

**Original Article**

**VANILLIC ACID INHIBITS LUNG CARCINOGENESIS BY MODULATES GLYCOPROTEIN ABNORMALITIES, MEMBRANE-BOUND ENZYMES, AND INFLAMMATORY MARKERS**

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**ABSTRACT**

**Objective:** The current plan was accompanied to explicate the possible protective role of vanillic acid (VA), on modification in lipid peroxidation, inflammatory cytokines, membrane-bound enzymes, and glycoconjugates during B(a)P induced lung cancer in Swiss albino mice.

**Methods:** Benzo(a)pyrene was administered orally (50 mg/kg b. wt) to induce lung cancer in Swiss albino mice. lipid peroxidation, serum marker enzymes, inflammatory cytokines, membrane-bound ATPases and protein-bound carbohydrate components (Hexose, hexosamine, sialic acid and fucose) and Mast cells and PAS staining were carried out.

**Results:** Lung cancer possessing animals exhibited increased levels of lipid peroxidation, ADA, AHH,  $\gamma$ -GT, 5'-NT, LDH, cytokines such as TNF- $\alpha$  and IL-1 $\beta$ , protein-bound carbohydrate components (protein-hexose, hexosamine, sialic acid, and fucose) also diminished activity of membrane-bound ATPases (Na<sup>+</sup>/K<sup>+</sup>ATPases, Ca<sup>2+</sup>ATPases, and Mg<sup>2+</sup>ATPase). Treatment with VA significantly ameliorated all these activities.

**Conclusion:** Overall, the present study evidence to the VA has effective anti-inflammatory in addition to free radical scavenging activity for the duration of lung carcinogenesis in Swiss albino mice.

**Keywords:** Lung cancer, Vanillic acid, Cytokines, Glycoconjugates, Benzo(a)pyrene

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**INTRODUCTION**

Lung malignancy is one of the most generally diagnosed cancers worldwide, it also ranks first as a foremost cause of cancer-related deaths both men and women [1]. Around 90% of all lung cancer is instigated through cigarette smoking [2, 3]. Oxidative stress-mediated lipid peroxidation (LPO) acting a substantial part of carcinogenesis and most studied relevant to the free radical chain reaction. Glycoproteins are the main constituents of the cell membrane which act as a significant part of cell differentiation, cell proliferation, and cell-cell interaction [4, 5]. Chemoprevention deals with a unique approach to regulate the incidence and mortality of lung malignancy [6]. In modern years, significant consideration was given to increase in dietetic consumption of phytochemicals because epidemiological, as well as experimental studies, gave an optimistic association between reduced risks of cancer due to intake of phytochemicals [7].

Vanillic acid (VA) is dietetic phenolic derivative natural substances found in vanilla bean and benzoic acid [8]. It is also found in wine and vinegar [9]. VA eliminates the ROS as well as hydroxyl radicals and lipid peroxide radicals and also protects the biological membrane by inhibiting lipid peroxidation [10, 11] It possesses antioxidant activity [10], anti-genotoxic [12]. VA has been connected with abundant pharmacological possessions to treat carcinogenesis [13, 14], apoptosis [15], and hepatoprotective effects [16]. VA also exhibits a chemopreventive effect in experimentally induced carcinogenesis in rats [17, 18]. Recently, we have stated the antioxidant and anticancer potential of VA during experimentally induced lung cancer [19].

The current plan was designed to focus the defensive effect of VA on the expression of inflammatory cytokines together with the lipid peroxidation, membrane-bound enzymes, and cell surface abnormalities by assessing the glycoconjugates levels during B(a)P induced lung cancer in albino mice.

**MATERIALS AND METHODS**

**Chemicals**

Benzo(a)pyrene, vanillic acid, BSA were bought from Sigma chemicals, St Louis, USA. The primary and secondary antibodies

obtained from Santa Cruz Biotech, USA. The other chemicals were procured of analytical grade from SRL Chemicals Pvt, Ltd, Mumbai, India. TNF- $\alpha$ , IL-1 $\beta$  enzyme-linked immunosorbent assay (ELISA) kits were obtained from Thermo Fisher Scientific.

