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EFFECT OF ACTIVE FRACTION OF *PUNICA GRANATUM* L EXTRACT ON TUMOR MARKERS IN BENZOPYRENE INDUCED LUNG CANCER IN SWISS ALBINO MICE

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ABSTRACT

Benzo(a) pyrene (BP), the polycyclic aromatic hydrocarbon which is highly carcinogenic present in tobacco smoke causes lung cancer. So nowadays a lot of attention on plant based products which act as chemopreventive agents are used to prevent lung cancer. So the present investigation focuses on the *Punica granatum* which is a medicinal plant and used in folk medicines. The present investigation was carried out to determine the effect of ethyl acetate fraction of *Punica granatum* rind extract on tumor markers in benzopyrene induced lung cancer in Swiss albino mice. The analysis on tumor markers revealed that the ethyl acetate fraction of *Punica granatum* rind extract possess antineoplastic effect on lung cancer induced in Swiss albino mice which may be due to the highest percentage of Pyrogallol in the active fraction.

Key words: *Punica granatum*, Benzopyrene, Swiss albino mice, Lung cancer, Pyrogallol.

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INTRODUCTION

Lung cancer is a common disease with high death due to late diagnosis. The incidence of lung cancer still remains very high. Tobacco smoke contains over 60 established carcinogens. Among the constituents of smoke the Polycyclic Aromatic Hydrocarbons (PAHs) such as benzo(a)pyrene, play a major role in lung carcinogenesis.

An alternative and novel approach for the management of lung cancer is chemoprevention through the recommended intake of health protective food especially those present in vegetables, fruits, beverages and spices in daily diet which inhibits or reverses the development of cancer. Therefore, the present investigation was undertaken to evaluate the protective activity of active fraction of *Punica granatum* rind extract on benzo[a]pyrene (BP)-induced lung carcinogenicity in Swiss albino mice.

MATERIALS AND METHODS

Chemicals

Benzopyrene was purchased from Sigma. All other chemicals used were of analytical grade.

Selection of animal and ethics

Swiss albino male mice (6 weeks old, 20-25g.) were purchased from Saveetha University, Chennai, India. Animals were housed in groups (3-5/cage) in polypropylene cages in a well ventilated room (air cycles: 15/min; 70:30) under an ambient temperature of 23±2°C and 40–65% relative humidity, with a 12-h light/dark artificial light cycle. They were provided with food (standard pelleted diet) and purified water ad libitum. All the animals were acclimatized at least for 7 days to the laboratory conditions prior to experimentation. Experimental animals were handled according to the University and Institutional Legislation, regulated by the committee for the purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Social Justice and Empowerment, Government of India **(IAEC.No Biochem BWC/010/10).**

Preparation of plant extract

Punica Granatum rind was collected from local commercial source. Herbarium specimen of the rind was prepared and preserved for future references. Fresh rind were washed thoroughly with water to remove the earthy matters and freed from debris. They were dried in shade and powdered mechanically. 30 gms of powdered *Punica granatum* rind was macerated with methanol and stored for 72 hours in ice cold condition for the extraction of phytochemicals. At the end of the third day extract was filtered using whatmann No. 1 filter paper and the organic layer was allowed to evaporate. The extracts were concentrated under vacuum, in rotary

evaporator, dried and stored in vacuum desiccators for future analysis. Methanolic extract was subjected to column chromatography and fractionated. The study was carried out with the ethyl acetate fraction obtained which was confirmed as active fraction by *in-vitro* study.

Induction of lung cancer

Lung cancer was induced orally by injecting benzopyrene [50 mg (dissolved in corn oil) kg^{-1} $b.wt^{-1}$] twice weekly for 16 weeks. [1]

Experimental design

The experimental animals were divided into five groups with six animals each.

Group I Normal vehicle control (corn oil)

Group II Cancer induced mice.

Group III After induction, treatment with active fraction of the extract (50 mg kg^{-1} $b.wt^{-1}$) for 1 month.

Group I Pretreatment with active fraction of the extract for 1 month and cancer induction

Group V Drug control mice treated with active fraction alone.

Collection of organ and homogenization

All the experimental animals were killed by cervical decapitation after the experimental period. The lung tissues were homogenized with motor driven Teflon coated homogenizer in ice-cold 0.1M Tris-HCl buffer pH 7.4 to obtain 10% homogenate and used for further analysis.

