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Original article

Anticancer and immunomodulatory effect of rhaponticin on Benzo(a) Pyrene-induced lung carcinogenesis and induction of apoptosis in A549 cells

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ABSTRACT

In worldwide, one of the most important cancer-related death is lung cancer. Also has the highest mortality rate between various cancer types. The count of lung cancer occurrence is increasing with an increased frequency by smoking. Proficient chemoprevention approaches are needed to prevent the occurrence of lung cancer. Therefore, the aim of this exploration is to determine the therapeutic impact on the immune modulatory effect of rhaponticin on lung tumorigenesis *in vivo* and *in vitro* cytotoxicity effect in A549 cells of human lung cancer. Lung cancer tumorigenesis in mice was challenged with benzo (a)pyrene (BaP) with 50 mg/kg bodyweight (b.wt) as oral administration for 6 weeks (two times/week). Rhaponticin were given orally 30 mg/kg b.wt (two times/week) in BaP induced mice from 12 weeks to 18 weeks. After treatment completes, the body weight was measured and then blood, lung tissue was collected for various parameters detection. The results evidenced that BaP induced mice decreased the bodyweight, increased lung weight, increased tumor markers (AHH, CEA and LDH), and increased the proinflammatory cytokines. The enzyme catalase, superoxide dismutase activity was decreased and increased lipid peroxidation in immune comprising cells compared with the control cells. Moreover, rhaponticin treatment improves in chemical assays and also the histopathological alteration of lung tissues. The present findings provide evidence about the therapeutic potentials of rhaponticin against BaP triggered lung tumorigenesis.

1. Introduction

Lung cancer is one of the predominant cancers that responsible for highest death rate worldwide. It was reported that 228,820 new incidences of lung cancer were identified in both sexes along with 135,720 deaths in 2020.(Siegel et al., 2020; Bray et al., 2018) About 85% of lung cancer from non-small cell lung cancer (NSCLC) and the common histotype is adenocarcinomas (ADCs).(Awasthi et al., 2018) In global settings, lung cancer death is increasing due to cancer bearing causes includes westernized diet, loss of physical activity and smoking.(Barta et al., 2019) The common therapy for cancer includes chemotherapy, surgery and radiotherapy. The patient's response to the chemotherapy is highly sensitive but after some period of time patients develop resistance even with combination therapeutic strategy. Although the available approaches for cancer treatment are not successful.(Chen et al., 2020) In fact, the development of resistance to the chemotherapy and metastasis are leads to failure of treatment approaches for cancers. Therefore, improved therapeutic procedures either by early diagnosis or target cancer with available or new drugs with fewer side effects are urgently needed to augment cancer therapy. Tobacco smoking is playing a significant factor with higher incidence for lung cancer (McCarthy et al., 2012); it is due to poly cyclic aromatic hydrocarbons (PAHs) present in the tobacco smoke.(Moorthy et al., 2015) The carcinogenic nature of PAHs is called as Benzo(a)pyrene (BaP) present in cigarettes and automobile exhausts.(Alexandrov et al., 2010) The initiating factor for carcinogenesis is the DNA

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adduct formation.(Zuo et al., 2014) The PAH prototype BaP possesses a strong carcinogenic potential and International Agency for Research on Cancer (IARC) classified the BaP as a carcinogen to humans. Though, carcinogenicity of BaP involves assorted mechanisms those are not fully studied yet. Following its bioactivation through cytochromes P450, BaP is a genotoxic, so it could lead to gene mutations.(Hernandez-Boussard and Hainaut, 1998) BaP could aggravate metastasis through the elevation of cell migration or via serving to the extracellular matrix expressions. (Ba, 2015)

Rhaponticin is a glucoside (aglycone) ingredient is isolated from a variety of herbs including *Rheum undulatum L* that is generally distributed in Asia.(Kim and Ma, 2018) Rhaponticin is a stilbenoid component present in the diet and also called as rhapontigenin that are produced from many plant species with potential pharmacological agents in the aerial and roots.(Kolodziejczyk-Czepas and Czepas, 2019) In traditional way is used for laxative, anti-inflammatory, purgative, anti-blood stasis and also used for dental diseases treatment. The chemicals present in the *Rheum undulatum* L includes many polyphenols mainly resveretrol, rhapontigenin and rhaponticin,(Ngoc et al., 2008) reported that hold antioxidant,(Matsuda et al., 2001) anti-allergic,(Matsuda et al., 2004) and anti-diabetic(Choi et al., 2005) activities. However, the anticancer activity of rhaponticin against the lung carcinogenesis was not scientifically proved yet. Therefore, in this current study we planned to explore the anticancer potential of rhaponticin against the BaP-provoked lung carcinogenesis and *in vitro* anticancer activity against the A549 cell line.

2. Materials and methods

2.1. Chemicals

Rhaponticin, and benzo(a)pyrene, were procured from Sigma (USA). All other analytical grade chemicals were attained from Sigma-Aldrich, USA. All the assay kits were obtained from Cell Signaling Technology, Danvers, MA, USA.

2.2. In vitro experiments

2.2.1. Cell line and culture

Lung carcinoma cell lines from human origin (A549) were cultivated in DMEM medium with 10% Fetal bovine serum and 1% of antibiotic/antimycotic solution (Gibco). The cells were grown in a standard CO_2 incubator condition for cell growth (37 °C, 5% CO_2, 95% humidity). After the confluence of the cells was separated by using 0.5% Trypsin EDTA (Gibco) and plated.

2.2.2. Cell viability by MTT assay

Rhaponticin mediated cell viability of A549 cancer cells was determined by MTT assay with standard cell culture assay protocol. The stock solution of Rhaponticin was diluted with 100% dimethyl sulfoxide (DMSO). Then final working concentration was prepared with<0.1% DMSO. The maximum work concentration of DMSO was used as the control (vehicle) for the present study. After cell confluence, cells were trypsinized and plated $5x10^3$ cells in 96 well cell culture plate, and sustained in a CO_2 incubator with standard condition for overnight at 37 °C. After the incubation period, fresh medium was replaced with rhaponticin 5, 10, 15, 20, 25, 50, 75 and 100 μM concentrations and incubated for 24 h. MTT (0.5 mg/ml) was added after 24 h and kept 2 h in CO_2 incubator. The MTT containing solution was removed and added DMSO to suspend the formed formazan crystals. The color development was read in a plate reader at 550 nm. The viability percentage of drug treatment was determined by three individual experiments and the calculated with untreated control (Abs T/Abs control × 100).

2.3. Reactive oxygen species detection

ROS detection in intracellular levels was examined by a non-fluorescent chemical 2′,7′-Dichlorofluorescin diacetate (DCF-DA). This lipophilic ester (non-fluorescent) can easily go through the plasma membrane into the cytosol and form DCF with ROS to generate green fluorescence that is proportional to the ROS. In brief, A549 cells ($5 \times l0^3$ cells/well) were added in a 96 well plate for overnight incubation. Rhaponticin 25 and 50 μM concentration was treated for 24 h. Later then 24 h, cells were cleansed with PBS and incubated DCF-DA in a medium at CO_2 incubator for 30 min and then washed with PBS. The ROS was detected by fluorescence excitation 485 nm and an emission of 525 nm wavelength. The data from the triplicate assays were calculated with % relative to control.

