-lactone, an α,β-unsaturated cyclopentenone or a conjugated ester capable of reacting covalently with biological nucleophiles, especially with the cysteine sulfhydryl group of proteins, in a Michael-type addition (Fig. 1), thereby inhibiting a variety of cellular functions which directs the cells into apoptosis [13–19].The differences in activity among individual SLs are attributed to the presence of different numbers of alkylating structural elements. Apart from this, other factors such as lipophilicity, molecular geometry and the chemical environment or the target sulfhydryl which may also influence the activity of these compounds.

Using helenalin and parthenolide as models, it has been well established that DNA binding of NF-kB is prevented by alkylation of cysteine in the p65/NF-kB subunit, which is considered to be the general mechanism for SL bearing α,β-unsaturated carbonyl structures [19]. Despite the plethora of experimental studies found in literature on the cytotoxicity of sesquiterpene lactones in general including few reports on parthenin in particular, little is known about the individual role of different alkylant structures on the cytotoxicity in terms of its SAR. This however, would be an important step in the direction of rational lead optimization.

Parthenin (**1**) has two active sites; the exocyclic double bond of α-methylene-γ-butyrolactone and double bond of cyclopentenone ring. Various structural modifications of parthenin skeleton have been carried out with a view to obtain more potent analogs with lower toxicity and better activity. In spite of the improved understanding of the role of each double bond present on the parthenin skeleton through such studies, the SAR of parthenin has not been unequivocally established so far and none of the literature reports revealed a rational approach to the modification of parthenin. Furthermore, monofunctional alkylants are known to possess low

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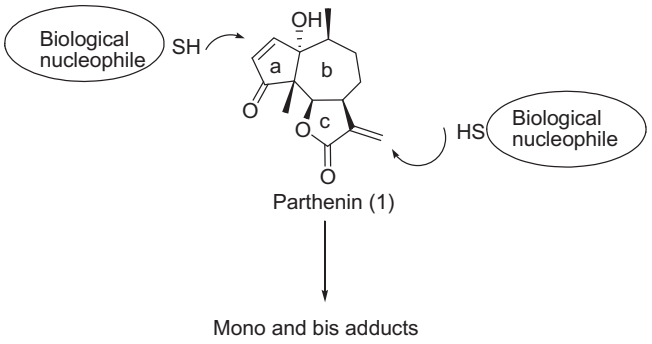
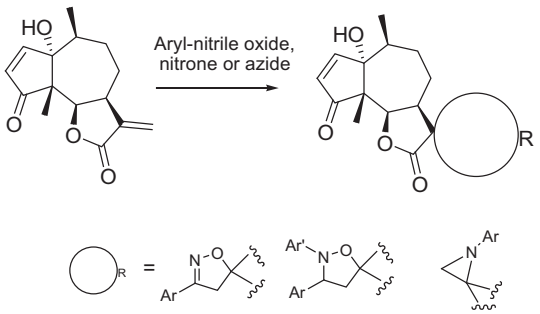


Fig. 1. Attack of biological nucleophiles on parthenin.

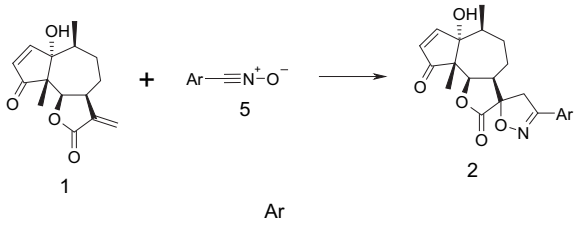
degree of side effects such as toxicity or contact dermatitis and generation of novel monofunctional analogs of parthenin would be undoubtedly an attractive method for new lead discovery with minimal toxicity. In this context, efforts from our group to generate the diversity around endocyclic unsaturation of parthenin through regioselective Baylis Hillmann adducts resulted in the derivatives with considerably reduced cytotoxicity [20]. This clearly demonstrated that tampering with the pharmacophoric cyclopentenone ring structure either leads to low or complete loss of NF-kB binding by the ligand and the observed low cytotoxicity in the derivatives was found to be due to inhibition of telomerase-a case of complete switch in the target preference possibly due to molecular overcrowding of the newly created cyclopentane moiety bearing exocyclic double bond [21].

To establish the role of exo- and endocyclic double bonds toward the anti-cancer activity, a strategy to selectively react one of these double bonds has been devised. Out of few chemical transformational possibilities available to achieve the above goal, selective addition of dipoles such as nitrile oxide, nitrone and azide to exocyclic double bond (Scheme 1) has been choosen, with an initial simple prediction that the newly incorporated nitrogen framework on the scaffold might also enhance the cytotoxicity.

Although both endocyclic and the exocyclic double bonds are active toward nucleophilic additions, dipolar cycloaddition takes place selectively across the exocyclic olefin possibly due to steric hindrance offered by the substitution at the cyclopentenone ring. Herein, we report the dipolar cycloaddition of nitrile oxide, nitrone and azide cycloaddition to the exocyclic double bond of parthenin to generate a focused library of novel spiro-isoxazolines (**2**), spiroisoxazolidine (**3**) and spiroaziridine (**4**) presented (Scheme 2–4). After screening the derivatives for their anti-cancer activity, we could easily establish the pharmacological importance of cyclopentenone ring over the α-methylene-γ-butyrolactone ring, which gives clear insight into the SAR of parthenin. The novel



Scheme 1. Synthesis of spiro derivatives of parthenin *via* **1,3**-dipolar cycloaddition of nitrile oxides, nitrones and azides to the exocyclic double bond.