Assay of tumor marker enzyme

Assay of γ-Glutamyl transpeptidase (EC 2.3.2.2)

The activity of γ-glutamyl transpeptidase was estimated according to the method of Orlowski and Meister.[2] The amount of p-nitroaniline in the supernatant was measured at 410 nm. The activity of γ-glutamyl transpeptidase was expressed as μmole of p-nitroaniline formed/min/mg protein

Assay of lactate dehydrogenase (EC 1.1.1.27)

The activity of lactate dehydrogenase was assayed by the method of King.[3] The color developed was read at 420 nm. The activity of lactate dehydrogenase was expressed as μmole of pyruvate liberated/min/mg protein.

RESULT AND DISCUSSION

Lung cancer was considered to be uncommon in the beginning of the century but has now reached almost higher proportions.[4] Lung cancer is the chief cancer leading to deaths in developed countries and in developing countries.[5] Deaths due to lung cancer are more than those due to colorectal, breast and prostate cancers. Frequency of death caused by lung cancer in

females is rising while it is declining in males in developed countries. This is the single most devastating cause of cancer-related deaths with approximately 1.5 million cases world-wide and more than 1.3 million cancer-related deaths in 2001. The five-year survival rate for lung cancer has improved only marginally from 5% in the late 1950s to 14% by 1994. This is in contrast to the five years survival rate of 52% for some other cancers. Lung cancer is responsible for about one million deaths per year and it rises to three millions per year by the year 2010. Cancer in lungs is the leading cause of cancer mortality in men and women in the US.[6] When there is an exposure to tobacco-related carcinogens it leads to lung cancer.[7, 8]

Tobacco smoking is the major risk factor for lung cancer and contains over 60 established carcinogens. Benzo(a)pyrene (BP), one of the polycyclic aromatic hydrocarbons which are highly carcinogenic present in tobacco smoke causes lung cancer in humans and in experimental systems.[9,10] This compound is ubiquitous in the environment, is mutagenic in both prokaryotic and eukaryotic test systems, and is an animal carcinogen.[11,12] Benzo(a)pyrene is unreactive compound, but it is converted to a highly reactive electrophile by enzymes which are involved in drug metabolism. The routes of metabolism of Benzo(a)pyrene are complex, the enzyme involved in the metabolism are the cytochrome P450 (CYP) system as a result it ends up in to a genotoxic metabolite, the anti isomer of Benzo(a)pyrene 7, 8-diol- 9, 10-epoxide. Three enzymatic reactions are required for its formation: initial epoxidation to yield the 7, 8-epoxide, hydrolysis of this epoxide to yield the (-)-Trans-7, 8-diol, and finally a second epoxidation of the diol to produce Benzo(a)pyrene -7,8-diol-9,10- epoxide (anti isomer).[13] This anti isomer produced during metabolism of benzo(a)pyrene may also cause activation of protooncogenes.[14]

Surgery, radiotherapy, chemotherapy and chemo-radiotherapy are the main treatment methods for most cancers but in case of lung cancer treatment outcome by these procedures are limited due to frequent recurrence. Therefore modern approaches for control of lung cancer focus on prevention. Although tobacco cessation is important for lung cancer prevention, ex-smokers are still at risk. Management of lung cancer by chemoprevention through recommended intake of selected food items and beverages is being studied.

Nowadays, a lot of attention focuses on plant based natural products which are used as chemopreventive agents and considered to be practically useful in certain *in vitro* systems and *in vivo* systems. It is vital to provide scientific proof to authenticate the use of a plant or its bioactive compounds for medicinal purposes.[15] Modern drugs, plants and plant extracts should be characterized for their pharmacological properties like pharmacokinetic and

pharmacodynamic properties by pharmacognostic screening and also toxicity of the plant or plant extracts should be screened before using as a drug to various systems.[16] Cancer chemoprevention is defined as the use of bio active compounds or dietary components to block, inhibit, or reverse the development of cancer in normal or preneoplastic tissue. A large number of potential chemopreventive agents have been identified and they act on all major stages of carcinogenesis and thereby prevent it.[17,18,19] Miranda *et al.*,[20] reported that many phytochemicals in plant extracts are proven to have anticancer activities and many are in use for cancer treatments. Minor incidences of cancer are seen in people who intake diets rich in fruits and vegetables.[21] So the present study was carried out to decrease the incidences of lung cancer by treating with active fraction which was obtained from *Punica granatum* rind. Analysis of lung weight, body weight and tumour markers were carried out to prove the antineoplastic effect of ethyl acetate fraction on benzopyrene induced lung cancer in Swiss albino mice model which are discussed below.