2.4. AO/EB staining

To find out the morphological changes for apoptosis after drug treatment performed (AO/EB) staining. A549 cells were supplemented with rhaponticin (25 and 50 μM) for 24 h. After the experiment completes at 24 h, cells were first fixed with methanol and glacial acetic acid (3:1 ratio) for 1 h and kept at room temperature. After that, AO and EB (1:1 ratio in PBS) solution were added and incubated for 5 min and washed with PBS. The stained cell images were taken by a fluorescence microscope.

2.5. The activity of caspases by ELISA

The human caspase 3 and caspase 9 activity was performed by a commercially available kit. This assay is performed with ELISA to detect and quantify the status of caspases. Rhaponticin (25 and 50 μM) treated for 24 h in A549 cells, and after 24 h extracted the cell lysates and used for caspase 3 and caspase 9 activity as per standard protocol available in the kit (Cell Signaling Technology, Danvers, MA, USA).

3. *In vivo* experiments

3.1. Animals

Healthy male Swiss albino mice weighing 20–25 g (7–8 weeks aged) were maintained and housed in standard conditions (Temperature 25° ± 2 °C), light (12 hr light/12 hr dark), and humidity with standard diet and drinking water. This animal research work was approved by the Institutional Animal Ethics Committee (IAECNO: 201904), Tangdu Hospital, Xi'an, China.

3.2. Experimental design

In four groups, each group consists of 6 animals. Group I: vehicle control is given corn oil. Group II: Animals were challenged with BaP (50 mg/kg b.wt) in corn oil through oral gavage for six week two times per day. Group III: animals received rhaponticin dissolved in corn oil (30 mg/kg b.wt) after BaP induction and started from twelfth week to end of the experiment. Group IV: animals were supplemented with rhaponticin alone from 12th week to 18 weeks of experiment (Fig. 1). After completion of the experiment, the mice were sacrificed by decapitation. The body weight, lung weight, and liver weight was measured and anticoagulant added blood was collected to count immunocompetent cells. Coagulated blood was also collected and used for immunoglobulins

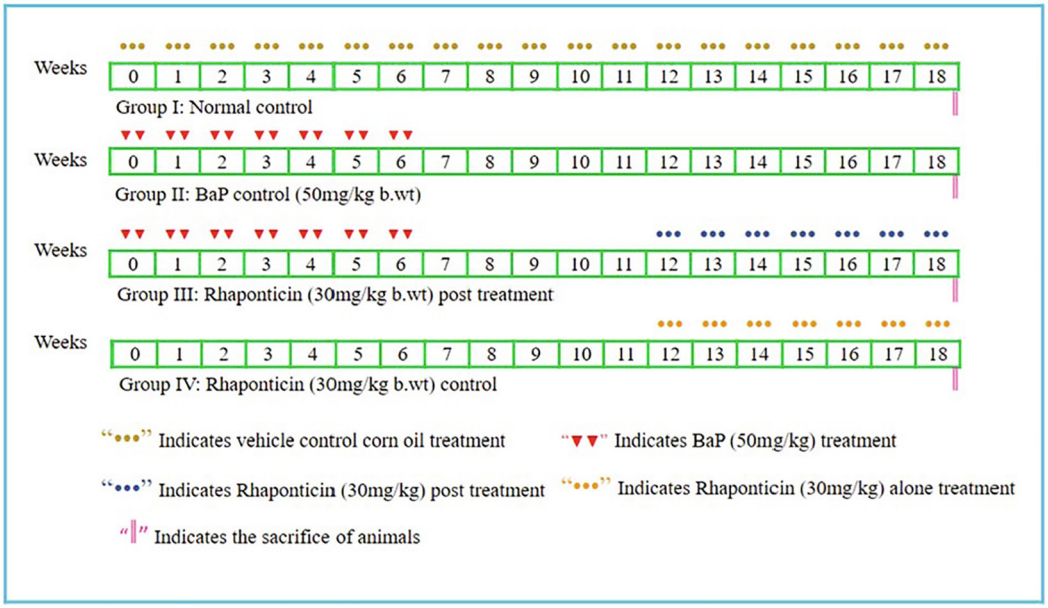

Fig. 1. Graphical representation of experimental design.

(IgG, IgA, and IgM) and cytokine levels estimation. The lungs were removed quickly and rinsed with saline (ice cold) solution, dry in a filter paper and weighed. One portion of the lung was homogenized with 0.1 M potassium phosphate buffer (pH 7.4) and then centrifuged for 10 min at 3000 rpm. Supernatant was used for biochemical analysis and/or stored at −80 °C until use. Another one part of tissue was fixed with 10% formalin for making tissue section for histology.

4. Biochemical analysis

4.1. Xenobiotic and marker enzymes in liver

The xenobiotic and liver function marker enzymes were analyzed in liver tissues. LDH, gamma-glutamyltransferase (γGT), aryl hydrocarbon hydroxylase (AHH) and 5′nucleotidase were assayed by ELISA kits. The assay used with the manufacturer's instructions.

4.2. Immunological oxidative stress in cells

Blood cells (Lymphocytes and neutrophils) were isolated by density-gradient centrifugation (Ficoll based method). Briefly, the collected blood centrifuged (500 × g) for 40 min to get buffy coat, and then it was separated in density-gradient centrifugation (500 × g) by using Ficol paque for 45 min. Lymphocytes present in the interface and neutrophils include the pellets was separated, mixed with PBS and washed. The peritoneal lavage cells were taken and wash by using PBS, then mixed with medium (RPMI-1640), the macrophages were isolated by slight modification of discontinuous percoll density-gradient centrifugation.(Rajendran et al., 2013; Vray and Plasman, 1994) The isosmotic Percoll was prepared with 0.9% saline solution with different specific gravities (1.030, 1.040, 1.050, and 1.070 g/ml). 1×10^6 peritoneal lavage cells were mixed in 1 ml of RPMI-1640 medium and placed in top of percoll (5 ml) prepared with different densities (four) and centrifuged for 20 min at 400 × g in 20 °C. The isolated macrophages viability was observed more than 95% by trypan blue cell counting method. Macrophage cells (5×10^6) were mixed with PBS and washed. Then suspended with 1 ml PBS and sonicated twice (20 s) at 80 W. After that, sonicated cells were centrifuged for 10 min (10000 × g) at 4 °C. The formed supernatants were used for enzyme assays such as SOD,(Madesh and Balasubramanian, 1998) catalase(Chance, 1954) and lipid peroxidation.(Ohkawa et al., 1979) The protein was analyzed by Lowry method.(Lowry et al., 1951)

