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Anticancerous Effect of Fruit pulp of *Aegle marmelos* against Benzo[A] pyrene Induced Lung Tumours in Rats

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Abstract

In the recent times, the lung cancer disease burden in the population has increased manyfolds globally. Among those carcinogen- Benzo[A]pyrene is one of the known which is directly responsible for the cause. Benzo(a)pyrene has been shown to inflict damage on the lungs as well as liver. Thus, the present study has been aimed to study the anticancer activity of *Aegle marmelos* fruit pulp extract on Benzo [A] pyrene induced lung cancer in rats. Thirty male Charles Foster rats, 6 weeks old weighing around (150-180 g) were used for the study and were induced Benzo[A] pyrene (25 mg/Kg dissolved in Olive oil) orally in two intervals (1st day and 14th day) and were left for 3 months. After 3 months, there was development of lung tumours, which were confirmed histopathologically. Thereafter, *Aegle marmelos* fruit pulp extract at the dose of 250mg/Kg body weight was administered to the rats for 5 weeks. After the treatment there was significant reduction in the lung tumour size was observed in the studied rats. All the parameters were studied and their data were analysed. The haematological parameters, the biochemical parameters and the histopathological parameters were also correlated for the efficacy of the drug.

Through the entire study, it can be concluded that, fruit pulp extract of *Aegle marmelos* possesses anticancerous effect against Benzo [A]pyrene induced lung cancer. This drug after various trials can be recommended as therapeutic drug for lung cancer disease in the future.

Keywords: Benzo[A]pyrene induced lung model; Tumour volume; Biochemical parameters study; Histopathological study; Fruit pulp extract of *Aegle marmelos*; Novel drug discovery

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Introduction

In state of Bihar, a large number of populations inhabit in villages with agriculture as major professional practice. A large number of rural families depend upon preparation of food in direct contact with fire, burning of wood or cow dung cakes which releases lot of smoke that results to various health related issues. The polycyclic aromatic hydrocarbons (PAHs) are usually released from burning of organic matters (Hyland et al. 2020). The PAHs are a group of large family of compounds that contain aromatic rings made up of only carbon and hydrogen atoms [1]. A large number of PAHs have been found to be toxic, which in longer duration of exposure causes gene alterations and leading to development of diseases like lung cancer (Balmes 2018; Hussain et al., 2014). The air pollution in the recent times has caused serious lung diseases in the older and younger age group such as asthma, bronchitis, recurrent cough and breathlessness and moreover the chronic exposure also causes the disease of lung cancer. Among the severe carcinogen is a Benzo(a)pyrene which are Poly Aromatic Hydrocarbons (PAHs) generated by partial burning of diverse organic material. Its metabolic activation occurs through cytochrome P_{450} to the 7, 8-dihydrodiol-9, to epoxide i.e. the eventual cancer causing agent which is considered for DNA damage and inducing carcinogenesis (Kasala et al. 2015) [2].

In the Indian medicine system *Ayurveda* there are plethoras of *Jadi Bootis* (medicinal plants) which have potent treatment for disease of lungs. Among them, *Aegle marmelos* or Wood apple has promising role against the various types of diseases such as gastrointestinal disorders, antimicrobial, antiviral, antipyretic, ulcer healing, diuretic, antimicrobial, anti-inflammatory, radioprotective and chemopreventive properties. The *A.marmelos* contains furocomarins, xanthotoxol, and methyl ester of alloimperatorinm flavonoids, rutin and marmesin. The leaves, barks, roots, fruits and seeds are extensively used by the people of Indian subcontinent as traditional or folk medicine for various ailments. Various parts of this tree are used for the ailment of disease including cancer (Akhouri et al. 2020, Baliga et al. 2013 and 2010, Manandhar et al. 2018, Ramakrishna et al.2015, Bhatti et al.2013, Agrarwal et al.2010, Rahman and Parvin 2014) [3-5].

Hence, the present study deals to develop Benzo[a]pyrene induced lung cancer model in Charles Foster rats and evaluate the efficacy of pulp extract of *A.marmelos* against the lung tumors [6].