**Experimental proposal**

All the experiments were considered and carried out according to the Institutional Animal Ethical Committee agreed guidelines (IAEC No.01/05/2018). Healthy male mice obtained from the veterinary college (Tanuvass), Chennai. The animals maintained in perfect, sanitized confines at (25 $\pm$ 2 °C) then were adjusted to the 12-h bright and dusky cycles. Animals were fed with commercially available rat pellet feed (Hindustan Foods Ltd, Bangalore, India).

Mice were randomly dispersed into four groups; each group contains 6 animals, groups classified as follows:

Group I served as control animals and was given corn oil orally for 16 w along with diet and drinking water. Group 2 animals were administered with B(a)P (50 mg/kg b. wt.) disbanded in corn oil orally twice a week for 4 subsequent weeks and remain for 16 w. Group 3 animals were treated with of VA (200 mg/kg b. wt.) alternate days a week orally for 16 w along with B(a)P. VA treatment was started one week earlier than to the beginning dosage of B(a)P administration and continued until the end of the experimental period. Group 4 animals were treated with of VA (200 mg/kg b. wt.) alternating days a week orally for 16 w. At the end of the experimental period, the animals were fasted throughout the night, anesthetized followed by cervical decapitation. The serum and supernatants were collected for evaluating different parameters. The protein was assessed in serum and tissue homogenate by the method [20]. Serum marker enzymes Adenosine deaminase (ADA) [21], AHH [22],  $\gamma$ -Glutamyl transpeptidase ( $\gamma$ -GT) [23], 5'-nucleotidase (5'-NT) [24], and lactate dehydrogenase (LDH) [25] were assayed. Erythrocyte membranes were isolated according to the standard protocol by the method [26] and LPO was evaluated according to [27].

**Determination of membrane-bound enzymes and Glycoprotein's**

The activity of Na<sup>+</sup>/K<sup>+</sup>ATPase by [28] Ca<sup>2+</sup>ATPase by [29] as well as Mg<sup>2+</sup>ATPase by [30] was estimated. Hexose, hexosamine, and fucose determination by [31]. Sialic acid was determined by the method [32].

**Inflammatory cytokines ELISA**

Serum TNF- $\alpha$  and IL-1 $\beta$  levels were measured by ELISA kits according to the manufacturer's protocol.

**Mast cell and PAS staining**

Mast cell staining investigation was performed by the standard procedure [33]. PAS staining investigation was performed by the standard procedure [34].

**Western blot analysis of inflammatory cytokines**

Western blot investigation of inflammatory cytokines (TNF- $\alpha$ , IL-1 $\beta$ ) was performed by the standard procedure [35]. Densitometry data are denoted in bar graphs as 'fold change' equalled to control.

**Statistical analysis**

The data were analyzed by using SPSS/10 software. Data were assessed by one-way analysis of variance (ANOVA) followed by least significance difference (LSD) test.  $P < 0.05$  were considered to indicate statistical significance. The entire outcomes were expressed as a mean  $\pm$  standard error (SE) for six animals in all groups.

**RESULTS**

**VA inhibits lipid peroxidation (LPO) in the lung and erythrocyte membrane of control and experimental animals**

Fig. 1a shows the B(a)P induced group II animals, there was a considerable increase in the levels of lung and erythrocyte membrane lipid peroxides while compared with group I animals. Whereas VA treated group III animals, there is a substantial reduction in the altitudes of lipid peroxides while compared to group II animals. However, there were no noticeable changes observed in group IV animals while compared to group I animals.

**VA decreased the levels of serum marker enzymes in experimental conditions**

Fig. 2 represents the effect of VA on the levels of serum marker enzymes in control and experimental animals. Serum marker enzymes ADA, AHH,  $\gamma$ -GT, 5'-NT and LDH were found to be noticeably elevated in cancer possessing group II animals. On VA (Group III) treatment, the levels of these enzymes were considerably retained to near normal level while compared to group II animals.

