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Synergistic growth inhibition of mouse skin tumors by pomegranate fruit extract and diallyl sulfide: Evidence for inhibition of activated MAPKs/NF-κB and reduced cell proliferation

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ABSTRACT

Limited outcomes from earlier chemopreventive studies have necessitated that some modifications be made to get better efficacy. It is proposed that cancer prevention is more feasible than treatment, and this could be achieved effortlessly with use of multiple agents competent of targeting multiple targets. This study was initiated to examine the chemopreventive efficacy of pomegranate fruit extract (PFE) and diallyl sulfide (DAS), alone and in combination, using 2-stage mouse skin tumorigenesis model. PFE and DAS alone delayed onset and tumor incidence by ~55% and ~45%, respectively, while their combination at low doses synergistically decreased tumor incidence more potentially (~84%, $p < 0.01$). In addition, regression in tumor volume was seen with continuous combinatorial treatment ($p < 0.01$). Mechanistic studies revealed that this inhibition was associated with decreased expression of phosphorylated ERK1/2, JNK1 and activated NF-κB/p65, IKKα, IκBα phosphorylation and degradation in skin tissue/tumor. Histological and cell death analysis also confirmed that combined PFE and DAS inhibit cellular proliferation and markedly induce apoptosis than the single agents. Altogether, our results suggest that PFE and DAS in combination impart better suppressive activity than either of these agents alone and provide support that development of novel combination therapies/chemoprevention using dietary agents will be more beneficial against cancer.

1. Introduction

Cancer chemoprevention using bioactive food agents, a cost-effective and non-toxic approach, has received considerable importance as a potential means to control the incidence of cancer. Bioactive food agents are comprised of a wide variety of phytochemicals that are ubiquitous in plants and many of them have been used as traditional medicines for thousands of years against diverse forms of cancers (Aggarwal and Shishodia, 2006).

Numerous studies performed so far, have initially screened cancer chemopreventive effects of a variety of single dietary agents administered separately however, evidences of their combinatorial effects, which mimic real life situations, are very scarce. Recent advancements in the field of cancer chemoprevention have shown that combination of two agents shows better efficacy as compared to any one constituent alone. In a recent study, Adhami et al. (2007) demonstrated an increased efficacy of selective cyclooxygenase-2 inhibitors in combination with polyphenols from green tea for inhibition of growth of human prostate cancer cells both *in vitro* and *in vivo*. Bhuvaneswari et al. (2005) in a different study identified the anti-genotoxic and anti-carcinogenic effects of tomato constituent lycopene and garlic extract through modulation of xenobiotic-metabolizing enzymes in hamster buccal pouch carcinogenesis. Other investigators found that constituents of tomato and garlic also exhibit combined protective effect on colon carcinogenesis in rats (Sengupta et al., 2003). Chemopreventive synergism was also observed using (−)-epigallocatechin-3-gallate and curcumin combination, curcumin being a major phenolic anti-oxidant and anti-inflammatory agent in the spice Curcuma longa (Khafif et al., 1998).

We have chosen to focus our experimental efforts on pomegranate and garlic, two foods frequently cited to protect humans from various carcinogenesis (Pantuck et al., 2006; Kim et al., 2002; Fleischauer et al., 2000; Dorant et al., 1993). Pomegranate (*Punica granatum* Linn.; Punicaceae) fruit is widely consumed fresh

Abbreviations: BrdU, bromodeoxyuridine; CNT, cumulative number of tumors; DAS, diallyl sulfide; DMBA, 7, 12-dimethylbenz[a]anthracene; ERK, extracellular signal regulates kinase; PFE, pomegranate fruit extract; H_2O_2, hydrogen peroxide; IHC, immunohistochemical; JNK, c-Jun NH2-terminal amino kinase; MAPKs, mitogen-activated protein kinase; NF-κB, nuclear factor kappa B; PCNA, proliferating cell nuclear antigen; PI, propidium iodide; TPA, 12-*O*-tetradecanoylphorbol 13-acetate.

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and in beverage forms as juice and wines (Gil et al., 2000). Pomegranate juice and peel possess marked anti-oxidant capacity (Kaur et al., 2006; Singh et al., 2002a; Gil et al., 2000) with high content of polyphenols, in particular, ellagitannins, condensed tannins and anthocyanins (Seeram et al., 2005;Gil et al., 2000). Pomegranate juice has been shown to have chemopreventive, chemotherapeutic, and anti-inflammatory efficacy (Adams et al., 2006; Malik et al., 2005). Afaq et al. (2005) showed that pre-treatment of mouse skin with pomegranate fruit extract (PFE) modulated the activation of mitogen-activated protein kinase (MAPKs) and nuclear factor kappa B (NF-κB), in the 12-*O*-tetradecanoylphorbol 13-acetate (TPA)- induced or ultra violet-B induced skin carcinogenesis model. Garlic (*Allium sativum*; Alliaceae) which has been used for flavoring and as a medicinal herb for centuries, exhibits anti-oxidant and anti-carcinogenic effects (Drobiova et al., 2009; Nagini, 2008). Diallyl sulfide (DAS), a component of garlic has been demonstrated to exert a potential chemopreventive activity against human cancers, such as colon and lung (Dausch and Nixon, 1990). Treatment with DAS caused cell cycle arrest, decreased cell proliferation and induced apoptosis by activation of caspase-3 and suppression of MAPK family protein, extracellular signal regulates kinase (ERK-2) activity in Colo 320 DM colon cancer cells (Sriram et al., 2008). Cell culture and animal studies have also supported that intake of pomegranate and garlic is associated with decreased skin cancer risk (Pacheco-Palencia et al., 2008; Arora et al., 2004). Studies conducted in our laboratory had also documented the anti-tumorigenic and anti-carcinogenic efficacy of DAS (Arora and Shukla, 2002; Singh and Shukla, 1998). Therefore, these widely consumed agents with the ability to modulate inflammation, cell proliferation, differentiation, and apoptosis-related pathways and no toxicity make attractive candidates for cancer prevention and therapy.

There is a growing interest in the use of a combination of chemopreventive agents at low doses rather than the administration of a single agent, as a means of increasing their efficacy against cancer (de Kok et al., 2008). Despite plenty of convincing data from laboratory studies which have demonstrated the anti-carcinogenic effects of PFE and DAS, nothing is known about their combinatorial roles in cancer chemoprevention. Therefore, the present study was undertaken to evaluate the efficacy of selected PFE and DAS in combination, as skin cancer-preventive and possibly skin cancer-therapeutic agents, using 7, 12- dimethylbenz[a]anthracene (DMBA) and TPA-induced mouse skin tumorigenesis.

2. Materials and methods

2.1. Chemicals

DMBA, TPA, DAS, bromodeoxyuridine (BrdU), β-actin (clone AC-74) and propidium iodide (PI) were purchased from Sigma Chemical Co. (St. Louis, USA). The mouse monoclonal total and phospho-specific: c-Jun NH2-terminal amino kinase (JNK) and p44/42 MAPK (Thr202/Tyr204) i.e. ERK1/2, IκBα (Ser32) antibodies were procured from Cell Signaling Technology (Beverly, USA). NF-κB/p65, IKKα, proliferating cell nuclear antigen (PCNA) and monoclonal anti-BrdU antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Hydroxylapatite and *N*, *N*-dimethylformamide was purchased from Sisco Research Laboratory (Mumbai, India). The respective horseradish peroxidase conjugate secondary antibodies were obtained from Bangalore Genei (Bangalore, India). The rest of the chemicals were of analytical grade of purity and were procured locally.

2.2. Preparation of PFE

Fresh fruit of pomegranate procured from the local market was washed and the outer skins were hand-peeled. Following peeling out, the edible portion (seed coat and juice) was squeezed in distilled water (1:10 by wt./vol.). The juice (extract) had a deep-red color. The red extract was filtered through filter paper (Whatman No. 1). PFE was prepared freshly thrice a week to be used for oral feeding to animals.