Materials and Methods

Chemicals and reagents: Benzo[a]pyrene (C20H12) manufactured by Sigma-Aldrich, USA, Product number B1760-100MG, (CAS Number: 50-32-8), Lot# SLBV8459, P code: 1002545809 was purchased from the Scientific chemical store of Patna, Bihar, India and was provided by the Research Department of Mahavir Cancer Sansthan and Research Centre, Patna, India. All the other solvents and chemicals used were of analytical grade 99% [7].

Medicinal plant: As a medicinal plant, *Aegle marmelos* is commonly known as Wood apple or Indian Bael was used in the study. The *Aegle marmelos* fruit were procured from the local market of Patna and were identified and certified by a botanist Prof. Ashok Kumar Ghosh. The seeds and fibre from *Aegle marmelos* fruit were removed and the pulp was extracted and dried in incubator at 37°C. Then grinded in to fine powder and later soaked in absolute ethanol for 48hrs and finally extracted with absolute ethanol using Rota vapour apparatus. The dose of the ethanolic pulp extract of *Aegle marmelos* was titrated to 75mg/kg body weight per day for 5 weeks after the estimation of LD_{50} value [8, 9].

Ethical approval: Before the use of animals, ethical approval was obtained from the Institutional Ethics Committee (IAEC) of the institute through CPCSEA (GoI) with CPCSEA Registration no. 1129/bc/07/CPCSEA. The research work was approved from the IAEC no. 2021/1B-06/10/21 dated 06/10/2021 [10].

Animals: Male Charles Foster rats were provided by the animal house of Mahavir Cancer Sansthan and Research Centre, Patna, India. The rats were housed in standard polypropylene cages housing 02 rats in each case. They were randomly divided into control and treatment groups. The room temperature was maintained at 22 ± 2 °C for rats with 12 hours light/dark cycle and the animals had free access to food and water *ad libidum* [11].

Lung tumor model development: For inducing the lung tumor, rats were treated with Benzo[a]pyrene at the dose of 25 mg/Kg dissolved in Olive oil orally in two intervals (1st day and 14th day) and were left for 3 months. After, 3 months the animals were development of lung tumors which were confirmed through fine needle aspiration and few animals were dissected for lung biopsy for final confirmation of the lung cancer [12].

Experimental design: Thirty male Charles Foster rats, 6 weeks old weighing (150-180 g) were divided into groups of n=6 animals in each.

Group I- Control (n=6)

Group II- Benzo[a]pyrene treated (n=12)

Group III- *Aegle marmelos* treated (n=6)- Upon Benzo[a]pyrene induced treated with *Aegle marmelos* ethanolic pulp extract (250mg/kg body weight per day) for 5 weeks (Group II rats).

Rats were anaesthetized and sacrificed after the completion of the dose. Blood samples were obtained through the orbital puncture of the experimental rats. For biochemical test and lipid peroxidation estimation serum were separated. Tissues of lungs and other organs were fixed in 10% formalin for histopathological studies [13-15].

Biochemical assay: Biochemical analysis were performed through the serum by standard kit process (Coral crest) on (UV - Vis) spectrophotometer (UV-10, Thermo Scientific, USA). The Liver function test (LFT) as serum glutamate pyruvate transaminase (SGPT) and serum glutamate oxaloacetate transaminase (SGOT) were measured according to the method Reitman and Frankel (1957), alkaline phosphatase (ALP) by using method of Kind and King (1954), total bilirubin activity by method Jendrassik and Grof (1938). The kidney function test (KFT) was analyzed through urea by the method of Fawcett and Scott (1960); Berthelot (1859), creatinine by the method of Bones and Tausky (1945) and uric acid by the method of Fossati and Prencipe (1980) [16, 18].