4.3. Estimation of enzymatic and non-enzymatic antioxidants in the lung tissues of BaP induced animals

The lung tissues were excised from both control and experimental animals homogenized with buffered saline. Then it was centrifuged at 10,000 rpm for 5 min and the supernatant was utilized for the estimation of antioxidants status. The status of superoxide dismutase (SOD) was studied via Kakkar et al. (Kakkar et al., 1984)(Kakkar et al., 1984) technique. The catalase (CAT) enzyme status was studied through the Sinha, (Sinha, 1972)(Sinha, 1972) technique. The status of glutathione peroxidase (GPx) was inspected via Rotruck et al. (Rotruck et al., 1973)(Rotruck et al., 1973) technique. The glutathione (GSH) status was studied by using the method of Ellman, (Ellman, 1959).(Ellman, 1959) The level of vitamin-C was inspected via the technique of Roe and Kuether, (Roe and Kuether, 1943).(Roe and Kuether, 1943)

4.4. Elisa

Concentration of immunoglobulin, cytokines, enzymes and CEA in serum and cell lysates was estimated by commercially available ELISA kits. The plate absorbance was read with respective wavelength of the assay in a microplate reader.

4.5. Histopathological analysis

The experimental animal's lungs were fixed with formalin and processed for making block with paraffin. The 5 μm thickness of paraffin sections were hematoxylin and eosin stained. Slides were monitored and photographed in a microscope (10X).

4.6. Statistical analysis

Data were analyzed by one-way ANOVA successively significance analyzed by Tukey's post hos test by using SPSS software

version 19. Data were portrayed as mean ± S.D of six mice. $p < 0.05$, and $p < 0.01$ was considered for significant.

5. Results

5.1. Cytotoxicity and AO/EB staining for apoptosis

Cell viability of A549 cancer cells decreased significantly ($p < 0.05$) after rhaponticin (5, 10, 15, 20, 25, 50, 75 and 100 μM) dose dependently treated for 24 h (Fig. 2A). IC50 of rhaponticin in A549 cancer cell was found that 25 μM.

The apoptotic detection in cells was eminent from fluorescence microscopy. The apoptotic cells usually emitted the fluorescence as orange color, whereas red color was emitted from necrotic cells and control live cells emitted green. Rhaponticin (25 and 50 μM) treated A549 cells were emitted more orange color compared than control represents the defined morphological modification that leads to apoptosis (Fig. 2B).

5.2. Effect of rhaponticin on ROS in A549 cells

ROS as an expected product in the cell metabolism, which can act as an anti-tumor by, encourage a range of pathways that regulates the cell death signaling mechanism, including apoptosis, necrosis and autophagy. Increased ROS can trigger changes in cell cycle-associated proteins. DCF-DA probe hydrolyzed by esterase and in membrane of the cells produce DCFH, and inside cells, ROS convert DCFH (non-fluorescent) into a fluorescent DCF. The present study, rhaponticin (25 and 50 μM) treatment remarkably ($p < 0.05$) augmented the DCF fluorescence in A549 cells after 24 h (Fig. 3A) that confirms rhaponticin may contribute for apoptosis related death of lung cancer through ROS generation.

5.3. Activity of caspases

Apoptosis or programmed cell death that is corresponding by member of the cysteine proteases such as caspases. There are series numbers of caspases involved during apoptosis. Caspase-3 plays a major role in executing the cell apoptosis. Apoptosis involves two major pathway, such as intrinsic (caspase 9) and extrinsic (caspase 8) and finally effect the activation of caspase 3 for destruction. In this study rhaponticin significantly ($p < 0.05$) increases the caspase 9 and caspase 3 activity at 25 and 50 μM concentration after 24 h in A549 cells (Fig. 3B). Therefore, the present study confirms that caspase 3 activation leads to apoptosis induction.

5.4. Effect of rhaponticin on body, lung, liver weight, and cancer incidence in experimental animals

The BaP (Group II) alone treatment was significantly decreased the body weight and liver weight ($p < 0.05$), increased lung weight compared than group I and tumor incidence occurs in all the 6 mice. But rhaponticin post treated mice (Group III) significantly ($p < 0.05$) retrieved the BaP induced effect on body, lung, and liver weight and also the cancer incidence (table 1). The drug alone treatment group found that it was similar to the control group.

5.5. Effect of rhaponticin on immunocompetent cells

In BaP induced (Group II) group animals exhibited reduction of leucocytes, lymphocytes, neutrophils, absolute lymphocyte count, and absolute neutrophil count compared than Control (Group I) animals. Post treatment with rhaponticin in BaP induced experimental animals (Group III) were showed significant ($p < 0.05$) retrieval of all the cells compared than BaP alone induced animals (Group II). Rhaponticin alone treatment (Group IV) animals showed no significant differences than control animals (Fig. 4). The rhaponticin effect on immune complexes such as phagocyte index, avidity complex, serum immune complexes (SIC) and nitroblue tetrazolium test (NBT) reduction in animals was depicted in Fig. 5. The immune complexes were decreased significantly in BaP induced group compared than group I.

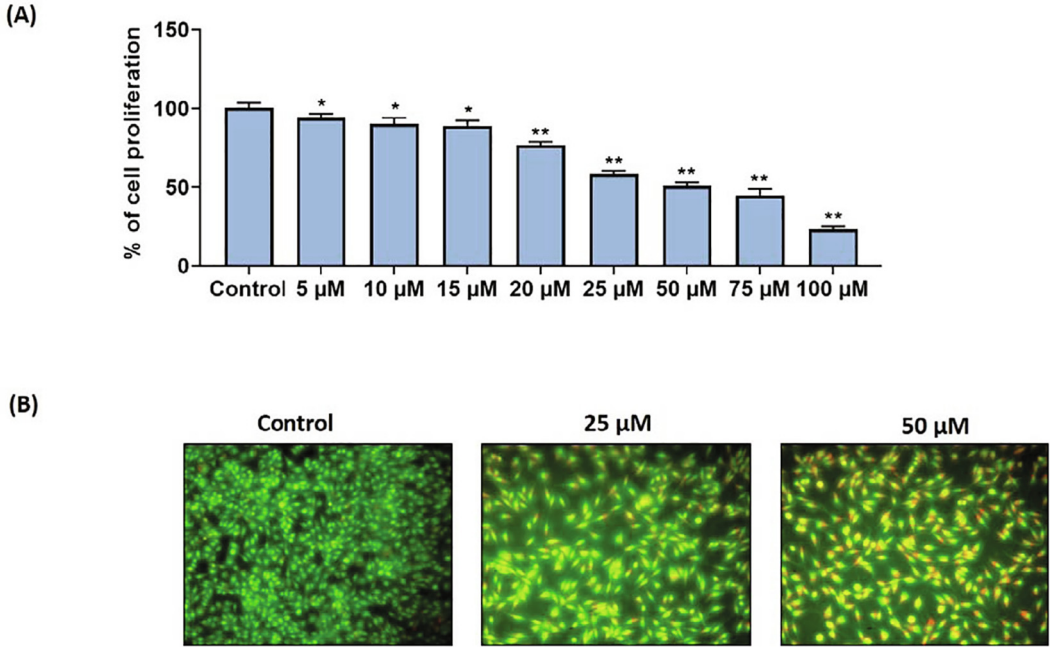

Fig. 2. Effect of Rhaponticin on cell viability and AO/EB staining after 24 h on A549 cells. (A) Cell viability by MTT assay. A549 cells were exposed with rhaponticin for 24 h and the absorbance was measured at microplate reader. Data depicted as mean ± SD. *denotes significance at $p < 0.05$; ** indicate significance at $p < 0.001$ compared with control. (B) AO/EB staining demonstrates morphological alterations after rhaponticin 25 and 50 μM supplementation.