Lung weight and body weight

The present study demonstrated that oral administration of ethyl acetate fraction of crude Methanolic *Punica granatum* extract has chemopreventive effects against Benzo(a)pyrene in Swiss albino mice. Different groups of mice showing their body and lung weights are depicted in figure 1 and figure 2 respectively. There was a significant decrease in the body weight of carcinogen treated (Group II) animals when compared with control (Group I) animals. Ethyl acetate fraction of methanolic *Punica granatum* rind extract post treated animals (Group III) were found to have a significant increase in the body weight when compared with cancer bearing animals. On pretreatment with ethyl acetate fraction of methanolic *Punica granatum* rind extract (Group IV), a significant increase in the body weight was noted when compared with cancer bearing animals. Ethyl acetate fraction supplementation also reduced lung weight in group III and IV animals when compared with group II animals.

The ethyl acetate fraction produced significant reduction in the lung tumor incidence and tumor multiplicity and significant increase in body weight. In lung cancer bearing (group II) animals, there was a sharp drop in their body weight. This may be due to the cancer cachexia. Cancer cachexia occurs most frequently in tumor and is associated with more than 20% of cancer deaths.[22] Cachexia is a complex metabolic status with progressive loss of weight and lessening of host reserves of adipose tissue and skeletal muscle. Weight loss and tissue wasting are the symptoms observed in cancer patients who imply poor prognosis and shorter survival time.[23]

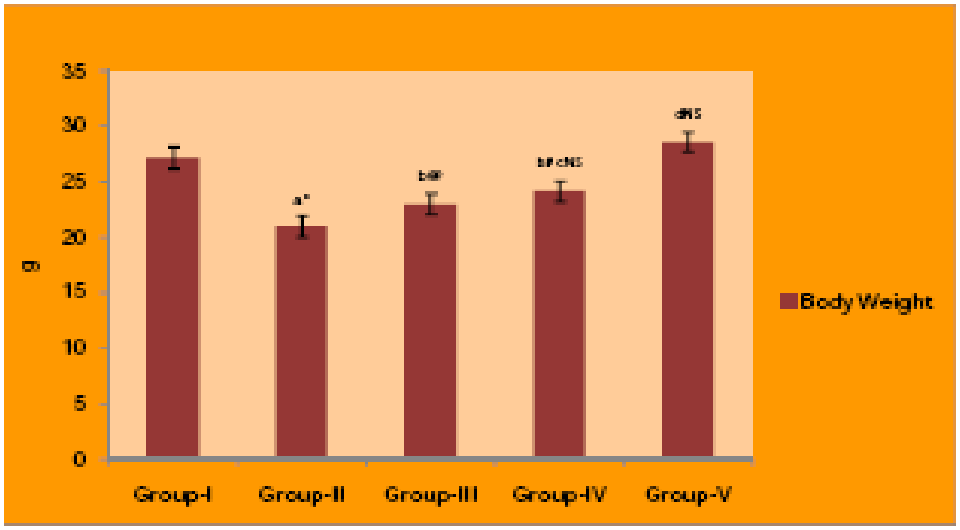

Figure 1 Effect of the extract on body weight in control and experimental animals

Each value is expressed as mean ± SD for six mice in each group. Body weight expressed as g. Group I- control animals, Group II- cancer bearing animals, Group III- Ethyl acetate fraction post treated and Group IV- Ethyl acetate fraction pre treated, and Group V- Ethyl acetate fraction alone. Statistical significance: *p<0.001, #p<0.01, @p<0.05 and NS – Not significant. a: as compared with group I; b: as compared with group II; c: as compared with group III; d: as compared with group I.