2a	2,6-Cl-C_6H_3	**2h**	2-NO_2-C_6H_4	**2o**	2-Me-C_6H_4
2b	4-MeO-C_6H_4	**2i**	4-NO_2-C_6H_4	**2p**	3-Me-C_6H_4
2c	4-Cl-C_6H_4	**2j**	3,4-OMe-C_6H_3	**2q**	4-Me-C_6H_4
2d	2-Br-C_6H_4	**2k**	3-OMe, 4-OH-C_6H_3	**2r**	4-F-C_6H_4
2e	-C_6H_5	**2l**	2-F-C_6H_4	**2s**	4-CN-C_6H_4
2f	4-NMe_2-C_6H_4	**2m**	3-NO_2-C_6H_4	**2t**	9-Anthracene-
2g	4-HO-C_6H_4	**2n**	2-Cl-C_6H_4		

Scheme 2. Synthesis of spiroisoxazoline derivatives of parthenin *via* **1,3**-dipolar cycloaddition of nitrile oxides to the exocyclic double bond.

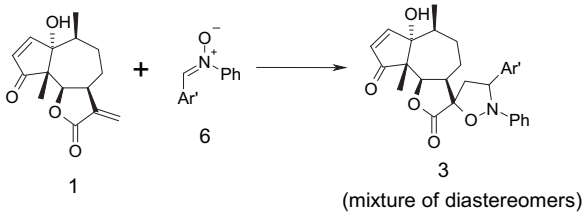
derivatives of parthenin thus generated have increased drug-likeness as revealed by the QSAR parameters and *in vivo* studies reveal reduced toxicity.

2. Results and discussion

2.1. Chemistry

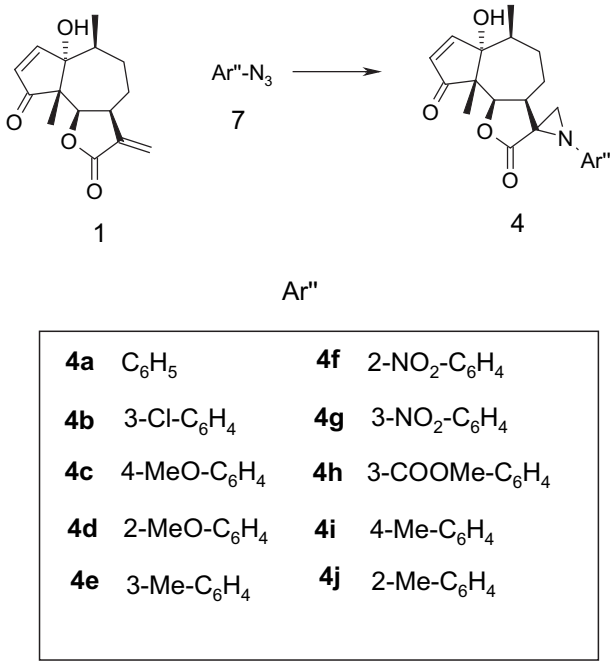
2.1.1. Synthesis of spiroisoxazoline derivatives of parthenin

As illustrated in Scheme 2, spiroisoxazoline derivatives of parthenin (**2**) were prepared through **1,3**-dipolar cycloaddition of



3a	4-Br-C_6H_4 (92:8)	**3g**	2-Me-C_6H_4 (94:6)	**3m**	3-Br, 4-MeO-C_6H_3 (95:5)
3b	4-Cl-C_6H_4 (93:7)	**3h**	C_6H_5 (97:3)	**3n**	2-Cl-C_6H_4 (93:7)
3c	4-F-C_6H_4 (94:6)	**3i**	2,6-Cl-C_6H_3 (65:35)	**3o**	2-F-C_6H_4 (92:8)
3d	4-CN-C_6H_4 (93:7)	**3j**	2,6-F-C_6H_3 (70:30)	**3p**	2,3,4,5,6-F-C_6 (90:10)
3e	4-MeO-C_6H_4 (95:5)	**3k**	3-Cl-C_6H_4 (90:10)	**3q**	3-MeO-C_6H_4 (91:9)
3f	4-Me-C_6H_4 (90:10)	**3l**	3-F-C_6H_4 (92:8)	**3r**	4-NO_2-C_6H_4 (90:10)

Scheme 3. Synthesis of spiroisoxazolidine derivatives of parthenin *via* **1,3**-dipolar cycloaddition of nitrone to the exocyclic double bond.



Scheme 4. Synthesis of spiroaziridine derivatives of parthenin *via* **1,3**-dipolar cycloaddition of azide to the exocyclic double bond.

various nitrile oxides (**5**) to parthenin [22–29]. The nitrile oxides (**5**) were prepared according to literature procedure in which various aromatic aldehydes converted to corresponding aldoximes (*syn* and *anti*), followed by the reaction with N-chlorosuccinimide in DMF. Nitrile oxide cycloaddition was selectively done to the double bond adjacent to lactone ring, which is clearly confirmed by the disappearance of the alkene protons adjacent to lactone ring. Spiroisoxazoline derivatives of parthenin were formed regiospecifically in all cases.

2.1.2. Synthesis of spiroisoxazolidine derivatives of parthenin

As illustrated in Scheme 3, spiroisoxazolidine derivatives of parthenin (**3**) were prepared through **1,3**-dipolar cycloaddition of various nitrones (**6**) to parthenin in dry benzene under reflux condition [29–32]. Nitrones (**6**) were prepared according to literature procedure in which nitrobenzene was reduced in the presence of Zn/NH_4Cl to get phenylhydroxylamine followed by the condensation of various aromatic aldehydes. Nitrone cycloaddition was selectively done to the double bond adjacent to lactone ring, which is clearly confirmed by the disappearance of the alkene protons adjacent to lactone ring. Interestingly may be due to the molecular complexicity of parthenin, two diastereomers were formed. Each diastereomer was isolated after column chromatography or sometimes by preparative TLC and characterized by ^1H NMR, ^{13}C NMR and Mass spectrometry. A clear chemical shift deviation of benzylic proton adjacent to nitrogen atom in isoxazolidine ring between two diastereomers was observed in ^1H NMR. In major isomer this proton appears as a triplet approximately at δ 5, but in the minor isomer this signal shifted toward more shielding region and appears approximately at δ 4. A series of nitrones were reacted with parthenin and both diastereomers of spiroisoxazolidines were isolated in each case.