2.3. Animals and treatment schedule

2.3.1. Long term in vivo studies

Male, Balb/c mice (15–18 gm body weight (b.wt.)) were obtained from Indian Institute of Toxicology Research (Lucknow, India) animal breeding colony and acclimatized for 1 week. The ethical approval for the experiment was obtained from institutional ethical committee. They were kept under standard laboratory conditions (temperature 23 ± 2 °C, relative humidity 55 ± 5%) and were fed with synthetic pellet basal diet (Ashirwad, Chandigarh, India) and water *ad libitum*. They were randomly divided into six groups of 25 animals each. Hair was clipped in the dorsal region with proper care in an area of 2 cm^2 using electrical clippers. Solvent used for DMBA, TPA and DAS was acetone. In brief, treatment was given as described below for 18 weeks of duration to attain the entire period of skin tumorigenicity:

Group I: Untreated control (no treatment was given to the animals).
Group II: DMBA + TPA (single topical application of DMBA, 52 μg/animal followed one week later by thrice a week application of TPA, 5 μg/animal).
Group III: PFE (10%) + DAS (PFE given as a sole source of drinking fluid followed by thrice a week topical application of DAS, 250 μg/animal).
Group IV: DMBA + TPA + PFE (single topical application of DMBA, 52 μg/animal followed one week later by TPA application as in Gr. II and PFE supplementation as in group III).
Group V: DMBA + TPA + DAS (single topical application of DMBA, 52 μg/animal followed one week later by TPA application as in Gr. II and DAS application as in group III).
Group VI: DMBA + TPA + PFE + DAS (single topical application of DMBA, as in group II followed one week later by TPA application as in Gr. II, PFE (5%) supplementation and DAS (125 μg/animal) application as in group III).

During treatment period animals were carefully observed for any change in b.wt., fluid/food intake and development of skin tumor. Tumors > 1 mm in diameter were considered in the cumulative number if they persist for 2 weeks or more.

After completion of 18 weeks of treatments duration, the regression of pattern of tumors in terms of both number and volume were recorded up to 26 weeks in the animals of group VI (bearing tumor or not). These were divided into 3 subgroups and treatment schedule was as follows:

Group VI A (non-tumor bearing): No further treatment was given to this group.
Group VI B (tumor bearing): TPA along with PFE and DAS were withdrawn.
Group VI C (tumor bearing): Only TPA was withdrawn.

After completion of the study period (26 weeks), all the animals of group VI B and group VI C were screened for tumor volume regression and sacrificed thereafter. Tumor volume was taken per mouse in each group using formula $V = D \times d^2 \times \pi/6$, where D = bigger dimension and d = smaller dimension. Skin from the painted area (with or without tumors) was excised out, cleaned, snap frozen in liquid nitrogen, and stored at −80 °C until further use.

2.3.2. Analysis of the effects of PFE and DAS combination

The nature of the combined effects of PFE and DAS was determined using the method described by Zhou et al. (2003) based on the principles described by Chou and Talalay (1984). In brief, the expected value of combination effect between agent 1 and agent 2 is calculated as [(observed agent 1 value)/(control value)] × [(observed agent 2 value)/(control value)] × (control value); and the ratio is calculated as (expected value)/(observed value). A ratio of >1 indicates a synergistic effect and a ratio of <1 indicates a less than additive effect.

2.3.3. Short term in vivo studies

30 animals (male, Balb/c mice, 15–18 gm b.wt.) were divided into six groups of 5 animals each and treatment was given for 2 weeks period as described below:

Group I: Untreated control (no treatment).
Group II: DMBA + TPA (single topical application of DMBA, 52 μg/animal followed one week later by 3 times a week application of TPA, 5 μg/animal).
Group III: PFE (10%) + DAS (PFE given as a sole source of drinking fluid followed by 4 times a week topical application of DAS, 250 μg/animal).
Group IV: DMBA + TPA + PFE (single topical application of DMBA, 52 μg/animal followed one week later by TPA application as in Gr. II and PFE supplementation as in group III).
Group V: DMBA + TPA + DAS (single topical application of DMBA, 52 μg/animal followed one week later by TPA application as in Gr. II and DAS application as in group III).
Group VI: DMBA + TPA + PFE + DAS (single topical application of DMBA, as in group II followed one week later by TPA application as in Gr. II, PFE (5%) supplementation and DAS (125 μg/animal) application as in group III).

At the end of the experiment, one animal of each group was injected intraperitoneally with BrdU (50 mg/kg b.wt.). After 2 h all the animals were sacrificed and

skin from the painted area was excised out, cleaned, snap frozen in liquid nitrogen, and stored at −80 °C until further use.

2.4. Preparation of lysates

Whole skin tissue/tumor homogenates (10%) were made as described by Kataoka et al. (2008) in both, the long term as well as the short term study. For the preparation of cytosolic and nuclear lysates, the skin tissue/tumor from both the studies were homogenized in lysis buffer and processed according to the method of Serpi et al. (1999). The supernatants were collected and stored at −80 °C till use.

2.5. Western blotting

Western blotting was carried out as described earlier (Arora et al., 2004). Protein concentration was measured following standard protocols (Lowry et al., 1951). Proteins (60 μg) were resolved on 10–12% SDS–PAGE and electroblotted onto polyvinylidene fluoride membranes (Millipore, Billerica, MA, USA). The blots were blocked overnight with 5% non-fat dry milk and probed with various monoclonal and polyclonal antibodies at dilutions recommended by the suppliers. Immunoblots were detected using chemiluminescence kit from Millipore, (Billerica, MA, USA). Equal loading in the blots were confirmed by stripping the membrane and reprobing it with β-actin. Densitometric analysis of the bands was done with digitalized scientific software program UN-SCAN-IT purchased from Silk Scientific Corporation (Orem, Utah, USA).

2.6. Histopathological analysis

Ten micrometer thick sections of the tissues were cut using cryostat microtome (Slee, Mainz, Germany) and were processed as per standard procedure, stained with haematoxylin and eosin, and examined under a microscope (Leica, Wetzler, Germany). Histopathological classification of the tumors was performed as described by Bogovaski (1973).

2.7. Immunohistochemical (IHC) staining

IHC staining, for determining PCNA and BrDU expression was carried out as described earlier by us (Arora et al., 2004), with slight modifications. In brief, frozen skin tissue/tumor sections (10 μm) (from long and short term study) were cut using cryostat (Slee, Mainz, Germany) and were fixed in neutral 10% formalin. The endogenous peroxidase activity was quenched with methanol: hydrogen peroxide (H_2O_2) solution and the non-specific binding were minimized with normal goat serum. The slides were incubated with anti-PCNA and anti-BrdU antibodies in a moist chamber for 72 h. After incubation, the sections were again incubated with normal goat serum and then with HRP-conjugated respective secondary antibodies. The slides were washed with phosphate buffer saline-triton X-100 in between the incubations. The color was developed using chromagen 3-methyl 9-ethyl carbazole in Tris (0.05 M, pH 7.3) buffer containing H_2O_2. The immunostained slides were analyzed on Leica Q Win500 image analysis system attached with high-resolution color digital charge coupled device camera (JVC). The immunoreactivity for PCNA and BrdU was assessed on the basis of the number of cells having positive reactivity in a skin section (count/mm^2 area).

2.8. Apoptosis analysis by annexin V/PI dual staining

Annexin-V and PI FITC detection Kit (BD, San Jose, CA, USA) was used for the differentiation between apoptotic and necrotic cell population. The single cell suspensions of treated and untreated skin tissues (from short term study) were prepared using Medimachine (Beckton Dickinson, San Jose, USA). For each sample, Annexin-V/PI fluorescence was analyzed, wherein fluorescence of cells was gated and counted using 'Cell Quest 3.1 software'.

2.9. DNA alkaline unwinding assay

Strand breaks in cellular DNA were quantified by alkaline unwinding assay using hydroxyapatite batch procedure as described by us previously (Nigam and Shukla, 2007). 100 μg of DNA from treated and untreated skin tissues sample (from short term study) was subjected to alkaline unwinding. The relative amount of duplex and single stranded DNA present at the end of the alkaline unwinding was quantified.

2.10. Statistical analysis

For the statistical analysis of skin tumor appearance dynamics, the Kaplan–Meier method of tumor-free survival estimation was applied. Statistically significant differences were determined between control and treatment groups using one-way ANOVA (GraphPad Prism software) followed by Dunnett post hoc test. Values with $p < 0.05$ were considered significant.