Lipid peroxidation (LPO): Thiobarbituric acid reactive substances (TBARS) are used as markers of LPO, was evaluated through the double heating method (Draper and Hadley 1990) applied on the principle of spectrophotometric measurement of colour reproduced during the reaction to thiobarbituric acid (TBA) with malondialdehyde (MDA). In this study, 2.5ml of 10% solution of Trichloroacetic acid (TCA) was mixed with 0.5ml serum in a centrifuge tube then heated in water bath for 15 minutes at 90°C. Then after, the mixture was left for cooling at room temperature, the mixture was further centrifuged at 3000rpm for 10 minutes. The 2ml supernatant was mixed with 1 ml of 0.675% TBA solution in test tube, which was again heated in water bath for 15 minutes at 90°C. The solution was left for cooling at room temperature. Absorbance was further measured by UV-Visible spectrophotometer (Thermo Scientific UV-10 USA) at 532nm [19-22].

Superoxide dismutase (SOD) activity: The epinephrine technique Misra et al., (1972) was used to measure SOD activity in the supernatant. The technique relies on measuring the rate of epinephrine auto-oxidation inhibition by SOD contained in the examined samples in 50 mM sodium carbonate buffer pH 10.2, within the linear range of auto-oxidative curve and U/mg protein units were used to express the SOD activity [23].

Catalase (CAT) activity: The catalase (CAT) activity was analysed

by using the technique of Goth et al., (1991). During this procedure serum samples were incubated in substrate containing 65 µmol/ml hydrogen peroxide in 60 mmol/l sodium-potassium phosphate buffer, pH 7.4 for 60s at 37 °C, Under these conditions one unit of CAT decomposed in 1 µmol of hydrogen peroxide (H2O2) per minute. Then and there 32.4 mM (NH4)2MoO4 (Ammonium molybdate) were used to stop enzymatic reaction and the yellow complex of molybdate and hydrogen peroxide was detected at 405 nm in comparison to a blank that contained all the components except the enzyme [24, 25].

Histopathological study: Lung tissues were fixed into 10% formalin for 24hours. Then tissues were dehydrated through graded series of ethanol and embedded into paraffin wax. The 4.5µm section were cut and stained by haematoxylin and eosin for histopathological study under light microscope (Cardiff et al., 2014) [26].

Statistical analysis: Results are presented as mean ± standard deviation (SD) for six rats in individual groups. Total variation represented in a set of data were analyzed through one-way analysis of variance (ANOVA) followed by Tukey's test with multiple comparisons ($p<0.05$ was statistically considered). Calculations were performed with the GraphPad Prism program (GraphPad 5 Software, Inc., San Diego, USA) [27].

Results

Morbidity and Mortality: In Benzo[a]pyrene treated group, there was mild mortality observed in the group. However, mild sluggishness was observed at the end of the treatment. Severe breathlessness was observed in every group which gradually decreased on administration of *Aegle marmelos.*

Effect of *A.marmelos* on liver functional test (LFT)

In comparison to the control group, in Benzo[a]pyrene treated group there was significant ($p<0.0001$) rise in the levels of SGPT, SGOT, ALP, and total bilirubin. But, after the treatment with the hydroxyethanolic pulp extract of *A.marmelos* there were significant ($p<0.0001$) decrease in the serum levels of SGPT, SGOT, ALP, and total bilirubin. The evidences suggests that *A.marmelos* has protective effect against Benzo[a]pyrene induced hepatotoxicity [28] (**Table 1**).

Effect of *A.marmelos* on Kidney functional test (KFT)

In comparison to the control group, in Benzo[a]pyrene treated group there was significant ($p<0.0001$) rise in the levels of urea, uric acid, creatinine, and albumin. But, after the treatment with the hydroxyethanolic pulp extract of *A.marmelos* there were significant ($p<0.0001$) decrease in the serum levels of urea, uric acid, creatinine, and albumin. The evidences suggests that *A.marmelos* has protective effect against Benzo[a]pyrene induced nephrotoxicity [29] (**Table 2**).

Table 1. Showing Liver function test data.