2.1.3. Synthesis of spiroaziridine derivatives of parthenin

As illustrated in Scheme 4, spiroaziridine derivatives of parthenin (**4**) were prepared through **1,3**-dipolar cycloaddition of various aromatic azides (**7**) to parthenin in dry toulene under refluxing condition [32]. Azide cycloaddition was selectively done to the double bond adjacent to lactone ring, which is clearly confirmed by the disappearance of the alkene protons adjacent to lactone ring.

All the products synthesized were characterized by IR, ^1H, ^{13}C NMR and mass spectroscopy data.

Table 1
IC_{50} values of various derivatives of parthenin in μM concentration.

Compound	Cell lines			Compound	Cell lines		
	SW-620	DU-145	PC-3		SW-620	DU-145	PC-3
2a	43	38	56	**3h'**	5.3	5.6	5.7
2b	41	20	35	**3h"**	6	6.5	9
2c	51	43	49	**3i'**	3	9	2.4
2d	44	18	51	**3i"**	5	6.3	4.2
2e	45.4	39.8	46	**3j'**	5	12	2.5
2f	5.1	4.5	6.3	**3j"**	7.2	9	2.3
2g	89	93	20	**3k'**	6.1	7.4	8.2
2h	40	34	37	**3k"**	10.5	6	12
2i	9	7	12	**3l'**	4.8	5	4.8
2j	86	84	25	**3l"**	5.1	8.1	9
2k	88	71	16	**3m'**	6.8	6.3	5.7
2l	11	24	8.2	**3m"**	9	5.4	8.1
2m	49	51	55	**3n'**	5.5	6	3.5
2n	9	19	12	**3n"**	9.2	4.1	6.3
2o	5	12	10	**3o'**	9	4.1	2.7
2p	15	28	66.6	**3o"**	10.2	6	2.5
2q	2.5	14	14	**3p'**	3	9.3	5.6
2r	3.6	13	27	**3p"**	5.5	6.1	5
2s	53	26	32	**3q'**	7.5	10	12
2t	4.3	4.6	4.9	**3q"**	6.6	11	9
3a'	6.7	5.3	5.8	**3r'**	5.7	5.6	8.4
3a"	8.1	4.2	2.3	**3r"**	5.1	6	9.3
3b'	7.5	7	7.8	**4a**	87.7	31.4	10.6
3b"	5.1	6	12	**4b**	48.4	39.5	86.1
3c'	7.3	6.4	5.7	**4c**	30	78	12
3c"	6	5	12	**4d**	47.3	27	52
3d'	4.8	5	4.8	**4e**	15	62.5	61.5
3d"	5	7	9.1	**4f**	33	28	10
3e'	7.3	6.4	5.7	**4g**	61	30	10.1
3e"	4.2	7.1	6	**4h**	30	18.5	10.1
3f'	8.7	8.1	8.5	**4i**	32	36	25
3f"	9	6.3	9.1	**4j**	22	30	12.5
3g'	12	4.5	15	**1**	38.7	31.1	40.3
3g"	9	5.1	15				

' major diastereomer, " minor diastereomer.

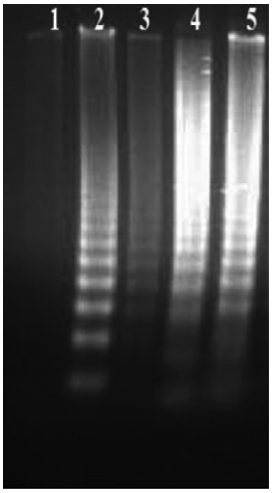


Fig. 2. DNA fragmentation assay of compound **2t** in SW-620 cells. Lane-1: Untreated Cells, lane-2: treated with 5 μM of Camptothecin, lane-3: treated with 10 μM of compound **2t**, lane-4: treated with 50 μM of compound **2t**, lane-5: treated with 100 μM of compound **2t**.