3. Results

3.1. Effects of PFE and/or DAS on tumorigenicity rate

Compared with the untreated control (group I), the experimental treatments (groups II–VI) did not alter food intake or final b.wt. (data not shown). The only exception being the final b.wt. of mice on PFE alone (group IV) which showed an increase of 16%. The mice recorded similar amounts of PFE consumption daily (data not shown) with no increase in mortality rate among all the control and experimental groups during the experiment. The first day of tumor incidence in the positive control group II (DMBA and TPA) was 44th day; however, it was 59th day in PFE treated group IV, 56th day in DAS treated group V and 92nd day in the PFE and DAS treated group VI. There was no tumor induction in group III. The chemopreventive potential of PFE and DAS was also evident by significant ($p < 0.01$) increase in tumor free survival of animals. 44%, 36%, 68% of animals of group IV, V and VI remained tumor-free until the termination of the experiment. However, by the end of the 18th week, 100% animals of the positive control group II developed tumors (Fig. 1(a)). The expected effect of the PFE and DAS combination on tumorigenicity rate (35.8%) was higher than the observed combination effect (32.0%) with a ratio of 1.12, suggesting that the PFE and DAS combination had a synergistic effect in inhibiting tumorigenicity (Table 1).

Protection was also seen in terms of reduction in tumor volume. The tumor volume was 127.0 ± 11.26 mm^3/mouse in DMBA and TPA group, which reduced to 60.3 ± 10.05, 81.2 ± 7.00 and 45.2 ± 7.01 mm^3/mouse in group IV, V and VI, respectively ($p < 0.01$). The expected effect of the combined PFE and DAS on average tumor volume (35.6%) was lower than the observed combined effect (47.5%) with a ratio of 0.85, suggesting that the PFE and DAS combination had a less than additive effect in reducing the tumor volume (Table 1).

The chemopreventive effect of PFE and DAS was also evident in terms of reduction in the cumulative number of tumors (CNT). While the positive control group showed 165 CNT, group IV, V and VI showed 74, 91, 27 CNT at the end of the 18th week (Fig. 1(b)). The observed effect (16.4%) in combination group VI was less than the expected value (24.7%), with ratio of 1.51, suggesting that PFE and DAS combination synergistically reduces CNT (Table 1).

Similarly, in terms of average number of tumors per tumor-bearing mouse, group IV showed 5.3 ± 2.11 tumors, group V showed 5.7 ± 1.47 tumors and group VI showed 2.4 ± 0.95 tumors, in comparison to DMBA and TPA treated group with 6.6 ± 1.15 tumors per tumor bearing mouse ($p < 0.01$, Fig. 1(c)). Comparisons of expected (69.3%) and observed values (36.4%), with ratio of 1.90 suggest that PFE combined with DAS synergistically reduced average number of tumors (Table 1).

3.1.1. Effects of PFE and DAS on tumor growth and regression

We further extended the work to observe regression offered by combined doses of PFE and DAS in tumor volume, if any. At 19th week, TPA treatment was ceased and morphological changes of the tumors were recorded in group VI B and VI C. Among the two groups, in Gr. VI C, commencement of regression was recorded, 23rd week onwards. Apparently, group VI B, from which PFE and DAS were withdrawn, showed no regression (Table 2). However, the tumor volume at 26th week was 229.7 ± 10.95 mm^3/mouse in DMBA + TPA group II and 42.7 ± 5.96 mm^3/mouse in group VI B which was a significant ($p < 0.01$) increase from the volume recorded for the 18th week (127.0 ± 11.26 and 40.2 ± 5.19 mm^3/mouse respectively). Also, the tumor volume decreased significantly ($p < 0.01$) to 28.9 ± 7.60 mm^3/mouse in the 18th week (50.2 ± 6.08 mm^3/mouse), in group VI C (Table 2, Fig. 1(d)).

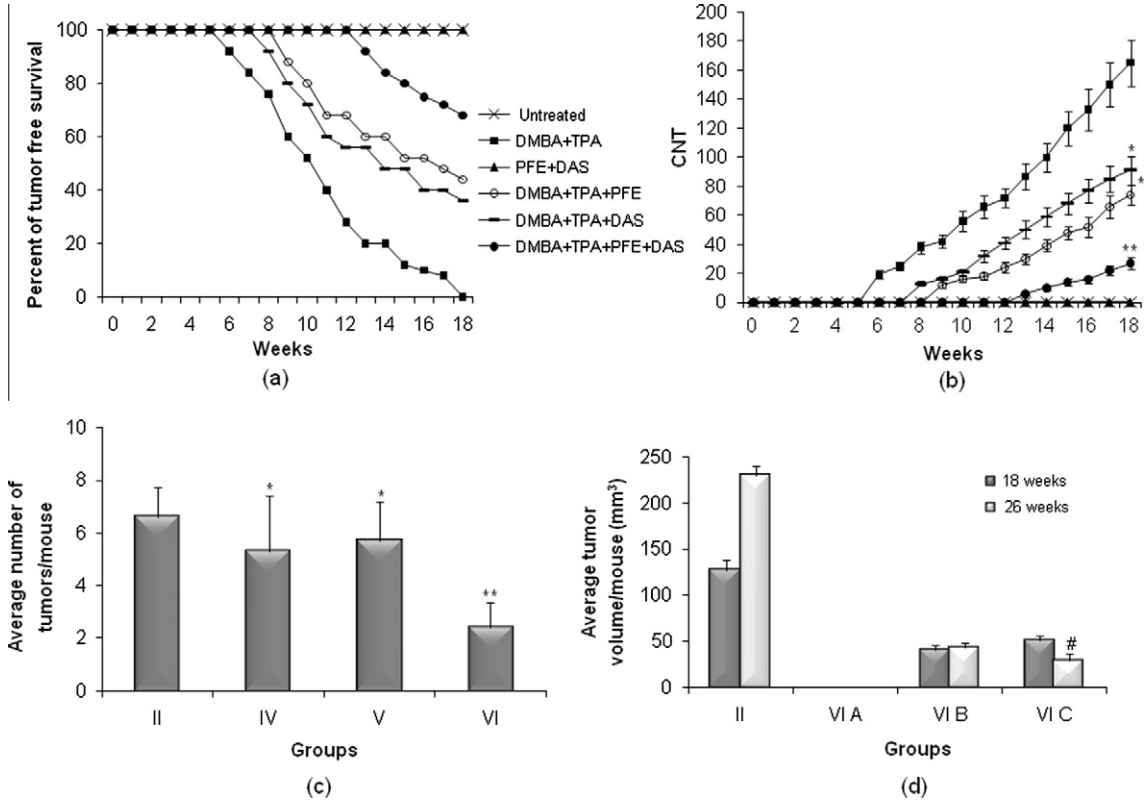

Fig. 1. Effect of PFE and/or DAS on DMBA and TPA-induced mouse skin tumors in terms of (a) percentage of tumor-free survival, (b) cumulative number of tumors per group, (c) reduction in average number of tumors/mouse and (d) reduction in tumor volume/mouse (mm^3). The data showed are of surviving animals up to 18th weeks in (a), (b) and (c) however, mean ± SD of surviving animals from 18th to 26th weeks in (d). *Indicates significant reduction over DMBA and TPA treated group II ($p < 0.01$). **Indicates significant reduction over PFE and DAS alone treated group IV and V ($p < 0.01$). #Indicates significant reduction after 26th weeks ($p < 0.01$).

Table 1
Possible combinatorial chemopreventive effect of PFE and/or DAS on DMBA and TPA-induced mouse skin tumorigenesis.