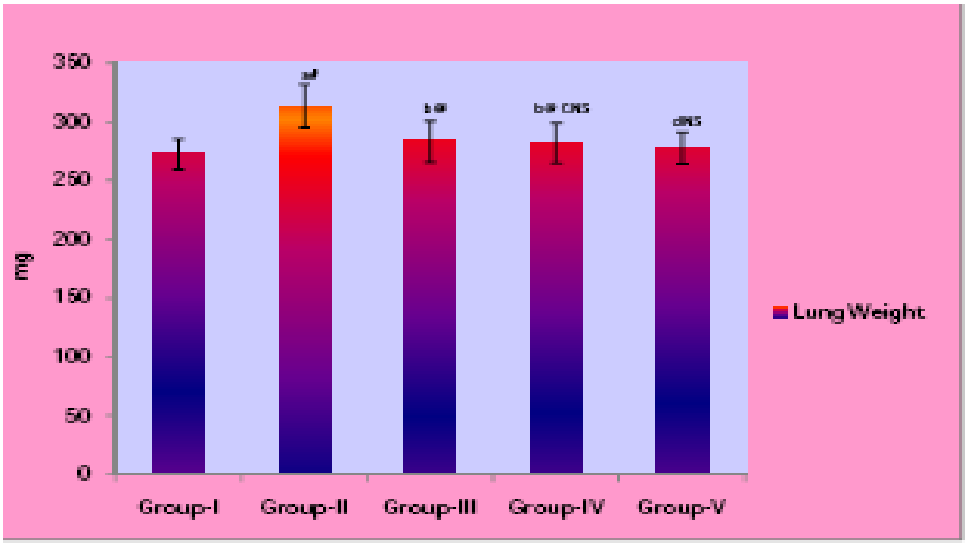

Figure 2 Effect of the extract on lung weight in control and experimental animals

Each value is expressed as mean ± SD for six mice in each group. Lung weight expressed as mg. Group I- control animals, Group II- cancer bearing animals, Group III- Ethyl acetate fraction post treated, and Group IV- Ethyl acetate fraction pre treated, and Group V- Ethyl acetate fraction alone. Statistical significance: p<0.001, #p<0.01, @p<0.05 and NS – Not significant. a: as compared with group I; b: as compared with group II; c: as compared with group III; d: as compared with group I.

It was reported by Pain *et al.*,[24] that the drop in body weight results due to the fewer amounts of food intake and poor absorption which may contributes to muscle wasting and weight loss in tumor cachexia. On drug treatment (Group III and Group IV), the gradual increase in body

weight indicates the antineoplastic property of the extract. Group V animals do not show any significant variation. Extract does not exert any side effects or toxic symptoms inferring its non toxic nature. No death was observed in group V.

In lung cancer bearing animals the significant tumour progression may be due to the enormous proliferation of the cancer cells. On treatment the significant tumour regression may be due to the inhibitory action of the drug on tumour growth. This indicates the positive nature of the ethyl acetate fraction of methanolic extract of *Punica granatum* as a potential anticancer agent.

Tumor incidence

Table 1 represents the effect of Ethyl acetate fraction of methanolic *Punica granatum* rind extract on B(a)P induced lung tumor incidence in mice. Group III and Group IV animals treated with ethyl acetate fraction show a significant decrease in tumor incidence when compared with cancer bearing animals. The percentage of tumor incidence was reduced in Group IV animals when compared with Group III animals.

Table 1 Effect of the extract on B(a)P induced lung cancer incidence in mice

Perticulars	Group I	Group II	Group III	Group IV	Group V
No of animals	6	6	6	6	6
No of tumor incidence	0	6	4	3	0

Group I- control animals, Group II- cancer bearing animals, Group III- Ethyl acetate fraction post treated, Group IV- Ethyl acetate fraction pre treated, and Group V- Ethyl acetate fraction alone.

Tumor marker enzymes

Tumor markers enzymes are measurable biochemical's that are coupled with malignancy which are either synthesized by tumor cells or by the body in response to tumor cells. They are typically substances that are released into the circulation of blood and thus measured in the blood during cancer. The overall metabolism is altered due to the uncharacteristic variations in the levels of marker enzyme which was observed during malignancy condition.[25] Disruption of many biochemical's, immunological and molecular properties of the host have been observed in benzopyrene mediated cancer conditions.[26] The tumour marker enzyme such as γ GGT, 5' ND and LDH are specific key markers of lung tissue damage.[27]

Table 2 depicts the activities of tumor marker enzymes such as γ-Glutamyl transpeptidase and Lactate dehydrogenase in lung of control and experimental animals. There was a significant increase in the enzyme activities in cancer bearing group II animals when compared with group I control animals. The increased enzyme activities was reduced significantly in group III and

group IV ethyl acetate fraction post and pre treated animals when compared with group II cancer bearing animals. Both post and pre treated (Group III and Group IV) animals, these changes were brought back to near normal but in Group IV it was more effective than Group III. However, ethyl acetate fraction alone treated animals (Group V) did not show any significant changes when compared with control (Group I) animals.