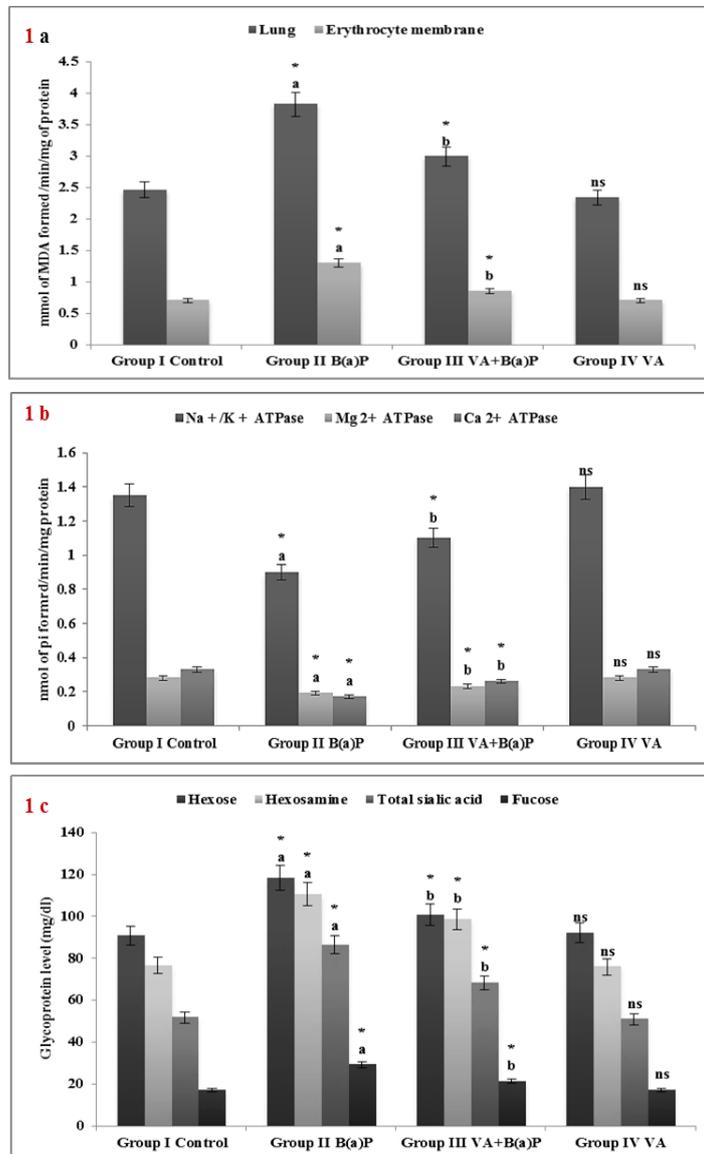


Fig. 1a: Levels of malondialdehyde (MDA) in lung and erythrocyte membrane, (fig. 1b) Levels of glycoconjugates, and (fig. 1c) Erythrocyte membrane ATPases activity in control and experimental animals, Results are expressed as mean  $\pm$  S. D for six animals in each group. Results are given as statistically significant at \* $p < 0.05$ ; <sup>a</sup>as compared with Group I; <sup>b</sup> as compared with Group II; <sup>ns</sup>: non-significant as compared to control. Units were expressed as mmol of MDA formed/min/mg of protein. Mg/ml. nmol of pi formed/min/mg protein

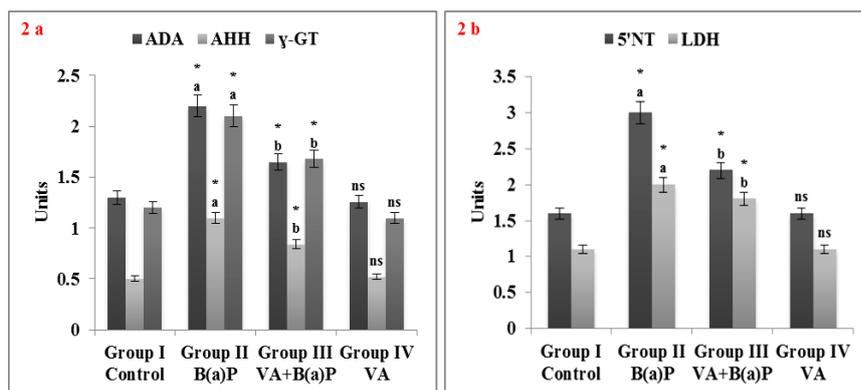


Fig. 2(a) and (b): Levels of serum marker enzymes in the control and experimental group of animals, Results are expressed as mean±S. D for six animals in each group. Results are given as statistically significant at \* $p < 0.05$ ; <sup>a</sup> as compared with Group I; <sup>b</sup> as compared with Group II; <sup>ns</sup>: non-significant as compared to control. Units: ADA–micro-moles of  $\text{NH}_3$  liberated/mg protein/hr.; AHH–micromoles of fluorescent phenolic metabolites formed/min/mg protein;  $\gamma$ -GT–nano-moles of p-nitroaniline formed/min/mg/protein; 5'-NT–nanomoles of Pi liberated/min/mg protein; LDH–micro-moles of pyruvate liberated/min/mg protein