Treatment (groups)	Tumorigenicity (%)			CNT (%)			ANT (%)			ATV (%)		
	Observed	Expected[a]	Ratio[b]	Observed	Expected[a]	Ratio[b]	Observed	Expected[a]	Ratio[b]	Observed	Expected[a]	Ratio[b]
Untreated control (I)												
DMBA + TPA (II)	100.0			100.0			100.0			100.0		
PFE + DAS (III)												
DMBA + TPA + PFE (IV)	56.0			44.8			80.3			47.5		
DMBA + TPA + DAS (V)	64.0			55.1			86.4			63.9		
DMBA + TPA + PFE + DAS (VI)	32.0	35.8	1.12	16.3	24.7	1.51	36.4	69.3	1.90	35.6	30.3	0.85

All the data are statistically significant, $p < 0.01$.
CNT, cumulative number of tumors; ANT, average number of tumors/mouse; ATV, average tumor volume/ tumor bearing mouse.
[a] Expected value of PFE and DAS combination = [(observed value of PFE)/(control value)] × [(observed value of DAS)/(control value)] × (control value).
[b] Ratio = (expected value/observed value). A ratio of >1 indicates a synergistic effect, and a ratio of <1 indicates a less than additive effect.

Although, the CNT and average number of tumors increased in positive control, they showed no change in PFE and DAS supplemented groups (Table 2). Moreover, animals of group VI A did not show any morphological changes and development of tumors till the termination of the experiment (Table 2). Thus, the combined supplementation of PFE and DAS resulted in regression in tumor volume but not in tumor number.

3.2. Effect of PFE and/or DAS on DMBA and TPA-mediated phosphorylation of ERK1/2 and JNK1 proteins

It has been reported that topical application of TPA in mouse skin results in a marked increase in the phosphorylated form of MAPKs (Afaq et al., 2005; Chun et al., 2003), affecting fundamental cellular processes like proliferation, differentiation, and survival, thus we further evaluated the expression levels of MAPK family proteins (Kennedy et al., 2007). In the present study, western blot analysis showed that the activation of MAPKs (ERK1/2 and JNK1) was gradually increased during progression of TPA-induced papillomagenesis in DMBA-initiated mouse skin over untreated control group ($p < 0.05$), in both long and short term studies (Fig. 2(a) and (b)). However, both the combined and either doses of PFE and DAS when supplemented with DMBA and TPA significantly ($p < 0.05$) down-regulated expression levels of ERK1/2 and JNK1 through inhibition of their phosphorylation as compared to animals given DMBA and TPA, in both the experiments, respectively. There was no effect on the total amount of ERK1/2 and JNK1 after any of the treatments (Fig. 2(a) and (b)). Interestingly, PFE was found to be more potent in decreasing ERK1/2 and JNK1 protein expression levels (Fig. 2(a) and (b)). It is clear from the Fig. 2(a) and (b) combined supplementation of PFE and DAS resulted in more significant ($p < 0.05$) decrease in the expression of phosphorylated ERK1/2 and JNK1.

Table 2
Combinatorial chemopreventive effect of PFE and/or DAS on tumor regression.

Groups	Treatment	Number of animals with tumors	CNT	ANT (mean ± SD)	ATV (mm^3) (mean ± SD)
At 18th week					
II	DMBA + TPA	25/25	165	6.6 ± 1.15	127.0 ± 11.26
VI A	(−) PFE + DAS-non-tumor bearing	0/14	–	–	–
VI B	(−) PFE + DAS-tumor bearing	5/5	15	3.0 ± 1.00[a]	40.2 ± 5.19[a]
VI C	(+) PFE + DAS-tumor bearing	6/6	16	2.6 ± 0.51[a]	50.2 ± 6.08[a]
At 26th week					
II	DMBA + TPA	25/25	178	7.1 ± 2.05	229.7 ± 10.95
VI A	(−) PFE + DAS-non tumor bearing	0/14	–	–	–
VI B	(−) PFE + DAS-tumor bearing	5/5	15	3.0 ± 1.00	42.7 ± 5.96
VI C	(+) PFE + DAS-tumor bearing	6/6	16	2.6 ± 0.51	28.9 ± 7.60[b]

(−) Indicates without treatment, (+) indicates with treatment.
CNT, cumulative number of tumors; ANT, average number of tumors/mouse; ATV, average tumor volume/tumor bearing mouse.
[a] Indicates significant reduction over DMBA and TPA treated group II ($p < 0.01$).
[b] Indicates significant reduction in ATV after 26 weeks ($p < 0.01$).

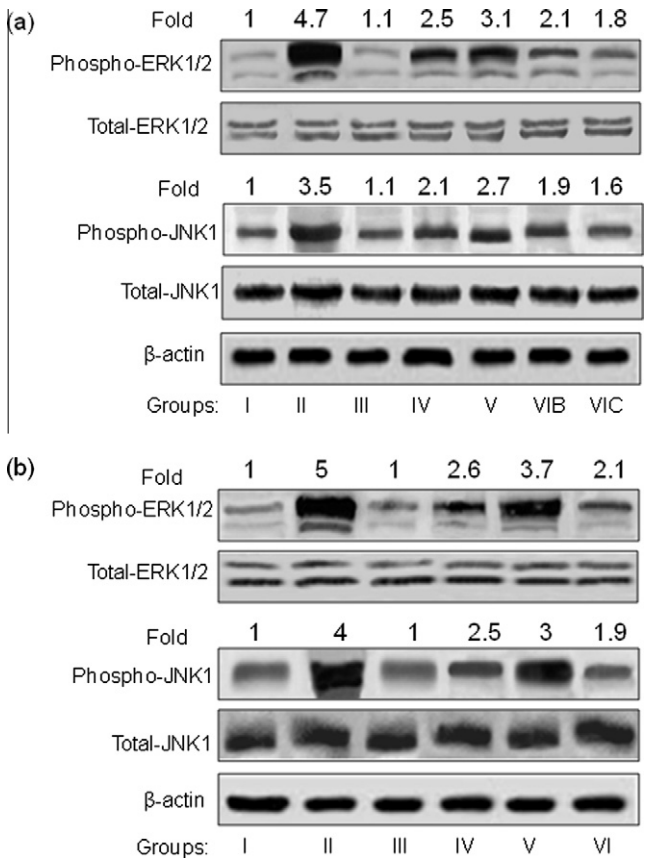

Fig. 2. Western blots showing the effect of PFE and/or DAS on the expression levels of both total and phospho-ERK1/2 and JNK1 in mouse skin tumors/skin in (a) long and (b) short term studies. Details for groups and treatments are described in Section 2. The bands shown here are from a representative experiment repeated three times with similar results with values as mean ± SD. β-Actin was used as a loading control. The pixel density of the specific immunoreactive bands was quantified by densitometry and expressed as a fold difference against β-actin.

3.3. Effect of PFE and/or DAS on DMBA and TPA-induced activation of NF-κB and IKKα and phosphorylation and degradation of IκBα protein expression

Studies have shown that one of the critical events in NF-κB activation is its dissociation with subsequent degradation of inhibitory protein IκBα via phosphorylation and ubiquitination (Takada and Aggarwal, 2004; Bharti and Aggarwal, 2002; Israel, 1995). Activation and nuclear translocation of NF-κB is preceded by the phosphorylation and proteolytic degradation of IκBα (Afaq et al., 2003; Bharti and Aggarwal, 2002). To determine whether the inhibitory effect of PFE and DAS was attributable to an effect on IκB degradation, we examined the cytoplasmic level of IκBα protein expression by western blot analysis. We found that DMBA and TPA application to mouse skin resulted in the degradation of IκBα protein expression in both long and short term studies. However, the combined and solitary treatments of PFE and DAS resulted in its inhibition, $p < 0.05$ (Fig. 3(a) and (b)). We next assessed whether DMBA and TPA application affects the phosphorylation of IκBα protein. As shown by western blot, DMBA and TPA induced a marked increase in the phosphorylation level of IκBα protein at Ser32 after treatment, which was synergistically inhibited by PFE and DAS treatments, ($p < 0.05$) (Fig. 3(a) and (b)). Studies have shown that IKKα activity is necessary for IκBα protein phosphorylation/degradation (Maniatis, 1997; Baldwin, 1996). To determine whether inhibition of DMBA and TPA-induced IKKα activation by PFE and DAS is attributable to suppression of IκBα phosphorylation/degradation, we also measured IKKα protein level. We found that combined treatments of PFE and DAS are more potent in inhibiting DMBA and TPA-induced increase expression of IKKα, ($p < 0.05$) (Fig. 3(a) and (b)). Next, we investigated whether alone and combined treatments of PFE and DAS inhibited TPA-induced activation and nuclear translocation of NF-κB/p65, the functionally active subunit of NF-κB in mouse skin. In the nuclear fraction, we found that DMBA and TPA application onto the mice skin resulted in the activation and nuclear translocation of NF-κB/p65. However, PFE and DAS inhibited NF-κB/p65 activation and nuclear translocation (Fig. 3(a) and (b)).