Table 2 shows the effect of the extract on the activities of some tumor marker enzymes in lung of control and experimental animals

Marker enzymes	Group I	Group II	Group III	Group IV	Group V
GGT	1.38 ± 0.12	2.14 ± 0.18^{a*}	1.82 ± 0.15$^{b@}$	1.69 ± 0.12$^{b\#CNS}$	1.42 ± 0.13^{dNS}
LDH	1.35 ± 0.09	1.98 ± 0.15^{a*}	1.72 ± 0.14$^{b@}$	1.46 ± 0.12$^{b\#C@}$	1.38 ± 0.10^{dNS}

Each value is expressed as mean ± SD for six mice in each group. Units: GGT - µmole of p-nitroaniline formed/min/mg protein; LDH - µmole of pyruvate liberated/min/mg protein. Group I- control animals, Group II- cancer bearing animals, Group III- Ethyl acetate fraction post treated, Group IV- Ethyl acetate fraction pre treated, and Group V- Ethyl acetate fraction alone. Statistical significance: $^{*}p<0.001$, $^{\#}p<0.01$, $^{@}p<0.05$ and NS – Not significant. a: as compared with group I; b: as compared with group II; c: as compared with group III; d: as compared with group I.

Gamma glutamyl transferase (GGT)[28] catalyzes the transportation of the gamma-glutamyl moiety of glutathione to an amino acid, a peptide or water (forming glutamate). Gamma glutamyl transferase plays a key role in the production and degradation of glutathione. The pathway of formation of glutathione known as gamma-glutamyl cycle. As a result it increase the glutathione synthesis[29] and Gamma glutamyl transferase may also be involved in drug and xenobiotic detoxification in which it converts the toxic substance to the non-toxic substanse.[30] Gamma glutamyl transferase is located in the membranes of the cell of many tissues and active site is present in the outer surface of the membrane. Gamma glutamyl transferase levels are augumented in cancer condition. Chemical carcinogens that enter the liver may initiate some systematic effects thereby synthesize certain tumor markers in the system which may lead to the induction of Gamma glutamyl transferase synthesis.[31] An elevation in Gamma glutamyl transferase was observed in cancerous condition.[32] These elevations of Gamma glutamyl transferase levels show the stages of carcinogenic process, since its levels are correlated with growth rate, histological differentiation and survival time of the host.[33] In lymphoma bearing mice, Gamma glutamyl transferase activity was considerably higher in lung tissue than in control mice.[34]

Lactate dehydrogenase (LDH or LD) is an enzyme (EC 1.1.1.27) present in a wide variety of organisms, including plants and animals. Lactate dehydrogenase catalyzes the interconversion of

pyruvate and lactate with concomitant interconversion of NADH and NAD+. LDH is an enzyme present throughout the body and it is elevated in several types of cancerous condition. It is used in monitoring the treatment of cancer. An augumented level of lactate dehydrogenase was observed in malignant cells which spread through out the organs of the body.[35] This may be due to the higher glycolysis rate occuring in malignant condition because this is the only energy yielding pathway. An enhanced level of lactate dehydrogenase was observed in lung cancer patients.[36] The elevated levels of the enzyme were also observed in ovarian cancer[37] and leak out of the cell due to the changes in permeability of cell membrane.

The activities of tumor markers were decreased in extract treatment and this may be due to the presence of high amount of pyrogallol[38] which has the anticancer effect**.** A superoxide anion generator, pyrogallol, inhibits the growth of HeLa cells via cell cycle arrest and apoptosis.[39]

CONCLUSION

Therefore the decrease in the activities of these marker enzymes was observed during ethyl acetate fraction treatment of crude methanolic *Punica granatum* extract offers some protection against the abnormal cell growth by changing the permeability this may be due to the antineoplastic effect of extract.

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