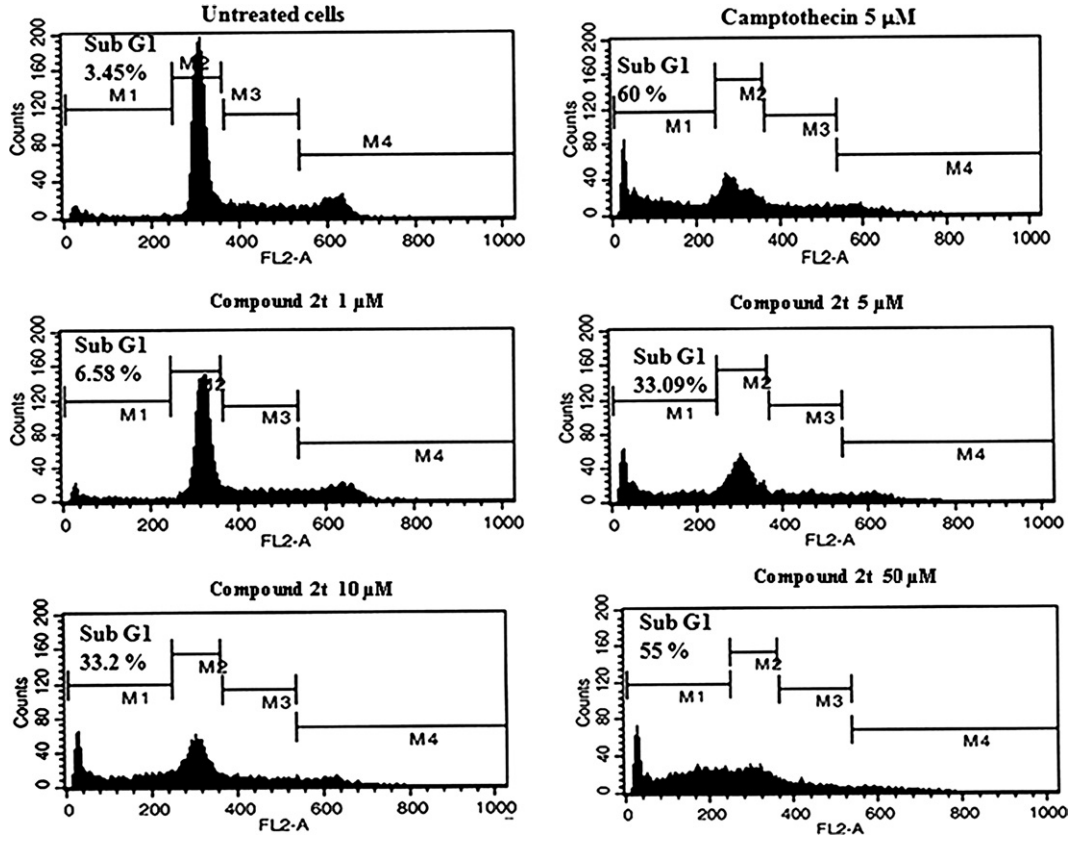


Fig. 3. Flow cytometric analysis of compound **2t** treated with SW-620 cells.

2.2. Evaluation of biological activity

2.2.1. In vitro anti-cancer activity

All derivatives of parthenin were screened for their anti-cancer activity against three human cancer cell lines (SW-620, DU-145 and PC-3). Most of the spiro derivatives of parthenin exhibited better anti-cancer activity than the parent parthenin. The results are summarized in Table 1. In spiroisoxazoline (**2**) series compounds **2f**, **2o**, **2p**, **2q**, **2r** and **2t** exhibited better cytotoxicity than parent molecule. In spiroisoxazolidine (**3**) series all the compounds showed better cytotoxicity than parent parthenin against all the cell lines. In spiroaziridine (**4**) series compound **4a**, **4c**, **4f**, **4e**, **4g**, **4h** and **4j** exhibited better cytotoxicity than parent parthenin. From the IC_{50} values it is clear that all the spiro derivatives of parthenin have anti-cancer activity and most of the compounds are having better anti-cancer activity than the parent parthenin.

2.2.2. DNA fragmentation assay

DNA fragmentation is the net result of apoptosis, and is observed at the late stage. DNA fragmentation assay showed that compound **2t** induced DNA fragmentation in SW-620 cells at 50 and 100 μM concentrations after 24 h of incubation. Camptothecin taken as a positive control showed significant fragmentation after 6 h at 5 μM concentration, however, no fragmentation was observed in untreated cells (Fig. 2).

2.2.3. Cell cycle analysis

To address the cell death caused by compound **2t**, the extent of apoptotic death was assessed using FACS flow cytometry through the determination of sub-G1 cell population by propidium iodide (PI) staining. The DNA cell cycle analysis of compound **2t** revealed a concentration dependent increase in the sub G_1 phase of cell cycle being 6.58, 33.09, 33.2 and 55% at 1, 5, 10 and 50 μM respectively and also complete blockage of G_1 phase at 50 μM concentration after 24 h of incubation in SW-620 cells was observed (Fig. 3).

2.2.4. In vivo study of compound *2t*

Solid tumor bearing mice treated with different doses of parthenin and its derivative compound **2t** exhibited dose dependent tumor growth inhibition against EAT tumor model. A highly significant ($p < 0.01$) tumor growth inhibition up to 35.11% was observed in EAT bearing mice treated with compound **2t** at 100 mg/kg i.p. dose whereas 200 mg/kg i.p. dose induced mortality of all the test animals by fourth day of the treatment, on the contrary a high level of toxicity without significant antitumor activity was recorded in parthenin treated groups as it caused mortality of all the animals by second and third day of treatment at 25 and 50 mg/kg i.p. doses respectively (Table 2).

In case of Ehrlich ascetic carcinoma (EAC) bearing mice same pattern of dose dependent tumor growth inhibition and toxicity was exhibited by both parthenin and its derivative Compound **2t**. At 100 mg/kg, i.p. dose of **2t** exhibited highly significant ($p < 0.01$) tumor growth inhibition up to 60% and its higher dose 200 mg/kg i.p. induced mortality of all the test animals by fourth day of the treatment. In this experiment also the parent compound parthenin induced mortality of all the test animals by 2nd and 3rd day of the treatment at 25 and 50 mg/kg i.p. doses respectively exhibiting high level of toxicity to the treated animals without any significant antitumor activity (Table 3). This clearly indicate the reduced

Table 2
Effect of parthenin and its compound **2t** on Ehrlich ascitic tumor (EAT) bearing mice.

Test group	Dose (mg/kg)	Tumor weight (mg)	% Tumor growth Inhibition
Control	0.2 mL	1708.57 ± 51.07	–
1	10	1542.86 ± 35.24	9.69
1	25	All animals died by 4th day	
1	50	All animals died by 2nd day	
2t	10	1547.14 ± 36.63	9.44
2t	25	1445.71 ± 41.52	15.38
2t	50	1332.14 ± 32.59*	22.03
2t	100	1108.57 ± 8.02**	35.11
2t	200	All animals died by 3rd day	
5-FU	20	832.28 ± 35.21**	51.28

$p > 0.05$ = Insignificant, * = $p < 0.05$ = Significant, ** = $p < 0.01$ = Highly significant.

toxicity and improved anti-cancer activity of the spiro derivative of parthenin.