3.4. Effect of PFE and/or DAS on histopathology of skin/tumor

Histologically skin tumor sections from DMBA and TPA applied group II exhibited varying degrees of structural and cytological changes as compared to untreated control group I (Fig. 4(a)). Tumors of group II animals were composed of focal proliferation of squamous cells, presence of some necrotic cells, keratinization (Fig. 4(a)). This clearly indicates the benign nature of tumors initiated and promoted by DMBA and TPA. However, skin sections from animals treated with PFE and DAS (group III), did not reveal any histopathological abnormalities (Fig. 4(a)). In group V, less pronounced preventive effect of DAS were noticed as compare to PFE supplemented group IV (Fig. 4(a)). The administration of both PFE and DAS led to suppression of DMBA and TPA-induced skin

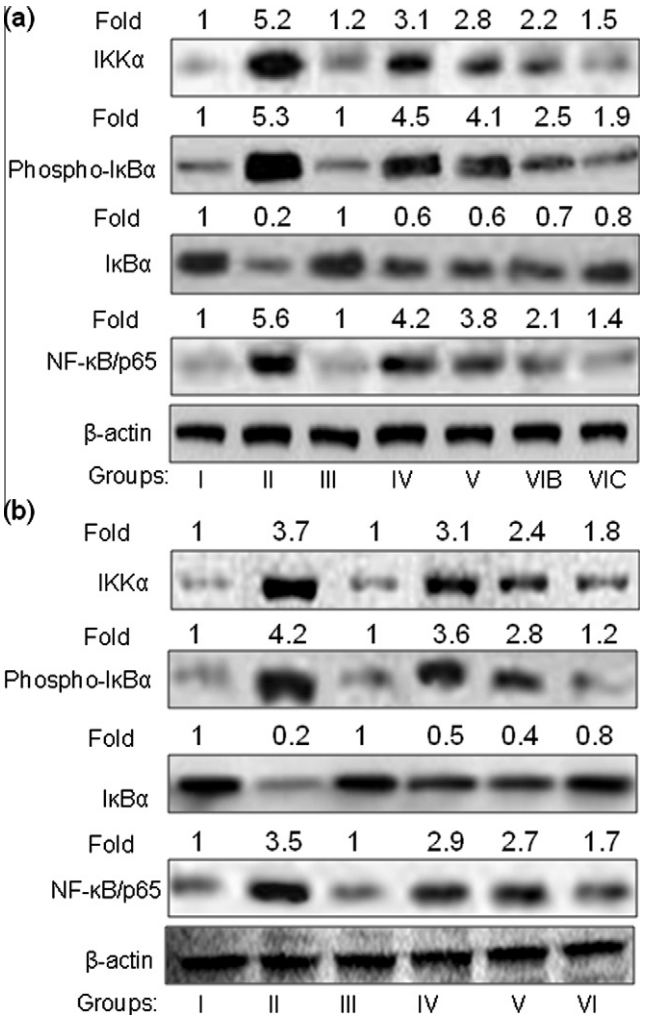

Fig. 3. Western blots showing the effect of PFE and/or DAS on the expression levels of activated NF-κB and IKKα, phosphorylation and degradation of IκBα in mouse skin tissue/tumors in (a) long and (b) short term studies. Details for groups and treatments are described in Section 2. The bands shown here are from a representative experiment repeated three times with similar results with values as mean ± SD. β-Actin was used as a loading control. The pixel density of the specific immunoreactive bands was quantified by densitometry and expressed as a fold difference against β-actin.

tumorigenesis. Skin tumor sections of group VI showed chances towards normalization of skin as compared to group II (Fig. 4(a)).

Similarly, in short term experimental groups' haematoxylin and eosin stained sections were showing pronounced preventive effects of PFE and DAS treatment when given in combination than alone over DMBA and TPA-induced changes in animals skin (Fig. 4(b)). In group II, disorganization of epithelium, presence of necrotic cells and focal proliferative area were noticed as compared to group I (Fig. 4(b)).

3.5. Effect of PFE and/or DAS on proliferation markers

IHC analysis of PCNA was used to assess the proliferation activity during tumor promotion (Fig. 5(a)) in long term assay. PCNA reactivity is associated with S phase of DNA replication (Mancini et al., 1994). PCNA reactivity was assessed by counting PCNA positive cells in 3 randomly selected fields and mean values were calculated for each of the group (Fig. 5(a)). A characteristic intense nuclear staining and higher number of PCNA positive cells were observed in animals treated with DMBA and TPA (2.8 folds) as compared to other groups (Fig. 5(a)). In untreated control group low positive staining and intensity was noted for PCNA (Fig. 5(a)). Treatment of PFE and DAS resulted in lowering of PCNA positive cells number ($p < 0.01$) as compared to Group II revealing that effects of combinational treatment of PFE and DAS were superior to alone treatment (Fig. 5(a)).

Both PCNA staining and BrdU incorporation were analyzed to determine the proliferative status of skin after treatment with DMBA and TPA and the respective preventive treatments in short term groups also (Fig. 5(b) and (c)). It was observed that the number of PCNA and BrdU positive cells were higher in animals of group II as compared to untreated animals (Fig. 5(b) and (c)). Treatment with either PFE or DAS or both in combination in DMBA and TPA applied animals resulted in significantly low levels of PCNA and BrdU positive cells ($p < 0.01$) (Fig. 5(b) and (c)).

3.6. Effect of PFE and/or DAS on apoptosis

Apoptosis is the most potent defense mechanism against cancer Lowe and Lin, 1990). We next quantified the extent of apoptosis by flow cytometric analysis of the skin cells labelled with Annexin V/PI. Results showed significant induction ($p < 0.05$) of apoptosis by either PFE/DAS or the two in combination over untreated and DMBA and TPA group II (Fig. 6). Percent increase in apoptotic population in PFE was 18.72 ± 1.43, DAS was 13.93 ± 1.72 and in combination group was 39.96 ± 1.03 as compared to group I and II where values was 2.32 ± 0.69 and 1.40 ± 0.10, respectively.

3.7. Effect of PFE and/or DAS on DMBA and TPA-induced nick formation

Based on the amount of duplex DNA remaining after alkali treatment, the number of strand breaks generated per unit DNA was determined in mouse skin tissues obtained from short term study. DMBA and TPA caused a significant DNA damage ($n = 1.55 \pm 0.40$) ($p < 0.01$) over untreated ($n = 0.007 \pm 0.001$). Inhibition of DMBA and TPA-induced DNA alkylation damage was recorded by either PFE or DAS or the two in combination in terms of reduction in strand breaks and found to be 51.57%, 59.07% and 67.48%, respectively (Table 3).

4. Discussion

It is speculated that combination of chemopreventive agents, which has the ability to intervene in critical pathways of the carcinogenesis, will have advantage over single agents. In the present study, by employing *in vivo* skin cancer model, we attempted to determine the chemopreventive combinatorial effects of PFE and DAS. This study clearly demonstrated that combined doses of the two agents exert more chemopreventive effects rather than either agent alone. Administration of PFE and DAS in combination was found to be synergistically effective in decreasing the rate of tumorigenicity, CNT and average tumor number (Table 1). Previous studies demonstrated chemopreventive efficacy of DAS and PFE against DMBA and TPA-induced mouse skin tumorigenesis (Afaq et al., 2005; Singh and Shukla, 1998) mainly attributed to their anti-oxidant and anti-inflammatory property (Drobiova et al., 2009; Nagini 2008; Adams et al., 2006; Gil et al., 2000). Our data exhibits significant regression of tumors volume where PFE and DAS supplementation continued till the termination of study (Table 2). Together with these, no increase in tumor volume or incidence was observed in animals (tumor bearing and non tumor-bearing) after withdrawal of the combined treatment (Table 2). Hence, combination of PFE and DAS had superior skin cancer chemopreventive efficacy as compared to either PFE/DAS.