Parameters	Control	Benzo [A] pyrene Treated	A.marmelos Treated for 5 Weeks
SGPT (U/mL)	35.89 ± 3.7	189.23± 8.67*	100.4 ± 7.25*
SGOT (U/mL)	37.56 ± 2.8	210.29± 9.32*	93.26± 1.67*
ALP (KA units)	9.57 ± 1.06	39.87 ± 1.56**	13.25± 1.74**
Bilirubin (mg/dL)	0.95 ± 1.79	3.12 ± 0.5*	1.9 ± 1.99**

Effect of *A.marmelos* on Lipid peroxidation (LPO)

The serum level LPO is significantly higher ($p<0.0001$) in Benzo[a] pyrene treated rats in comparison to the control group. However, there was substantial ($p<0.0001$) decrease observed after the hydroxyethanolic pulp extract of *A.marmelos* administration had significant changes compared to control. This suggests the antioxidant potential of pulp extract of *A.marmelos* [30] (**Figure 1**).

Effect of *A.marmelos* on Superoxide Dismutase (SOD)

The SOD activity significantly decreased ($p<0.0001$) in Benzo[a] pyrene treated rats in comparison to the control group. However, there was substantial ($p<0.0001$) increase observed after the hydroxyethanolic pulp extract of *A.marmelos* administration had significant changes compared to control. This suggests the antioxidant potential of pulp extract of *A.marmelos* [31] (**Figure 2**).

Effect of *A.marmelos* on Catalase (CAT) activity

The CAT activity significantly decreased ($p<0.0001$) in Benzo[a] pyrene treated rats in comparison to the control group. However, there was substantial ($p<0.0001$) increase observed after the hydroxyethanolic pulp extract of *A.marmelos* administration had significant changes compared to control. This suggests the antioxidant potential of pulp extract of *A.marmelos* [32] (**Figure 3**).

Histopathological study

The histopathological study shows normal architecture of alveolar sacs in the tissue of lung. The parietal and visceral layers appear to be with normal functioning of the lung (Figure 4A). In the Benzo[A]pyrene treated rats shows lung cells with papillary tubulo-alveolar carcinoma (Figure 4B). But, after the treatment with the pulp extract of *A.marmelos* there has been significant reversal in the cells of lung. However, the residual disease is mildly still persistent. (Figure 4C and 4D).

Showing microphotograph of lung tissue sections of **(A)** Control lung section showing normal architecture of alveolar sacs x 500 **(B)** Benzo[a]pyrene treated lung showing papillary tubulo-alveolar carcinoma x 500 **(C) & (D)** *A.marmelos* treated group showing significant normalization in the lung tissue with least residual disease x500 [33-36].

Discussion

Benzo[A]pyrene is the most common environmental carcinogen

Table 2. Showing Kidney function test data.

Parameters	Control	Benzo [A] pyrene Treated	A.marmelos Treated for 5 Weeks
Urea (mg/dL)	32.15 ± 2.67	70.18 ± 2.67**	46.33 ± 1.78**
Uric acid (mg/dL)	3.74 ± 0.84	17.95 ± 2.55**	9.67 ± 2.06*
Creatinine (mg/dL)	0.68 ± 0.92	5.27 ± 2.05*	1.97 ± 1.28*