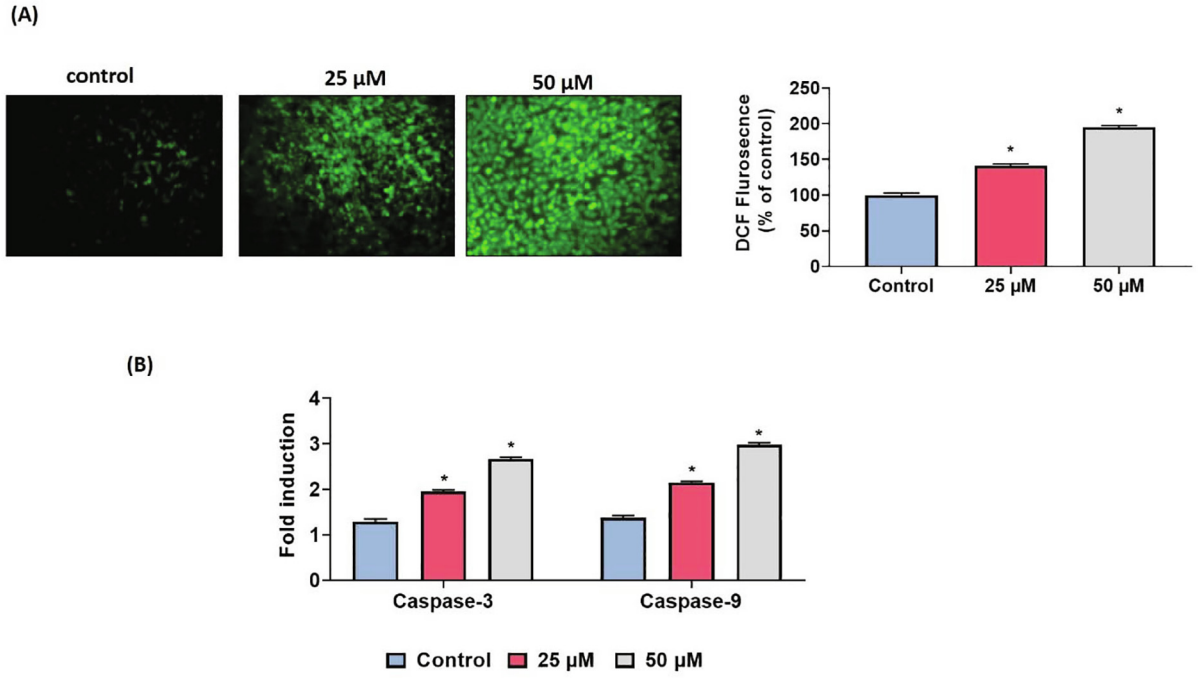

Fig. 3. Effect of rhaponticin on ROS analysis and caspases activity by ELISA after 24 h on A549 cells. (A) The ROS production examined after DCF-DA. A549 cells were exposed with rhaponticin for 24 h and then examined under the fluorescence microscope. (B) Apoptosis was detected using a caspase-3 and caspase-9 activity assay. Data correspond to as mean ± SD. * denotes significance at p < 0.05; ** indicate significance at p < 0.001 compared with control. * indicates significance at p < 0.05 compared to control.

Table 1
Effect of Rhaponticin on bodyweight, lung weight, liver weight, and tumor incidence in BaP induced animals.

Parameters	Bodyweight (g)	Lung weight (mg)	Liver weight (g)	Tumor incidence (%)
Group I	28.95 ± 8.96	243.66 ± 68.63	2.18 ± 0.69	0
Group II	23.99 ± 6.12*	382.33 ± 91.52*	1.24 ± 0.12*	100*
Group III	27.42 ± 7.99#	306 ± 82.74#	1.89 ± 0.34#	50#
Group IV	29.52 ± 11.47#	246.66 ± 70.08#	2.12 ± 0.57#	0

Data were displayed as meanS.D of six mice. Data not sharing a common superscript letter differ significantly at $p < 0.05$. Note: Group I: Control; Group II: BaP-induced animals; Group III: BaP-challenged animals treated with 30 mg/kg of rhaponticin; Group IV: Animals administered with 30 mg/kg of rhaponticin alone.

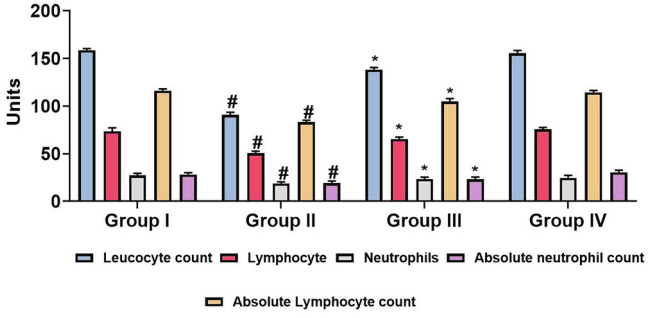

Fig. 4. Effect of Rhaponticin on the hematological counts in BaP induced experimental animals. The unit is presented as mean ± S.D of triplicates. Statistical significance considered as p < 0.05. # indicates compared with group I; * indicates p < 0.05 compared with group II. Note: Group I: Control; Group II: BaP-induced lung cancer bearing animals; Group III: BaP-challenged animals treated with 30 mg/kg of rhaponticin; Group IV: Animals administered with 30 mg/kg of rhaponticin alone.

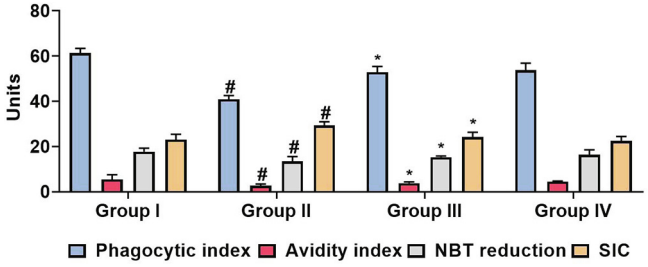

Fig. 5. Effect of Rhaponticin on the phagocyte index, avidity index, NBT reduction, and SIC in BaP induced experimental animals. Data expressed as mean ± S.D of triplicates. Significance considered as P < 0.05. #: compared with group I; *: as compared with group II; #: as compared with group II. Note: Group I: Control; Group II: BaP-induced animals; Group III: BaP-challenged animals treated with 30 mg/kg of rhaponticin; Group IV: Animals administered with 30 mg/kg of rhaponticin alone.