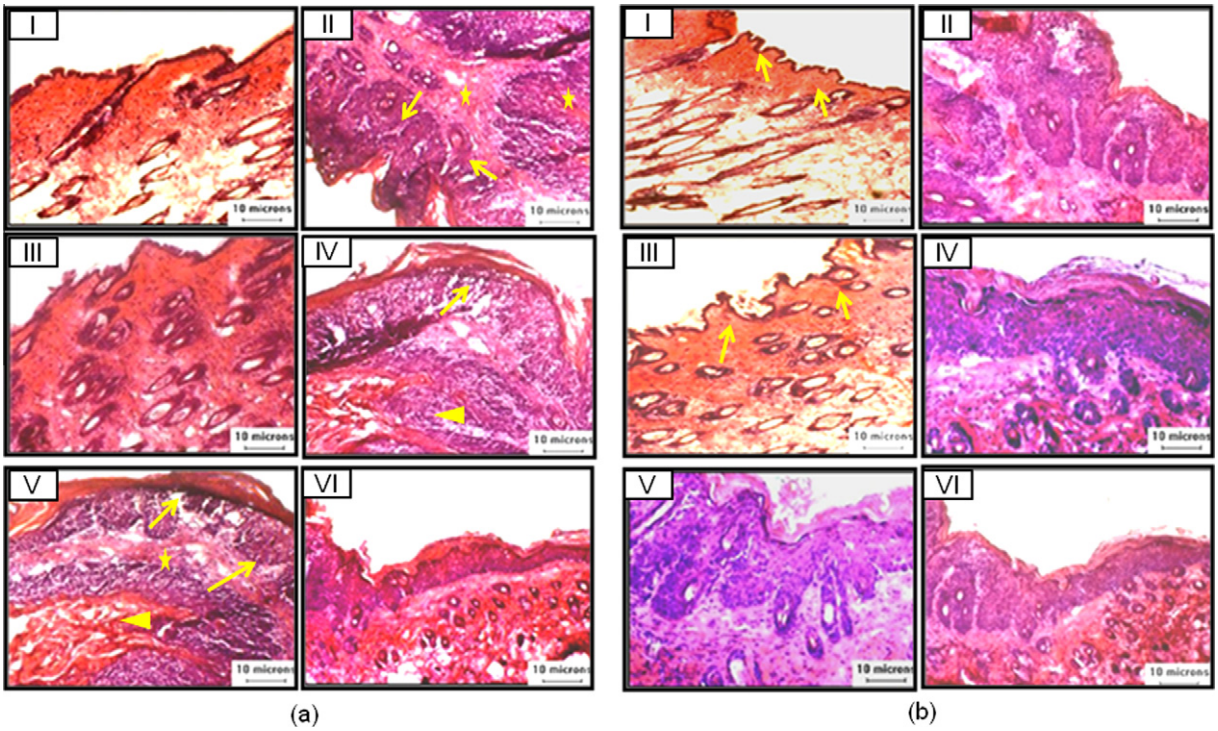

Fig. 4. Histological study of mouse skin/tumors from (a) long and (b) short term experimental groups. Details for groups and treatments are described in Section 2. Representative sections stained with haematoxylin and eosin showed-arrow: squamous cells, asterisk: presence of some necrotic cells, arrow head: keratinisation. All magnifications are at 10x.

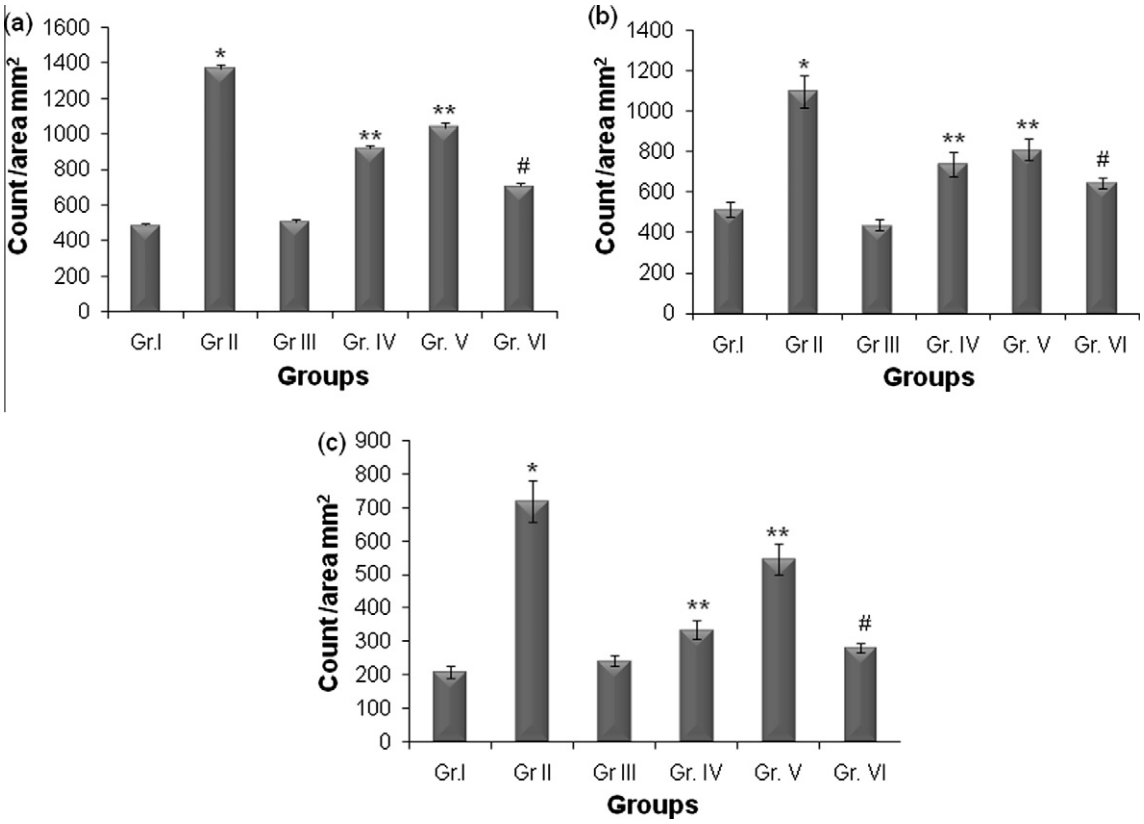

Fig. 5. Effects of PFE and DAS treatment on modulation of: (a) PCNA expression in different groups from long term experiment, (b) PCNA expression in different groups from short term experiment, and (c) BrdU incorporation in different groups from short term experiment. Details are described in Section 2. Bar diagram shows mean ± SD of three independent sets analysis. *Significantly increased in comparison of group I ($p < 0.01$), **significantly decreased when compared with group II ($p < 0.01$). #Significantly decreased when compared with groups IV and V ($p < 0.01$).