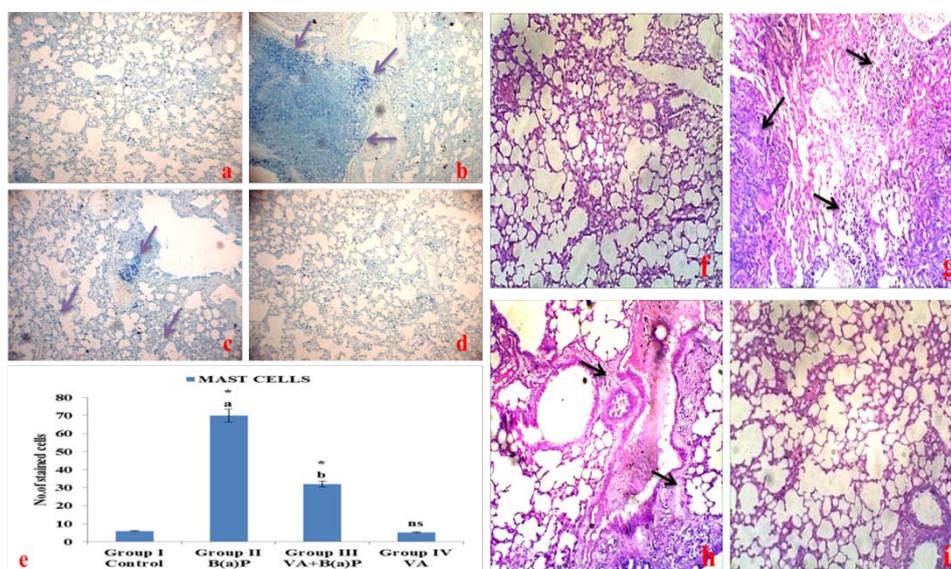


Fig. 3: Histochemical analysis of mast cells by toluidine blue staining and PAS staining in the lung of control and experimental animals (10X), fig. 3 (a) shows the section from control mice lung exhibits normal architecture with minimal mast cells. (b) Section from B(a)P induced mice lung showing an abnormally increased number of mast cell density (c) section from VA treated mice lung showing a reduced number of mast cell density and infiltration. (d) Section from drug control mice lung showing normal architecture as similar to that of normal control lung. Violet color arrows indicate positively stained mast cells. (e) Represents the average bar graph of the total number of mast cells in ten different fields/slides in control and experimental groups. The data expressed as mean±SD for six mice in each group. Statistically significant at \* $p < 0.05$ ; <sup>a</sup> as compared with Group I; <sup>b</sup> as compared with Group II; <sup>ns</sup>: non-significant as compared to control. Fig. 3 (f) Control group shows normal glycoconjugate expression. (g) B(a)P induced lung cancer-bearing mice lung section (Black arrow) indicates overexpression of glycoconjugates. (h) Decreased expression of glycoconjugates in VA+B(a)P treated animals. (i) The Drug control group shows normal glycoconjugates expression as compared to the control group

#### VA treatment reduced the mast cell infiltration

Fig. 3 (a to d) shows the toluidine blue staining of control and experimental animals. Tumor possessing animals of group II showed the increased number of mast cells while compared to control animals. Whereas, VA treated animals revealed a substantial decrease in the number of mast cells while compared to cancer possessing animals. VA alone treated animals did not show any significant change when compared to control animals.

#### VA reduces the levels of inflammatory cytokines

Fig. 4 a, b and c depict the levels of  $\text{TNF-}\alpha$  and  $\text{IL-1}\beta$  was markedly increased tumor possessing animals when compared to control animals. Whereas VA treated animals, revealed significantly reduced

the protein expression of  $\text{TNF-}\alpha$  and  $\text{IL-1}\beta$  when compared to lung cancer possessing animals.