Post-initiation of rhaponticin (Group III) in BaP induced animals showed increment of the immune complex levels compared than BaP (Group II) induced animals. In animals, rhaponticin alone treatment (Group IV) has no variations compared than control animals (Group I).

5.6. Effect of rhaponticin on immunoglobulin levels

The levels of IgG, IgA and IgM measured in serum. BaP induced experimental animals showed significant decreased IgG, IgM and increased IgA levels (Group II). Rhaponticin post treatment in BaP induced animals showed significant ($p < 0.05$) retrieval of IgG, IgM and IgA levels compared than control animals (Fig. 6).

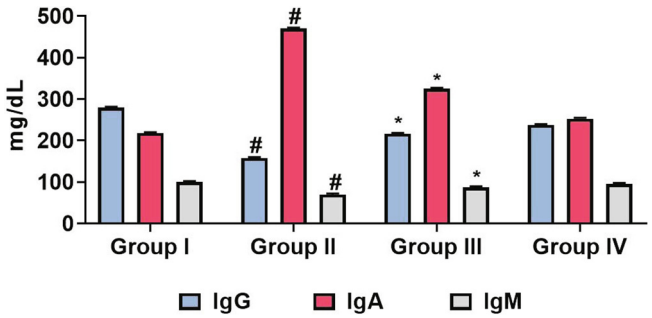

Fig. 6. Effect of Rhaponticin on immunoglobulin levels in the serum of BaP induced experimental animals. Data expressed as mean ± SD of triplicates. Statistical significance considered as $p < 0.05$. #: as compared with group I; *: as compared with group II. Note: Group I: Control; Group II: BaP-induced animals; Group III: BaP-challenged and 30 mg/kg of rhaponticin treated animals; Group IV: Animals administered with 30 mg/kg of rhaponticin alone.

5.7. Effect of rhaponticin on immunological oxidative stress

The BaP (Group II) treated mice show evidence of oxidative stress (immunological) as increase of lipid peroxidation (malondialdehyde level) in cells of lymphocytes, macrophages and neutrophils compared than group I mice. This is due to decreases of levels of catalase and superoxide dismutase enzyme activities (Fig. 7). Rhaponticin post treatment (Group III) animals significantly ($p < 0.05$) ameliorated the BaP provoked oxidative stress compared than BaP (Group II) induced animals. The decreased lipid peroxidation (malonaldehyde level) in terms increase of enzymes catalase and SOD activities in lymphocytes, macrophages and PMN.

5.8. Effect of rhaponticin on antioxidants status in the lung tissues of BaP-challenged mice

The BaP-challenged mice (Group II) reveal that the reduced statuses of antioxidants like CAT, SOD, GPx, GSH, and vitamin-C in the lung tissues (Fig. 8). Rhaponticin (30 mg/kg) post supplemented animals (Group III) displayed the notable ($p < 0.05$) improvement in the antioxidants status of BaP provoked (Group II) animals. The augmented levels of antioxidants were noted in the rhaponticin administered animals.

5.9. Effect of rhaponticin on xenobiotic and liver function marker enzymes

The levels of xenobiotic and liver function marker enzymes determined in serum of experimental animals. BaP induced experimental animals showed significant decrease of aryl hydrocarbon hydroxylase (AHH), lactate dehydrogenase (LDH), gamma-glutamyl transferase (γGT) and 5′ Nucleotidase activity (Group II). Rhaponticin post treatment in BaP induced animal's shows significant ($p < 0.05$) retrieval of AHH, LDH, γGT and 5′ Nucleotidase activity compared than control (Group I) animals (Fig. 9).

5.10. Effect of rhaponticin on CEA and cytokines

Fig. 10A represents the tumor marker CEA levels in the serum of rhaponticin effect on BaP induced experimental animals. CEA has significant increase in BaP-induced animals compared than with control animals. Whereas, rhaponticin post treatment (Group III) animal showed significant ($p < 0.05$) retrieval of CEA. In Fig. 10B represents the cytokines levels of TNF-alpha, IL6, and IL1β in the serum of rhaponticin effect on BaP induced experimental animal. All the cytokines were increase in BaP induced animals compared