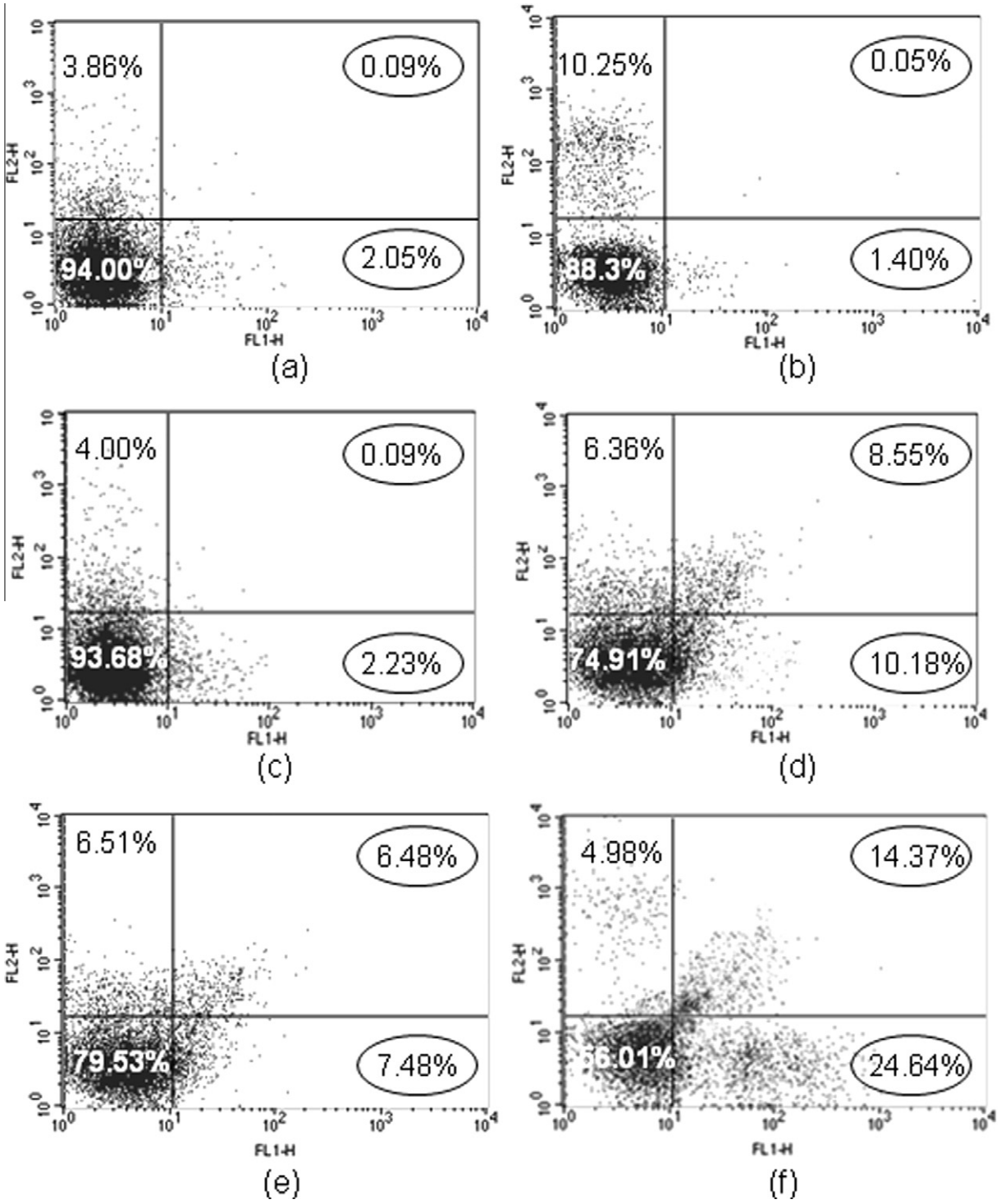

Fig. 6. Representative figure of flow cytometric analysis of apoptosis by using Annexin V and PI dual staining method in mouse skin on treatment with PFE and DAS. The data is representative of three independent experiments ($p < 0.05$). Details are described in Section 2. Figure showing cells in the upper right (UR), upper left (UL) and lower right (LR) portions portion of the picture indicates late apoptotic cells, necrotic and pre-apoptotic cells, respectively whereas cells present in lower left (LL) portion of the picture indicate percentage of live cells.

We further investigated the expression of proteins involved in MAPK pathway. The MAPK family represents an important group of signaling proteins that can regulate fundamental cellular processes like proliferation, differentiation, and survival (Kennedy et al., 2007). Moreover, it is one of the most well documented signaling cascades highly relevant in the process of tumor promotion and progression induced by chemical carcinogens in mouse skin carcinogenesis, affecting not only cell proliferation, but also apoptosis by promoting the survival of the tumor cell (Afaq et al., 2005; Xiao et al., 2005; Chun et al., 2003; Zoumpourlis et al., 2003). Activation of ERKs and JNK1 are found in most skin tumors and involved in skin carcinogenesis (Sarfaraz et al., 2008). In accordance with above mentioned studies, treatment of DMBA and TPA in mouse skin resulted in marked increase in the phosphorylation

Table 3
Number of DNA strand breaks inhibited by PFE or/and DAS against DMBA and TPA-induced DNA alkylation damage.

Groups	Treatment	Number of DNA strand breaks (*n*)
II	DMBA + TPA	1.55 ± 0.40
IV	DMBA + TPA + PFE	0.75 ± 0.20[a] (51.57%)
V	DMBA + TPA + DAS	0.63 ± 0.15[a] (59.07%)
VI	DMBA + TPA + PFE + DAS	0.50 ± 0.13[b] (67.48%)

Data are expressed as mean ± SD of five animals. '*n*' Represents number of strand breaks of duplex DNA over untreated control group.

[a] Significant prevention over DMBA and TPA-induced DNA strand breaks ($p < 0.01$).

[b] Significant prevention over PFE and DAS alone treated groups IV and V ($p < 0.01$). Values in parenthesis represents the percentage prevention offered by PFE or/and DAS against DMBA and TPA-induced DNA strand breaks.

of ERK1/2 and JNK1; however, PFE and/or DAS treatment synergistically inhibited this phosphorylation (Fig. 2). Supplementation of PFE was found more potent than DAS in decreasing the increased levels of phosphorylated ERK1/2 and JNK1 when given for both longer and shorter study durations. These findings suggest a possibility that as an initial response PFE and DAS (in combination) modulate ERK1/2/JNK1 activation which leads to inhibition of tumor growth and regression by inducing apoptotic cell death (Sarfaraz et al., 2008; Xiao et al., 2005).

Studies have shown that ERK1/2 is involved in the transcriptional activation of NF-κB (Adderley and Fitzgerald, 1999). NF-κB has emerged as one of the most promising molecular targets in the prevention of cancer. We next investigated the effect of PFE/DAS on the pattern of NF-κB activation and its nuclear translocation by DMBA and TPA in mice skin. NF-κB resides in the inactive state in the cytoplasm as a heterotrimer consisting of p50, p65 and IκBα subunits. An IκBα kinase, IKKα, phosphorylates serine residues in IκBα at position 32 and 36 (Karin and Ben-Neriah, 2000). Upon phosphorylation and subsequent degradation of IκBα, NF-κB activates and translocates to the nucleus, where it binds to DNA and activates the transcription of various genes (Garg and Aggarwal, 2002; Karin and Ben-Neriah, 2000). Several lines of evidence suggest that proteins from the NF-κB and IκB families are involved in carcinogenesis. Afaq et al. (2005) have shown that PFE treatment inhibits skin tumorigenesis in CD-1 mice by suppressing inducible and constitutive NF-κB activation. In the present study, we found that treatment of PFE and DAS to mice significantly inhibited DMBA and TPA-induced NF-κB and IKKα activation and phosphorylation and degradation of IκBα protein (Fig. 3). Other study reported previously that DAS caused down-regulation of NF-κB and apoptosis in human malignant neuroblastoma SH-SY5Y cells (Karmakar et al., 2007). Thus, this study suggests that the possible mechanism behind synergistic effect of PFE and DAS in inhibiting DMBA and TPA-induced mouse skin tumors include their capability of inhibiting NF-κB pathway.

PCNA is potentially useful molecular marker of cellular proliferation kinetics and fits to the expected biological mechanisms where it can be correlated to the decreased/increased cancer incidence (Hall et al., 1990). Enhanced expression of PCNA, a 36 kDa co-factor of DNA polymerase δ, is one of the downstream effects of the activation of MAPK/ERK1/2 signaling and well correlated to the status of cellular proliferation (Singh et al., 2002a; Singh et al., 2002b). In this study both PCNA staining and BrdU incorporation was analyzed to study the proliferative status of mouse skin following DMBA and TPA treatment. Results showed inhibition of DMBA and TPA-induced skin tumors by PFE and/or DAS was accompanied with decreased PCNA and BrdU positivity. Besides these, sections of skin tissues/tumors of DMBA and TPA treated animals displayed major epithelial proliferation in their histopathology. However, skin tissue/tumor sections of mice given PFE and/or DAS displayed reversal of DMBA and TPA-induced cellular abnormalities which again support our above findings. Altogether, inhibition of ERK1/2 and JNK1 activation, suppression of PCNA expression and BrDU incorporation was more pronounced in combination supplementation than alone, which suggests the superiority of synergistic effects.