#### Effect of VA administration on the activity of lung and erythrocyte ATPases in the control and experimental animals

Table 1, fig. 1b respectively shown the lung and erythrocyte ATPases, such as  $\text{Na}^+/\text{K}^+$ ATPase,  $\text{Mg}^{2+}$ ATPase, as well as  $\text{Ca}^{2+}$ ATPase of control and experimental animals. Noticeable significant decrease in the levels of  $\text{Na}^+/\text{K}^+$ ATPase,  $\text{Mg}^{2+}$ ATPase, and  $\text{Ca}^{2+}$ ATPase was perceived in group II cancer possessing animals while compared to group I animals. Treatment of VA with B(a)P mice normalized the levels of lung and erythrocyte ATPases in group III animals. However, there were no significant changes in group IV animals while compared to control animals.

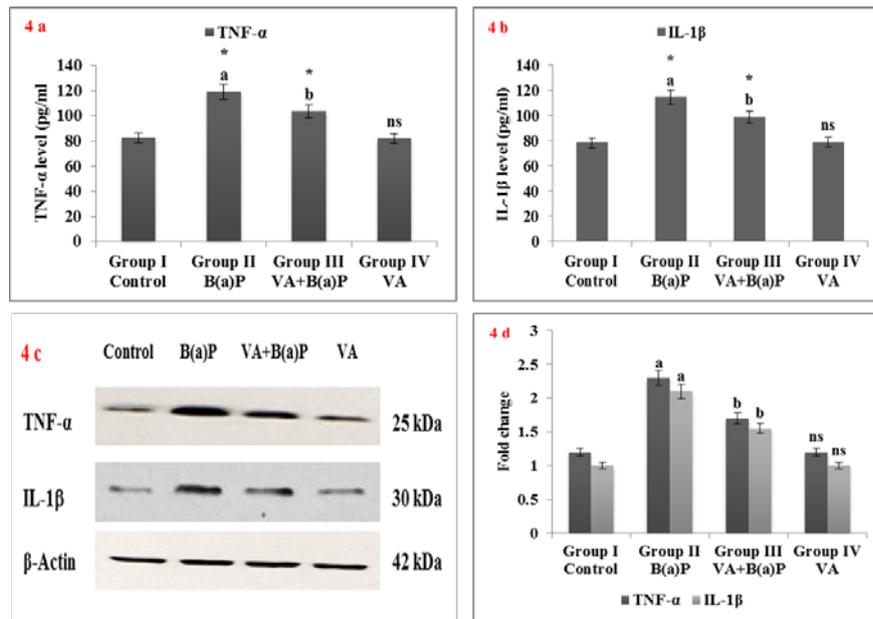


Fig. 4a and b: Effect of VA on TNF- $\alpha$  and IL-1 $\beta$  ELISA and fig. (4c) Immunoblot expression levels in experimental groups of animals, results are expressed as mean $\pm$ S. D for six animals in each group. Results are given as statistically significant at \* $p$ <0.05; <sup>a</sup> as compared with Group I; <sup>b</sup> as compared with Group II; <sup>ns</sup>: non-significant as compared to control. Fig. (4d) Quantitative data stating the equivalent protein levels were assessed using densitometry and is uttered as "fold change" as compared with control. Units were expressed as pg/ml

Table 1: Effect of VA administration on the activity of ATPases of lung tissue in the control and experimental groups

Particulars	Group I control	Group IIB(a)P	Group IIIVA+B(a)P	Group IVVA
Na <sup>+</sup> /K <sup>+</sup> ATPase	6.22 $\pm$ 0.26	3.33 $\pm$ 0.32 <sup>a</sup>	4.53 $\pm$ 0.39 <sup>b</sup>	6.05 $\pm$ 0.23 <sup>ns</sup>
Mg <sup>2+</sup> ATPase	10.98 $\pm$ 0.4	6.76 $\pm$ 0.49 <sup>a</sup>	7.83 $\pm$ 0.44 <sup>b</sup>	10.87 $\pm$ 0.41 <sup>ns</sup>
Ca <sup>2+</sup> ATPase	9.62 $\pm$ 0.31	4.86 $\pm$ 0.44 <sup>a</sup>	6.51 $\pm$ 0.59 <sup>b</sup>	9.46 $\pm$ 0.35 <sup>ns</sup>