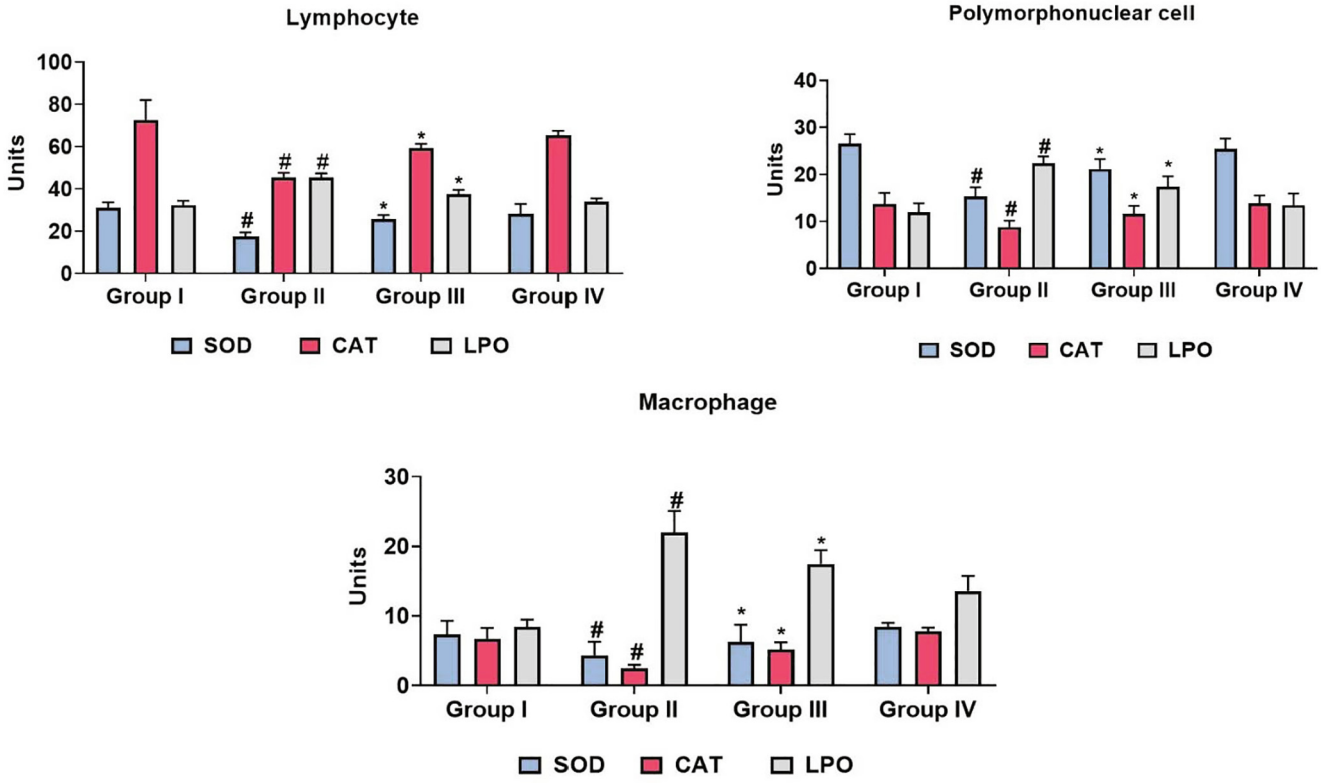

Fig. 7. Effect of Rhaponticin on the hematological counts in BaP induced experimental animals. The unit is presented as mean ± SD of triplicates. Statistical significance considered as $P < 0.05$. #: as compared with group I; *: as compared with group II. Note: Group I: Control; Group II: BaP-induced animals; Group III: BaP-challenged animals treated with 30 mg/kg of rhaponticin; Group IV: Animals administered with 30 mg/kg of rhaponticin alone.

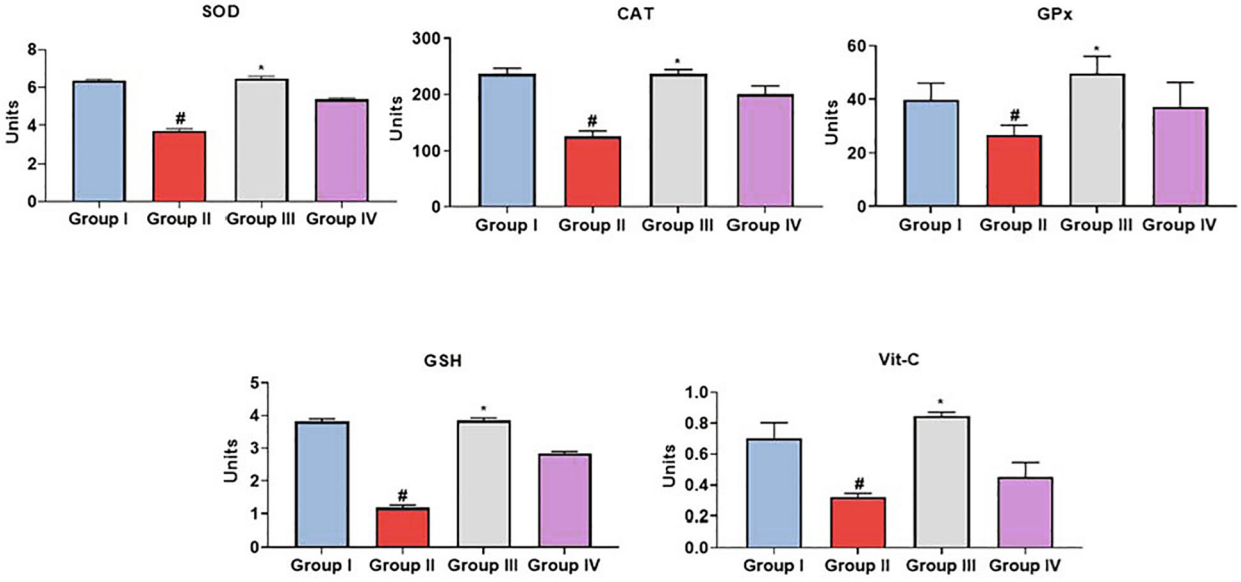

Fig. 8. Effect of Rhaponticin on the antioxidants in lung tissues of BaP induced experimental animals. The unit is presented as mean ± SD of triplicates. Significance considered as P < 0.05. #: as compared with group I; *: as compared with group II. Note: Group I: Control; Group II: BaP-induced lung cancer bearing animals; Group III: BaP-challenged animals treated with 30 mg/kg of rhaponticin; Group IV: Animals administered with 30 mg/kg of rhaponticin alone.

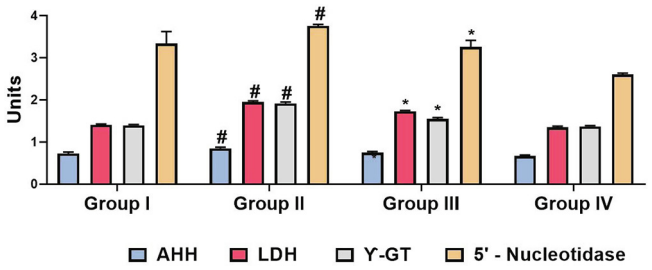

Fig. 9. Effect of Rhaponticin on the activities of xenobiotic and liver function marker enzymes in the liver of BaP induced animals. The unit is presented as the mean ± SD of triplicates. Significance considered as P < 0.05. *: as compared with group I; #: as compared with group II. Group I control, group II: BaP treated, Group III Rhaponticin Post initiation, Group IV: Rhaponticin alone. Note: Group I: Control; Group II: BaP-induced animals; Group III: BaP-challenged animals treated with 30 mg/kg of rhaponticin; Group IV: Animals administered with 30 mg/kg of rhaponticin alone.

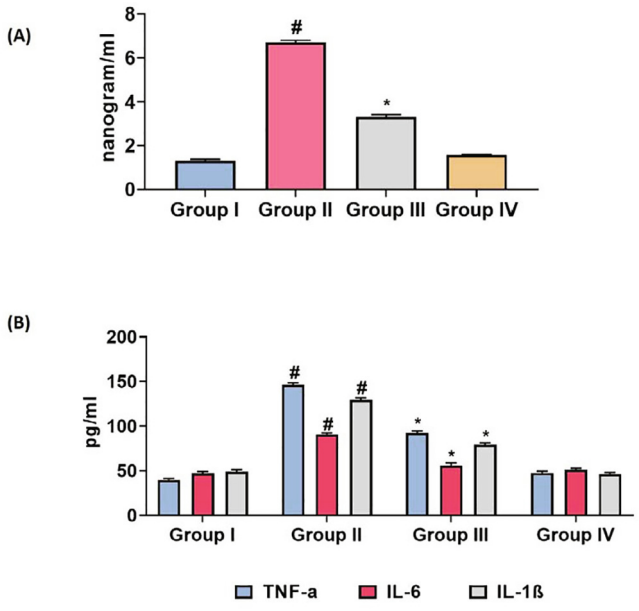

Fig. 10. Effect of Rhaponticin on CEA and pro-inflammatory cytokines in serum of BaP induced experimental animals. (A) Serum levels of CEA. (B) Serum levels of pro-inflammatory cytokines. Each value is expressed as mean ± SD. Significance considered as p < 0.05. #: as compared with group I; *: as compared with group II. Note: Group I: Control; Group II: BaP-induced animals; Group III: BaP-challenged animals treated with 30 mg/kg of rhaponticin; Group IV: Animals administered with 30 mg/kg of rhaponticin alone.

than with control animals but rhaponticin post treatment (Group III) animal showed that retrieval of cytokines levels.