*2.2.5. Western blot analysis of the inhibition of NF-kB-P65 expression by compound **2t***

NFkB-P65 expression is completely inhibited by compound **2t**. It can be presumed that expression is inhibited either on transcriptional level or translational level.

NF-kB is a central mediator of the human immune response. In the majority of cell types this protein is composed of a p50 and a p65 subunit. It is retained in an inactive cytoplasmic complex by binding to IκB, its inhibitory subunit. A large variety of inflammatory conditions, such as bacterial and viral infections as well as inflammatory cytokines, rapidly induce NF-κB activity.

Active NF-kB is released from the cytoplasmic complex by phosphorylation, ubiquitination and degradation of the IκB subunit. The activated factor then translocates to the nucleus where it stimulates the transcription of its target genes. NF-kB regulates the transcription of various inflammatory cytokines, such as IL-1, IL-2, IL-6, IL-8 and TNF-α as well as genes encoding cyclo-oxygenase-II, nitric oxide synthase, immunoreceptors, cell adhesion molecules, hematopoietic growth factors and growth factor receptors. Pharmacological inhibition of NF-kB *in vivo* may thus substantially attenuate inflammatory processes. The inhibition of NF-kB-P-65 by compound **2t** is shown in Fig. 4.

Western blot analysis of NFkB-p65 in compound **2t** treated SW-620 cells showed decreased expression of protein with the treatment time. It was observed that compound **2t** caused early inhibition of translocation of protein in to the nucleus starting from 6h treatment that was completely inhibited with prolonged exposure. Interestingly, expression of the protein was also decreased in cytosolic fraction, thereby indicating that compound **2t** doesn't only inhibit the translocation of protein from cytosol to nucleus but may also regulate it at transcriptional levels.

Table 3
Effect of Parthenin and its derivative **2t** on Ehrlich ascitic carcinoma (EAC) bearing mice.

Test group	Dose (mg/kg)	Astic fluid volume (mL)	Tumor cells in ascetic fluid ($\times 10^7$)	%Tumor growth inhibition
Control	0.2 mL	4.57 ± 0.39	85.00 ± 4.75	–
1	10	3.92 ± 0.38	74.71 ± 2.77	12.01
1	25	All animals died by 4th day		
1	50	All animals died by 2nd day		
2t	10	4.07 ± 0.35	78.42 ± 2.90	7.74
2t	25	3.62 ± 0.32	68.71 ± 3.16	19.16
2t	50	2.85 ± 0.37	61.85 ± 4.20*	27.23
2t	100	2.07 ± 0.33**	33.57 ± 2.60**	60.50
2t	200	All animals died by 3rd day		

$p > 0.05$ = Insignificant, * = $p < 0.05$ = Significant, ** = $p < 0.01$ = Highly significant.

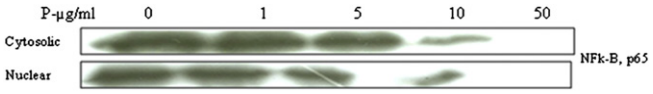


Fig. 4. Inhibition of NF-kB-P65 by spiro derivative of parthenin.

3. Conclusion

The present set of data clearly reveals that spiro derivatives of parthenin presented here induce concentration dependent apoptosis in cancer cells. Spiroheterocyclic derivatives of parthenin exhibited improved cytotoxicity as indicated by the *in vitro* cell based assay whereas *in vivo* data of select derivative clearly indicate reduced mammalian toxicity when tested in mice models. Isoxazolidine series shown superior cytotoxicity as compared to other two series *viz.* spiroisoxazoline and spiro aziridines. Select spiro derivatives of parthenin completely inhibited the NFkB-P65 expression confirmed that these derivatives block p65 subunit of NFkB. SAR of parthenin could be established with clarity as it has been established through the present study that conservation of α,β-unsaturated ketonic moiety of parthenin is crucial for retaining the anti-cancer activity of the ligand whereas the modification of the α-methylene-γ-butyrolactone would facilitate better protein modulation thus enabling improved activity and bioavailability through fine tuning of hydrophilic lipophilic balance. Furthermore, the exocyclic double bond can be advantageously utilized to incorporate appropriate structural entities that may enhance the cytotoxicity of the parent molecule.

4. Experimental protocols

Melting points were recorded on Buchi Melting point apparatus D-545 and IR spectra (KBr) on Bruker Vector 22 instrument. NMR spectra were recorded on Bruker DPX500 instrument in $CDCl_3$ with TMS as an internal standard. Chemical shift values are reported in d (ppm) and coupling constants in hertz. Mass spectra were recorded on ESI-esquire 3000 Bruker Daltonics instrument. The progress of all reactions was monitored by TLC on 2 × 5 cm precoated silica gel 60 F254 plates of thickness 0.25 mm (Merck). The chromatograms were visualized under UV 254–366 nm and iodine.

4.1. Synthesis

4.1.1. Synthesis of spiroisoxazolines derivatives of parthenin

In a typical procedure, to a solution of anthracene nitrile oxide (0.577 g, 1.2 mmol) in THF (10 mL) stirred over a period of 10 min, maintaining the temperature between 0–5 °C, added parthenin (0.262 g, 1 mmol) and stirred the reaction mixture at same temperature for 15 min followed by stirring at ambient temperature for 2 h. The solvent was evaporated in *vaccuo* and the crude was subjected for flash chromatography. The pure product (**2t**) was characterized on the basis of ^{1}H NMR, ^{13}C NMR and mass spectrometry.