Results are expressed as mean $\pm$ SD for six animals in each group. Results are given as statistically significant at \* $p$ <0.05; <sup>a</sup> as compared with Group I; <sup>b</sup> as compared with Group II; <sup>ns</sup>: non-significant as compared to control. Units: Na<sup>+</sup>/K<sup>+</sup>ATPase, Mg<sup>2+</sup>ATPase and Ca<sup>2+</sup>ATPase are expressed micromole ( $\mu$ mol) of inorganic phosphate formed/min (mg/protein).

#### Effect of VA administration on cell surface glycoconjugates abnormalities

Fig. 1c, table 2 shows the levels of glycoconjugates (Protein-bound hexose, hexosamine, total sialic acid, then fucose) in the serum and lung tissues respectively. The levels of glycoconjugates in the serum and lung tissue significantly increased in administered with B(a)P

animals while compared to control animals. Oral administration of VA+B(a)P treated mice brought back the levels of the glycoconjugates to a nearer normal level. No significant difference was noticed in the levels of serum and lung glycoconjugates in VA alone group animals while compared with control animals. Fig. 3 (f to i) shows the PAS staining analysis also confirms the VA treatment during B(a)P administration.

Table 2: Effect of VA on glycoproteins like hexose, hexosamine, total sialic acid and fucose of lung tissue in the control and experimental animals

Particulars	Group I control	Group IIB(a)P	Group IIIVA+B(a)P	Group IVVA
Hexose	85.41 $\pm$ 3.45	109.65 $\pm$ 9.27 <sup>a</sup>	97.32 $\pm$ 7.08 <sup>b</sup>	84.92 $\pm$ 3.26 <sup>ns</sup>
Hexosamine	38.6 $\pm$ 1.98	67.2 $\pm$ 6.91 <sup>a</sup>	53.61 $\pm$ 5.22 <sup>b</sup>	39.1 $\pm$ 1.78 <sup>ns</sup>
Total sialic acid	15.72 $\pm$ 1.08	28.56 $\pm$ 3.78 <sup>a</sup>	22.45 $\pm$ 2.07 <sup>b</sup>	16.02 $\pm$ 1.12 <sup>ns</sup>
Fucose	13.3 $\pm$ 1.21	21.26 $\pm$ 2.78 <sup>a</sup>	18.5 $\pm$ 2.16 <sup>b</sup>	13.63 $\pm$ 1.29 <sup>ns</sup>

Results are expressed as mean $\pm$ SD for six animals in each group. Results are given as statistically significant at \* $p$ <0.05; <sup>a</sup> as compared with Group I; <sup>b</sup> as compared with Group II; <sup>ns</sup>: non-significant as compared to control. Units: hexose, hexosamine, total sialic acid, and fucose units are expressed mg/g tissue protein.

#### DISCUSSION

During malignancy, the cell membrane plays a crucial role in the control of cell adhesiveness, cell death, as well as cell proliferation. Lipid peroxidation generally takes place fewer in normal physiological circumstances. The B(a)P is a very effective carcinogen with an ability to induce enormous amounts of free radicals generation, which in turn reacts with lipids causing LPO. In nature, there is a dynamic balance the number of free radicals generated in the body and antioxidant defense systems that reduce or scavenging

them and protect the body against their adverse effects [36]. In the existing study, it is evident that an increased level of LPO was established in cancer possessing animals. However, the administration of VA decreased the LPO, which may be due to the free radical scavenging movement [19, 37].

ADA is an essential enzyme in purine metabolism as it catalyzes the translation reaction of adenosine to inosine [38]. AHH metabolizes some of the polycyclic hydrocarbons to phenols, dihydrodiols, quinines, and epoxides [22]. High levels of AHH were established in

pulmonary in the serum and tissues of animals exposed to B(a)P [39]. GGT activity assists as a specific marker of the prediction of the carcinogenic process [40]. 5'-NT are enzymes that hydrolyze nucleotides with a phosphate group on carbon atom 5 of ribose. Increased activities of 5'-NT in tumorous animals appear to have originated from the proliferating tumor cells [41, 42]. LDH is a fairly sensitive marker for solid neoplasms, also raised activity of the enzyme was designated in the serum of patients with lung cancer [43]. In the existing study, there was a substantial elevation in the levels of these enzymes in cancer possessing animals. VA treatment brought down the levels of these marker enzymes close to normal level shows that VA possesses defensive agents against carcinogenesis.