5.11. Histological changes in lung

Histological evaluation of the lung from control (vehicle) and group IV (drug alone) treated animals show normal histomorphology with small nuclei. The lung tissues of BaP-challenged animals (Group II) possessed proliferative lesions with focal bronchial and alveolar epithelial hyperplasia. BaP-challenged animals also displayed the diminution of tissue architecture with distorted alveoli as seen from augmented amount of hyperchromatic nuclei in the cells of alveolar wall with extensive proliferation of alveolar epithelium. The animals supplemented with rhaponticin (Group III) demonstrated diminished alveolar injury with near normal arrangements (Fig. 11).

6. Discussion

Natural components have been extensively experiment as chemopreventive effect with less or no side effects. Studies investigated that bioactive component from natural products for managing cancer is also one of the established strategy. (Arumugam et al., 2019; Sharma et al., 2012) In our study rhaponticin treatment in A549 lung cancer cells has cytotoxic effects for 24 at various concentrations (5, 10, 15,20,25, 50, 75, 100 μM) compared than control are shown in Fig. 1A. Apoptosis is called as programmed cell death mechanism for maintaining a stable internal environment in multicellular organisms. Apoptosis can be turn on by the modification of membrane unity, cell clumping and

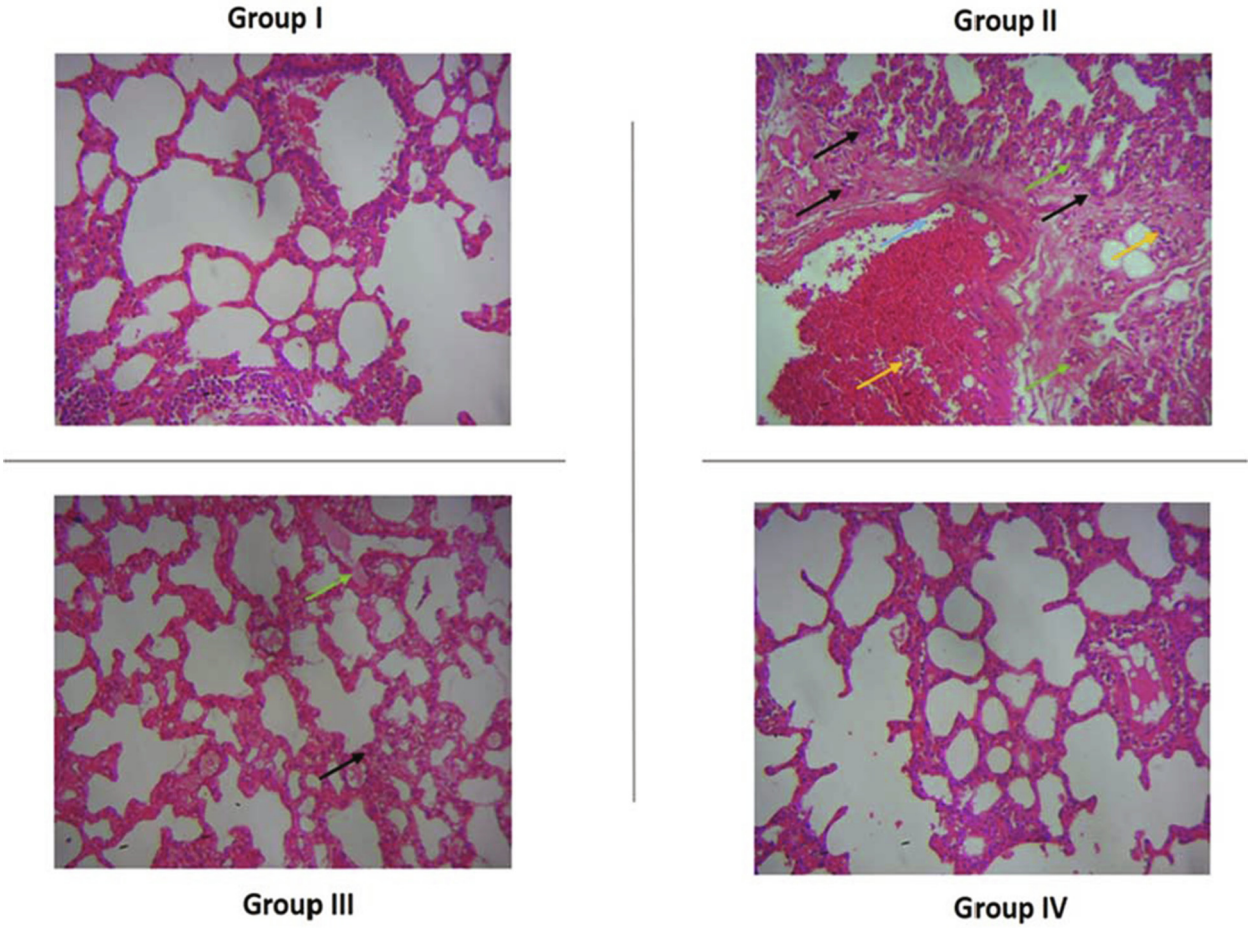

Fig. 11. Effect of Rhaponticin on histopathological changes of lung on BaP induced animals. (Group I) Normal morphology of control (Group II) BaP challenged animals displaying alveolar damage and irregular nuclei with hyper chromatic cells. (Group III) Rhaponticin post supplemented animals showing decreased alveolar injury and decreased hyperchromatic cells. (Group IV) Rhaponticin alone treated animal's shows normal morphology like that of control animals. The images were taken photograph at 10 × magnification by camera microscope.

cytoplasmic contraction suppression of cell development.(Koff et al., 2015) Inducing apoptosis has a known methodology for tumor therapy to evaluate the anticancer drug efficacy.(Renault and Chipuk, 2014; Pistritto et al., 2016) Caspases family is involved in the apoptosis mechanism of action. The apoptosis pathway begins with caspase initiators (2, 8, 9, and 10) and complete with caspase executors (3, 6 and 7) for the cleavage of cellular components.(Thomberry and Laxebnik, 1998; Pant et al., 2016) ROS mediated apoptosis can cause by collapse in membrane potential and thereby inhibit cell cycle. Once, ROS entered in to cytoplasm and again enter in to the nucleus that leads damage of DNA. In addition, stimulate internal apoptotic pathway, and eventually mediates cell death.(Yang et al., 2010; Rajendran et al., 2008) The morphological changes of apoptosis by AO/EB staining shows more orange fluorescence after 25 and 50 μM concentration of rhaponticin (Fig. 2B) that confirm the indication of apoptosis. Also, ROS (DCF fluorescence) content (Fig. 3A) was increased after 24 h of rhaponticin treatment that was due to apoptosis on A549 cells through ROS. Also we confirmed that rhaponticin significantly augmented the activity of initiator caspase 9 and executor caspase-3 (Fig. 3B). Therefore, this *in vitro* study confirms that rhaponticin induces cytototoxicity and apoptosis of lung cancer cells.