*4.1.1.1. Compound **2t**.* Colorless solid, m.p: 162 °C; $[\alpha]_D^{25}$ −27 (c 0.6, $CHCl_3$); ^{1}H NMR ($CDCl_3$): δ 8.54 (s, 1H), 8.15 (d, J = 8 Hz, 2H), 8.02 (d, J = 8 Hz, 2H), 7.61–7.36 (m, 5H), 6.20 (d, J = 5.7 Hz, 1H), 5.36 (d, J = 5.5 Hz, 1H), 3.80 & 3.77 (s, together integrating to diastereomeric 1H), 3.52 & 3.48 (s, together integrating to diastereomeric 1H), 3.44–3.41 (dd, J = 7.22, 5.51 Hz, 1H), 2.43–2.37 (p, J = 6.74, 1H), 2.35–2.32 (dd, J = 7.04, 6.05, 1H), 1.98–1.9 (q, J = 13.68, 1H), 1.8–1.76 (dd, J = 8.2, 5.51, 1H), 1.54–1.50 (dd, J = 8.71, 5.89, 1H), 1.39

(s, 3H), 1.15 (d, J = 7.7 Hz, 3H); ^{13}C NMR ($CDCl_3$): δ 212.0, 168.6, 164.3, 163.6, 154.2, 132.3, 131.5, 130.1, 130.4, 129.6, 128.4, 127.5, 126.9, 124.0, 90.1, 86.8, 83.4, 78.1, 71.2, 60.2, 49.2, 41.2, 40.4, 31.0, 22.1, 20.4, 18.3; IR (KBr): 3442, 2926, 2870, 1774, 1722, 1626, 1593 cm^{-1}; ESI MS (m/z): 482 $(M + H)^+$; Anal. calcd. for $C_{30}H_{27}NO_5$: C, 74.83; H, 5.65; N, 2.91; Found. C, 74.80; H, 5.60; N, 2.89.

4.1.2. Synthesis of spiroisoxazolidine derivative of parthenin

In a typical procedure, to a solution of parthenin (0.5g, 1.9 mmol) in dry benzene (6 mL), was added appropriate nitrone (1.58g, 2.4 mmol) in dry benzene and refluxed the reaction mixture for 8 h. The solvent was evaporated in *vaccuo* and the crude was subjected for flash chromatography. The pure products (**3a'** and **3a"**) was characterized on the basis of ^{1}H NMR, ^{13}C NMR, I.R. and mass spectrometry.

4.1.2.1. Compound 3a'. Pale yellow color solid; mp: 185 °C; $[\alpha]_D^{25}$ −12 (c 0.9, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.52–7.48 (m, 3H), 7.39 (d, J = 8.41 Hz, 2H), 7.21-7-17 (t, J = 7.98 Hz, 2H), 6.94–6.92 (t, J = 7.32 Hz, 1H), 6.86 (d, J = 8.20 Hz, 2H), 6,26 (d, J = 5.86 Hz, 1H), 5.26 (d, J = 5.59 Hz, 1H), 5.16–5.13 (t, J = 8.01 Hz, 1H), 3.21–3.17 (dd, J = 6.51, 5.6 Hz, IH), 2.94–2.90 (dd, J = 6.9, 5.6 Hz, 1H), 2.5–2.45 (dd, J = 9.24, 3.26 Hz, 1H), 2.37–2.31 (p,J = 7.35 Hz, 1H), 2.24–2.17 (td, J = 6.37 Hz, 1H), 1.95–1.86 (q, J = 13.62 Hz, 1H), 1.69–1.65 (dd, J = 5.67, 8.49 Hz, 1H), 1.47–1.43 (dd, J = 9.44, 5.01 Hz, 1H), 1.34 (s, 3H), 1.09 (d, J = 7.79 Hz, 3H); ^{13}C NMR ($CDCl_3$): 211.46, 174.42, 164.06, 151.11, 140.03, 132.1, 131.57, 128.84, 128.5, 122.48, 121.65, 114.83, 86.54, 84.19, 79.64, 69.97, 58.99, 49.45, 42.73, 39.9, 31.44, 21.74, 20.12 17.97; IR (KBr): 3451.68, 2962.07, 2925.92, 1774.56, 1754.00, 1722.69, 1596.79, 1488.03, 1011.38, 800.35, 754.89, 713.22 cm^{-1}; ESI MS (m/z): 539 $(M + H)^+$; Anal. Calcd for $C_{28}H_{28}BrNO_5$: C, 62.46; H, 5.24; N, 2.60. Found: C, 62.40; H, 5.27; N, 2.63.