The inflammatory microenvironment of cancerous tissue is distinguished by the occurrence of host leukocytes both in the supportive stroma and between tumor cells, with macrophages, dendritic cells, T cells, and MCs [44]. Mast cells can secrete numerous proangiogenic factors, which can start the tumor angiogenesis switch and they have been shown to store in tissues undergoing angiogenesis through tumor progression [45]. The increased mast cells were related to reduce survival rates in patients through pulmonary adenocarcinoma [46]. VA supplementation animals exhibited significantly diminished in the mast cell density proves its chemopreventive nature.

Acute inflammation generated by exogenous administration of TNF- $\alpha$  and IL-1 $\beta$  has been known under certain malignancy and metastasis [47]. TNF- $\alpha$  is predominately activated by macrophages and induces the variability of cellular response including apoptosis [48]. TNF- $\alpha$  has several possessions including lysis, hemorrhagic necrosis of tumor cells, and tissue destruction through adhesive molecules [49], which may stimulate the growth of fibroblasts and certain tumor cells [50]. IL-1 $\beta$  is a significant pro-inflammatory cytokine connected with chronic inflammation and is radically elevated in the levels of adenocarcinoma patient's serum [51, 52].

The membrane protection is in need during the treatment of adverse conditions of the disease progression [53]. The membrane-bound enzymes such as Na<sup>+</sup>/K<sup>+</sup>ATPases, Mg<sup>2+</sup>ATPase, and Ca<sup>2+</sup>ATPase are liable for the transport of sodium/potassium, magnesium and calcium ions transversely on the cell membranes at the outflow of ATP by way of hydrolysis. Na<sup>+</sup>/K<sup>+</sup>ATPase movement is liable for a large part of the energy spending which constitutes the essential metabolic enzyme activity and the changes in the membrane dynamic lead to pathological conditions [4, 5, 54]. Ca<sup>2+</sup>ATPase is a reflection of energy-dependent calcium transport across the cell membrane less deformable. Mg<sup>2+</sup>ATPase, along with the other ATPase is also involved in energy-requiring processes in the cell. The protective effect of VA in this system could be either due to scavenging peroxides before attacking the membrane and or due to blocking the oxidation of membrane lipids.

Glycoproteins exert a significant part in contributing to the surface properties, tumorigenesis, and as mediators of immunological specificity. Malignant transformation is frequently associated with molecular variations such as glycoproteins and glycolipids. Increased levels of tissue and serum glycoprotein's hexose, hexosamine, sialic acid, and fucose during malignancy reflect moreover a local or systemic tissue response of the tumor [55]. Evidence from animal experimentation recommends that the presence of malignant tumors invoke the rise in the synthesis of glycoproteins, which subsequently enter into the circulation [56, 57]. These results were also agreed with our present examination from which we noticed an increased level of tissue glycoconjugates in lung cancer-induced animals. VA treatment considerably reduces the level of these glycoproteins to a near-normal state. These decreases in the levels of glycoprotein components signify that the VA can restrain malignancy by modulating cellular transformation to protect the cell wall abnormalities.

## CONCLUSION

Our present investigation indicates that VA supplementation improved the levels of the inflammatory cytokines TNF- $\alpha$ , IL-1 $\beta$  protein expression, disruptions in lipid peroxidation and membrane-bound enzyme activity, as well as glycoprotein levels during B(a)P

induced lung cancer, thus confirms its chemopreventive effect. From all the above results, we conclude that the VA has a potential anti-inflammatory, chemotherapeutic agent against B(a)P induced lung carcinogenesis in Swiss albino mice.

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Nil

## AUTHORS CONTRIBUTIONS

All the authors have contributed equally.

## CONFLICT OF INTERESTS

The authors wish to approve that there are no identified conflicts of interest.

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