Further we performed the *in vivo* efficiency of rhaponticin on BaP induced experimental animal model. We found that the decrease in body weight of BaP induced lung cancer animals showed in consist with previous studies and increased the lung weight.(Velli et al., 2019; Malmgren et al., 1952) Rhaponticin post-treated in BaP induced animals showed that body weight was increased and decreased lung weight indicated its defensive efficacy, which can be ascribed by inhibition of rhaponticin on proliferation, inflammation, and tumor growth. In PAH family, BaP is an established carcinogen, and the PAH-induced humoral immunosuppression was identified as a wealth of encouraging finding about immune modulation.(Herr et al., 2011) BaP induced experimental animals (Group II) were decreased in lymphocyte, neutrophil, total leukocyte and absolute lymphocyte, neutrophil counts (Fig. 4) that possibility of decreased ATP contents in cancerous animal. The immune cells turn on the supply of adequate cellular energy and reduced glycemic condition. In the rhaponticin post-treated animals in BaP induced animal increased the cell counts of lymphocyte, neutrophil, total leukocyte and absolute lymphocyte, neutrophil counts. The neutrophils killing capability was noted with reduction of NBT, and phagocytic skill of the neutrophils, as designated by the phagocytic, avidity index and SICs were decreased significantly in the BaP induced animals compared than control animals. The availability of excess antibodies or antigen, SIC, give as a sign of immune response. This reduction of antibodies was due to the decrease of antibody generation in during cancer condition. Post-treatment of rhaponticin were ameliorate the immune responses (Fig. 5).

The two major immunoglobulins are IgG and IgM and involved in humoral immune responses such as neutralization of toxins,

opsonization and complement activation.(Chandy et al., 1983) IgA levels are elevated during failure in the clearance mechanism of damaged liver.(Eräsalo et al., 2018) BaP treatment animals caused changes in immune responses; therefore the immunoglobulins (IgG and IgM) were decreased and increased immunoglobulin IgA levels observed compared than control animals. In the rhaponticin-treated animals given BaP showed increased IgG and IgM, whereas decreased IgA levels (Fig. 6) confirm that ameliorative effect of rhaponticin. In addition, rhaponticin effect through the regulation of antioxidant capacity was performed in lymphocytes, polymorphonuclear cells and macrophages. BaP treated animals SOD, and catalase was significantly decreased and increased lipid peroxidation in lymphocytes, PMN cells and macrophages (Fig. 7). Study speculated that many naturally occurring stilbenoids (pinosylvin, monomethylpinosylvin, piceatannol, resveratrol and rhapontigenin) have effective inhibition on pro-inflammatory reaction of the macrophages.(Sadeck et al., 2014) The immunoprotective role of rhaponticin action on BaP induced animal involved in the stimulation of SOD and CAT; in this manner improve the damage caused by lipid peroxidation of lymphocytes, macrophages and PMN. The rhaponticin treatment also improved the antioxidants status in the lung tissues of BaP-induced mice (Fig. 8).

AHH is a cytochrome *P*-450 dependent (cytochrome P4501A1) carcinogen-metabolizing enzyme, important tool for the lung cancer diagnosis and used with a marker enzymes. The AHH activity and expressions are induced through the receptor (AhR) activation by PAHs.(Rao and Kumar, 2015; Amir et al., 2007) In serum and tissues AHH activity was increased in the of BaP exposed animals.(Miao et al., 2013) In this study, also AHH activity increased in BaP induced group and rhaponticin treatment decrease in AHH which results may be decreased the BaP metabolism. LDH is a prognostic biomarker and increased LDH activity in serum was observed in numerous malignant tumor types.(Asokkumar et al., 2012) Also have an important role in glycolysis pathway for energy production used for accelerated tumor growth (Xie et al., 2014) and vital for cancer cell survival and growth.(Ngo and Nutter, 1994) In the present study, BaP induced animals were observed increase LDH activity in the serum but rhaponticin treatment retrieved the LDH levels showed that defensive against carcinogenesis. The activity of γGT aids as a specific marker in prediction of the carcinogenic process.(Erdemli et al., 2004) The enzymes 5′-NT hydrolyze a phosphate group of ribose in carbon atom 5. An activity of 5′-NT was increased in tumorous animals that emerge to proliferate tumor cells.(Dao et al., 1980; Kanna et al., 2019) In our study, γGT and 5′-NT activities were increased in BaP induced animals and it was retrieved by rhaponticin treatment (Fig. 9).

Cancer chemoprevention and cancer therapy is deeply involved in the discovery of tumor markers. CEA is a dependable marker for early types of cancer as well as prognostic indicator.(Anandakumar et al., 2009) CEA is frequently expressed and increased in cell outside of malignant epithelial tumor types and an survival benefit and promotes metastasis by permitting connection to other cells. (Veronesi et al., 2005) In the current investigation, BaP induced animals increased the CEA levels but rhaponticin treatment were decreased the CEA levels (Fig. 10A). The finding of CEA is a tremendous experimental analysis for tumor diagnosis and metastasis. Chronic inflammation is a key risk factors for cancer development (Jafri et al., 2013) that through series of molecular pathways regulation; among one pathways is NF-κB, activated in carcinogenesis at early stages.(Naugler and Karin, 2008) The inflammation generated by the various cytokines has been recognized in certain cancer and metastasis.(Balkwill and Mantovani, 2001) TNF-α is mainly activated by macrophages and regulates the cellular response including apoptosis.(Ben, 2003) Chronic inflammatory condition, the pro inflammatory cytokines IL-1β was elevated in adenocarcinoma patient's serum.(Cheng-cheng et al., 2017) BaP induced animals in our study significant increment of cytokines, it is also one of the reason for cancer development with chronic inflammation. The histopathological study of lung tissue observations (Fig. 11) suggests that rhaponticin decreases the tumor induction and ameliorate the biochemical parameters with immune regulatory function therefore, it could be useful for lung cancer therapy.

7. Conclusion

The current study reports evidenced that rhaponticin has the promising role as a chemotherapeutic drugs against BaP induced lung tumorigenesis in mouse model. The chemopreventive effect against BaP induced animal, rhaponticin may have ability to decrease of oxidative stress through the amelioration and also the immune modulation. This *in vitro* study noted that rhaponticin decreased the cell viability it is due to increase in ROS, and increased the caspase-3,-8 activity mediated apoptosis clearly revealed that the cytotoxic activity of rhaponticin in A549 cancer cell line. Together, the findings have displayed that efficiency of rhaponticin for management of lung cancer. However, further investigation with molecular mechanism of pathway mediated apoptosis, metastasis, and angiogenesis was needed in the future.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Further Reading

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