4.1.2.2. Compound 3a". Pale yellow colour solid; mp: 179 °C; $[\alpha]_D^{25}$ −5.5 (c 0.75, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$): 7.51–7.48 (m, 5H), 7.19–7.16 (t, J = 7.55 Hz, 2H), 6.98–6.95 (t, J = 7.38 Hz, 1H), 6.89 (d, J = 7.74 Hz, 2H), 6.21 (d, J = 5.86 Hz, 1H), 5.2 (d, J = 5.48 Hz, 1H), 4.33–4.3 (t, J = 8.21 Hz, 1H), 3.13–3.1 (dd, J = 5.47, 6.2 Hz, 1H), 2.92–2.88 (dd, J = 9.17, 3.95 Hz, 1H), 2.69–2.65 (dd, J = 7.96, 5.06 Hz, 1H), 2.38–2.32 (p, J = 7.35 Hz, 1H), 2.25–2.18 (td, J = 6.30 Hz, 1H), 1.94–1.86 (q, J = 13.51 Hz, 1H), 1.75–1.71 (dd, J = 5.67, 8.42 Hz, 1H), 1.58–1.54 (dd, J = 9.55, 4.55 Hz, 1H), 1.37 (s, 3H), 1.1 (d, J = 7.77 Hz, 3H); ^{13}C NMR ($CDCl_3$): 211.21, 174.55, 164.001, 152.01, 140.33, 132.12, 131.42, 128.64, 128.45, 122.46, 121.66, 114.63, 86.51, 84.78, 79.75, 70.09, 59.12, 49.54, 42.72, 40.21, 31.68, 21.91, 20.06 18.05; IR (KBr): 3452.09, 2962.32, 2925.54, 1774.66, 1754.20, 1722.69, 1596.52, 1488.0, 1012.11, 800.91, 754.9, 715.23.cm^{-1}; ESI MS (m/z): 561 $(M + Na)^+$; Anal. Calcd for $C_{28}H_{28}BrNO_5$: C, 62.46; H, 5.24; N, 2.60. Found: C, 62.41; H, 5.27; N, 2.63.

4.1.3. Synthesis of spiroaziridine derivatives of parthenin

In a typical procedure, to a solution of parthenin (0.05 g, 0.19 mmol) in dry toluene (3 mL), was added phenyl azide (0.045 g, 0.4 mmol) and refluxed the reaction mixture for 15 h. The solvent was evaporated in *vaccuo* and the crude product was subjected for flash chromatography to afford pure product. The pure product (**4a**) was characterized on the basis of ^{1}H NMR, ^{13}C NMR, DEPT and mass spectrometry.

4.1.3.1. Compound 4a. White colour solid; m.p: 245–247 °C; $[\alpha]_D^{25}$ −1 (c 0.75, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.48 (d, J = 5.36, 1H,), 7.15–7.12 (t, J = 7.81 Hz, 2H,), 7.0–6.95 (t, J = 7.72, 1H), 6.75 (d, J = 7.66 Hz, 2H), 6.18 (d, J = 5.3 Hz, 1H), 5.21 (d, J = 5.66 Hz, 1H), 3.03–2.97 (td, J = 6.54 Hz, 1H), 2.72 (s, 1H), 2.63 (s, 1H), 2.21–2.15 (p, J = 7.11, 1H), 2.12–2.08 (dd, J = 7.86, 6.12, 1H), 2.03–1.96 (q, J = 13.68, 1H), 1.69–1.65 (dd, J = 8.1, 5.48, 1H), 1.52–1.48 (dd, J = 8.63, 5.24, 1H), δ 1.33 (s, 3H), 1.12 (d, J = 7.6 Hz, 3H); ^{13}C NMR ($CDCl_3$): δ 210.51, 162.23, 144.51, 130.5, 124.03, 119.1, 116.1, 84.6, 77.82, 60.21, 49.1, 41.12, 35.27, 30.54, 22.48, 20.54, 17.06; IR (KBr): 3439, 2961, 2925, 1779, 1751, 1721, 1590, 1562 cm^{-1}; ESI MS: 376 $(M + Na)^+$. Anal. Calcd for $C_{21}H_{23}NO_4$: C, 71.37; H, 6.56; N, 3.96. Found: C, 71.30; H, 6.61; N, 4.0.

4.2. Evaluation of in vitro anti-cancer activity

The effect of spiro derivatives of parthenin on the growth of cancer cell lines was evaluated according to the procedure adopted by the National Cancer Institute for *in vitro* anti-cancer drug screening that uses the protein-binding dye sulforhodamine B to estimate cell growth [33]. Briefly, cells in their log phase of growth were harvested, counted and seeded (10^4 cells/well in 100 mL medium) in 96-well microtitre plates. After 24 h of incubation at 37 °C and 5% CO_2 to allow cell attachment, cultures were treated with varying concentrations (0.1–100 mM) of test samples made with 1:10 serial dilutions. Four replicate wells were set up for each experimental condition. Test samples were left in contact with the cells for 48 h under same conditions. Thereafter cells were fixed with 50% chilled TCA and kept at 4 °C for 1 h, washed and air-dried. Cells were stained with sulforhodamine B dye. The adsorbed dye was dissolved in tris-buffer and the plates were gently shaken for 10 min on a mechanical shaker. The optical density (OD) was recorded on ELISA reader at 540 nm. The cell growth was calculated by subtracting mean OD value of the respective blank from the mean OD value of experimental set. Percentage of growth in the presence of test material was calculated considering the growth in the absence of any test material as 100% and the results are reported in terms of IC_{50} values.

4.3. DNA gel electrophoresis

DNA fragmentation was determined by electrophoresis of extracted genomic DNA from human colon cancer cell line. Cells (2×10^6/6 mL medium/60 mm tissue culture plate) were treated with Compound **2t** at 10, 50 and 100 μM for 24 h. Cells were harvested, washed with PBS, pellets were dissolved in lysis buffer (10 mM EDTA, 50 mM tris pH 8.0, 0.5% w/v SDS and proteinase K (0.5 mg/mL) and incubated at 50 °C for 1 h). Finally the DNA obtained was heated rapidly to 70 °C, supplemented with loading dye and immediately resolved on to 1.5% agarose gel at 50 V for 2–3 h.

4.4. DNA cell cycle analysis

Effect of Compound **2t** on DNA content by cell cycle phase distribution was assessed using SW-620 cells by incubating the cells 1×10^6 mL/well with Compound **2t** (1, 5, 10 & 50 μM each) for 24 h. The cells were then washed twice with ice-cold PBS, harvested, fixed with ice-cold PBS in 70% ethanol and stored at −20 °C for 30 min. After fixation, these cells were incubated with RNase A (0.1 mg/mL) at 37 °C for 30 min, stained with propidium iodide (50 μg/mL) for 30 min on ice in dark, and then measured for DNA content using BD-LSR flow cytometer (Becton Dickinson, USA) equipped with electronic doublet discrimination capability using blue (488 nm) excitation from Argon laser. Data were collected in list mode on 10,000 events for FL2-A vs. FL2-W.