It is well established that apoptosis has profound effect on the progression of benign to malignant phenotypes thus targeted for the prevention and/or therapy of skin cancer (Thompson, 1995; Lowe and Lin, 1990). In this study, induction of apoptosis was identified as another preventive mechanism exerted by both PFE and DAS against DMBA and TPA-induced mouse skin cancer. The results of annexin V and PI staining showed that combinatorial effect of the two, leads to enhanced apoptotic population (annexin V positive) than either of them alone. Moreover, it is also evident that PFE when given alone, after DMBA and TPA exhibited greater cell death than DAS.

DMBA/TPA is known to generate reactive oxygen species that contribute to DNA damage (Frenkel et al., 1995). Although a number of studies provide insight into the anti-DNA damaging potential of PFE and DAS when given separately (Guo et al., 2007; Nigam and Shukla, 2007), yet reports addressing their ability in combination are not available. In the present study, we noted that treatment of PFE and/or DAS inhibited the DMBA and TPA-induced DNA damage as revealed by the reduction in strand breaks, however, combination doses imparted more strand breaks reduction than alone. This might be occurring because both are known to possess reactive oxygen and other free radical species scavenging properties (Noda et al., 2002; Wu et al., 2002).

The central finding of the present study is that combination of PFE and DAS synergistically inhibited mouse skin tumor growth which was accompanied by regression of tumor volume, reduction in nick formation, decrease in proliferation markers, inhibition of MAPKs and NF-κB signaling and induction of apoptotic cell death. Therefore, we assert that the use of combination therapy in management of skin cancer prove to be more beneficial over individual agents. In conclusion, changes in dietary and/or nutritional patterns might have profound impact on reducing cancer incidence which could be achieved by replacing single one to their combinations.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Acknowledgments

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Corrigendum

Corrigendum to "Synergistic growth inhibition of mouse skin tumors by pomegranate fruit extract and diallyl sulfide: Evidence for inhibition of activated MAPKs/NF-κB and reduced cell proliferation" [Food Chem. Toxicol. 49 (2011) 1511–20]

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The authors regret to inform that there were unknowing errors in Figs. 2 and 6. The corrected images are given below. These figures are not affecting the results and conclusion of the manuscript. Hence, the text in original paper remains unchanged.

The authors would like to apologise for any inconvenience caused.

Fig. 2

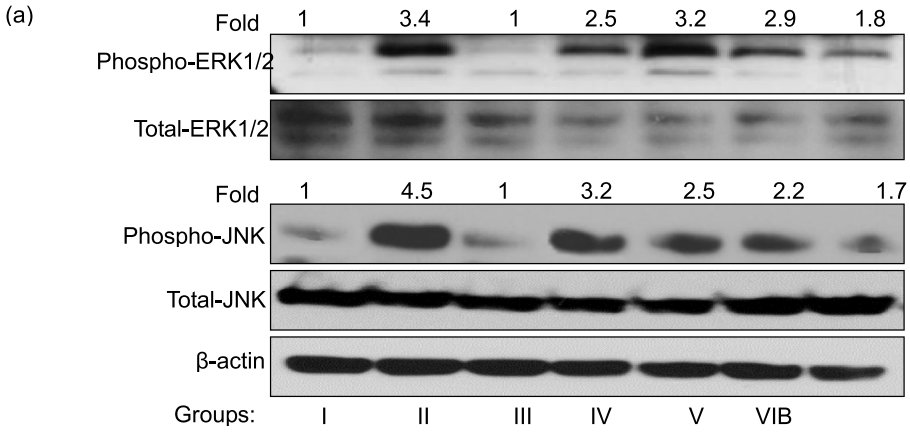

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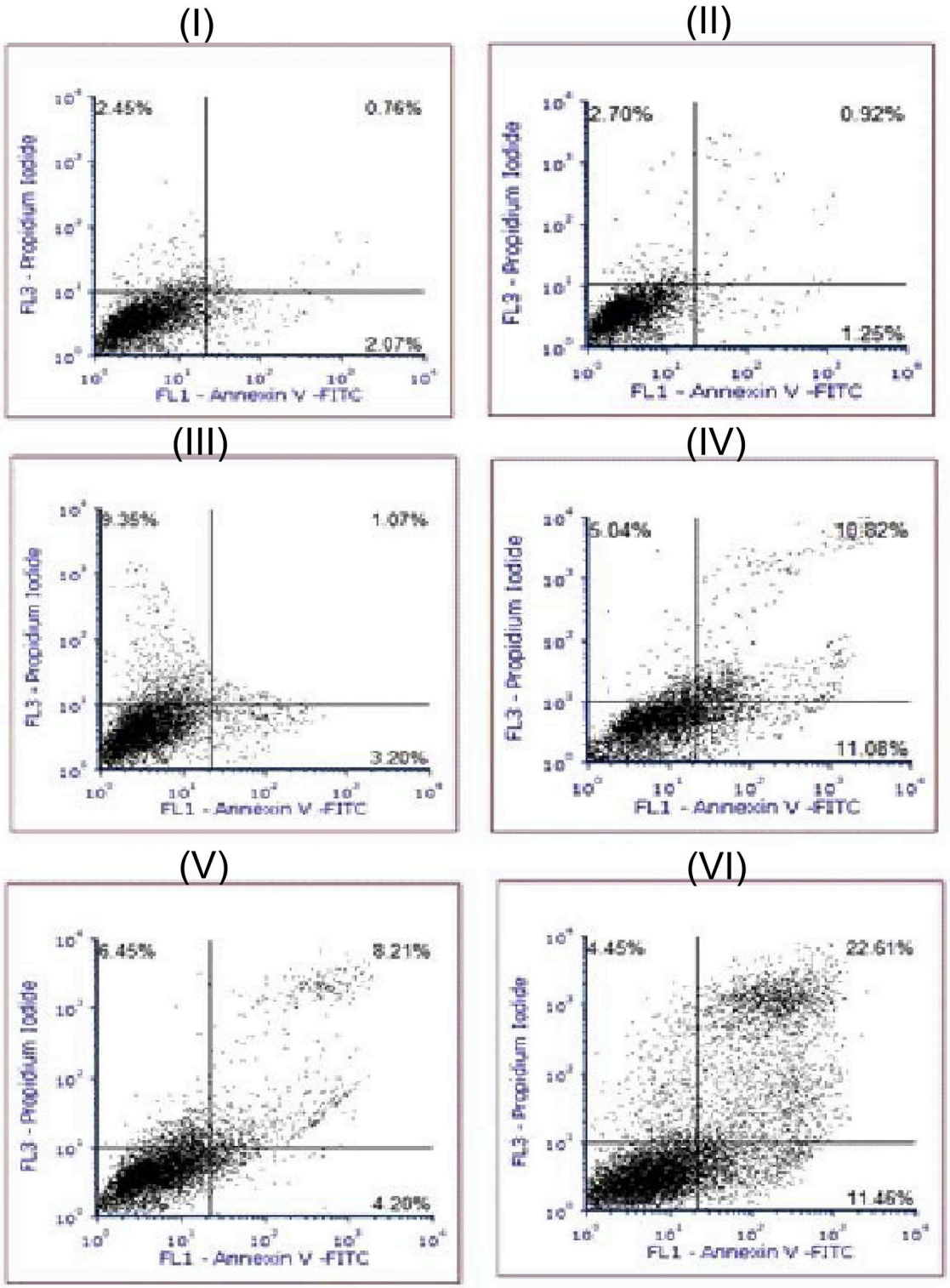
Fig. 6


(I)
2.45%
0.76%
2.07%
FL3 - Propidium Iodide
FL1 - Annexin V -FITC
(II)
2.70%
0.92%
1.25%
FL3 - Propidium Iodide
FL1 - Annexin V -FITC
(III)
1.07%
3.20%
FL3 - Propidium Iodide
FL1 - Annexin V -FITC
(IV)
5.04%
11.08%
FL3 - Propidium Iodide
FL1 - Annexin V -FITC
(V)
6.45%
8.21%
4.20%
FL3 - Propidium Iodide
FL1 - Annexin V -FITC
(VI)
4.45%
22.61%
11.46%
FL3 - Propidium Iodide
FL1 - Annexin V -FITC