4.5. In vivo anti-cancer activity

4.5.1. Animal care and housing

Non-inbred Swiss albino mice from an in-house colony were used in the present study. The breeding and experimental animals were housed in standard size polycarbonate cages providing internationally recommended space for each animal. Animals were fed balanced mice feed supplied by M/s Ashirwad Industries, Chandigarh (India) and autoclaved water was available *ad libitum*. Animals were cared as per the guide for the care and use of laboratory animals (1996), ILAR, Washington DC. They were housed in controlled conditions of temperature (23 ± 2 °C), humidity (50–60%) and 12:12 h of light: dark cycle. The study and the number of animals used were approved by the institutional animal ethics committee, IIIM-Jammu, India. The study was conducted as per the protocols of National Cancer Institute (NCI), USA [34].

4.5.2. Anti tumor activity of parthenin and its derivative compound **2t** on Ehrlich ascites tumor (EAT)

Parthenin and its derivative **2t** were evaluated against solid Ehrlich Ascites Tumor (EAT) models at different doses. A standard procedure for experiment was as followed: Animals of the same sex weighing 20 ± 3 g were injected 1×10^7 cells collected from the peritoneal cavity of non-inbred swiss mice, bearing 8–10 days old tumor cells, into the right thigh, intramuscularly (on Day 1). On next day animals were randomized and divided into test groups (7 animals in each test group) and one tumor control group (10 animals). Test groups were treated with different doses of Parthenin (10 mg/kg, 25 mg/kg and 50 mg/kg) and its derivative Compound **2t** (10 mg/kg, 25 mg/kg and 50 mg/kg, 100 mg/kg and 200 mg/kg) suspension in 1% gum acacia in their respective group intra-peritonealy for nine consecutive days. Another test group was administered 5-FU @ 20 mg/kg i.p and served as positive control. The tumor control group was similarly administered normal saline (0.2 mL i.p). The percent tumor growth inhibition was measured on day 13 with respect to their tumor weight. Shortest and longest diameters of the tumor mass were measured with the help of Vernier caliper and tumor weight (mg) was calculated by using following formula:

$$\text{Tumor weight(mg)} = \text{Length(mm)} \times \text{Width(mm)}^2/2$$

The average tumor weight for each group was calculated and the percent tumor growth inhibition in treated groups was calculated as follows:

$$\%\text{Tumor Growth Inhibitor} = 100 \times (\text{Average tumor weight of control group} - \text{Average tumor weight of test group}) / \text{Average tumor weight of control group}$$

4.5.3. Anti tumor activity of parthenin and its derivative **2t** on Ehrlich ascites carcinoma (EAC)

Ehrlich Ascites Carcinoma (EAC) was propagated by transplanting 1×10^7 cells from an animal bearing 8–10 days old Ehrlich Ascites Carcinoma, into the peritoneal cavity of non-inbred swiss mice. For testing, mice of the same sex weighing 20 ± 3 g bearing ascites tumor were selected. 1×10^7 cells obtained from an animal bearing 8–10 days old ascites tumor were injected into peritoneal cavity of all animals used for testing (0 Day). On next day animals were randomized and divided into test groups (7 animals in each test group) and one tumor control group (10 animals). Test groups were treated with different doses of Parthenin (10 mg/kg, 25 mg/kg and 50 mg/kg) and its derivative compound **2t** (10 mg/kg, 25 mg/kg and 50 mg/kg, 100 mg/kg and 200 mg/kg) suspension in 1% gum acacia in their respective group intra-peritonealy for nine consecutive days. Another test group was administered 5-FU @ 20 mg/kg i.p and served as positive control. The tumor control group was similarly administered normal saline (0.2 mL i.p). The percent tumor growth inhibition was measured on day 12 with respect to volume of ascitic fluid and the number of tumor cells in the ascitic fluid of peritoneal cavity [35].

The percent tumor growth inhibition was calculated as follow.

$$\%\text{Growth inhibitor} = 100 \times (\text{Average no. of cells in control group} - \text{Average no. of cells in test group}) / \text{Average no. of cells in control group}$$

4.5.4. Preparation of cytosolic and nuclear extracts for the evaluation of NF-kB by western blotting

SW-620 cells (5×10^6) were treated with 2t (5 μM) for indicated time periods. Cells were washed with ice-cold phosphate-buffered saline and centrifuged. Cytosolic and nuclear lysates were prepared as described earlier [35]. Cell pellets were homogenized in 200 μl of buffer A (10 mM Hepes, pH 7.9, 1 mM EDTA, 1 mM EGTA, 100 mM KCl, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 5 mM NaF, 1 mM $NaVO_4$ and 10% protease cocktail inhibitor). The tubes were placed in ice for 10 min. Nonidet P-40 was added (0.5%, v/v), tubes vortexed briefly and centrifuged at 8000 × g for 15 min. The cytosolic supernatants were stored at −80 °C. The pellets obtained were resuspended in 50 μL of buffer A supplemented with 20% glycerol, 0.4 M KCl, kept on ice for 30 min and centrifuged at 13,000 × g for 15 min. The supernatants were stored at −80 °C for analysis of nuclear NF-kB and AIF. All steps of fractionation were carried out at 4 °C. Western blot analysis for NF-kB-p65 was performed by as described by Malik et al., 2007.

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Appendix. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejmech.2011.04.030.

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