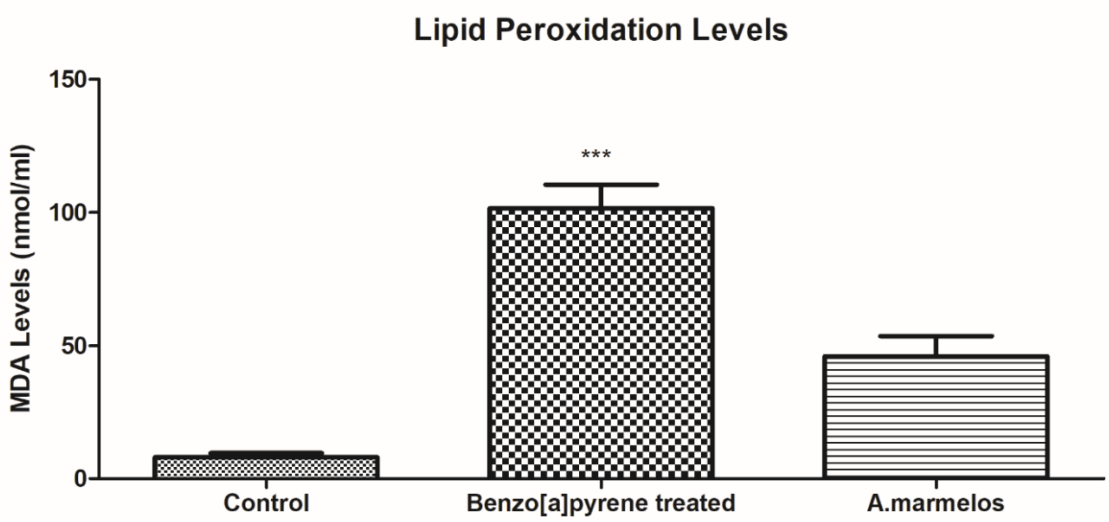

Figure 1 Showing levels of lipid peroxidation activity in different treatment groups.

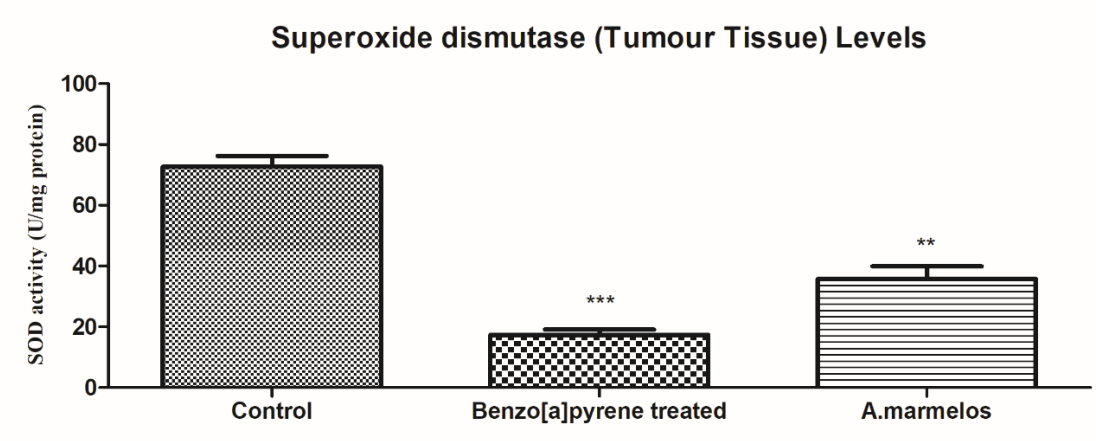

Figure 2 Showing levels of Superoxide dismutase activity in different treatment groups.

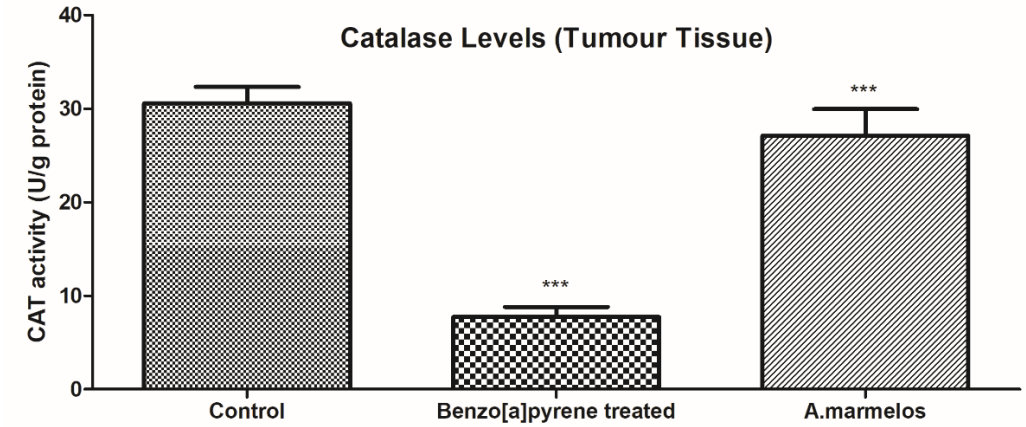

Figure 3 Showing levels of Catalase activity in different treatment groups.

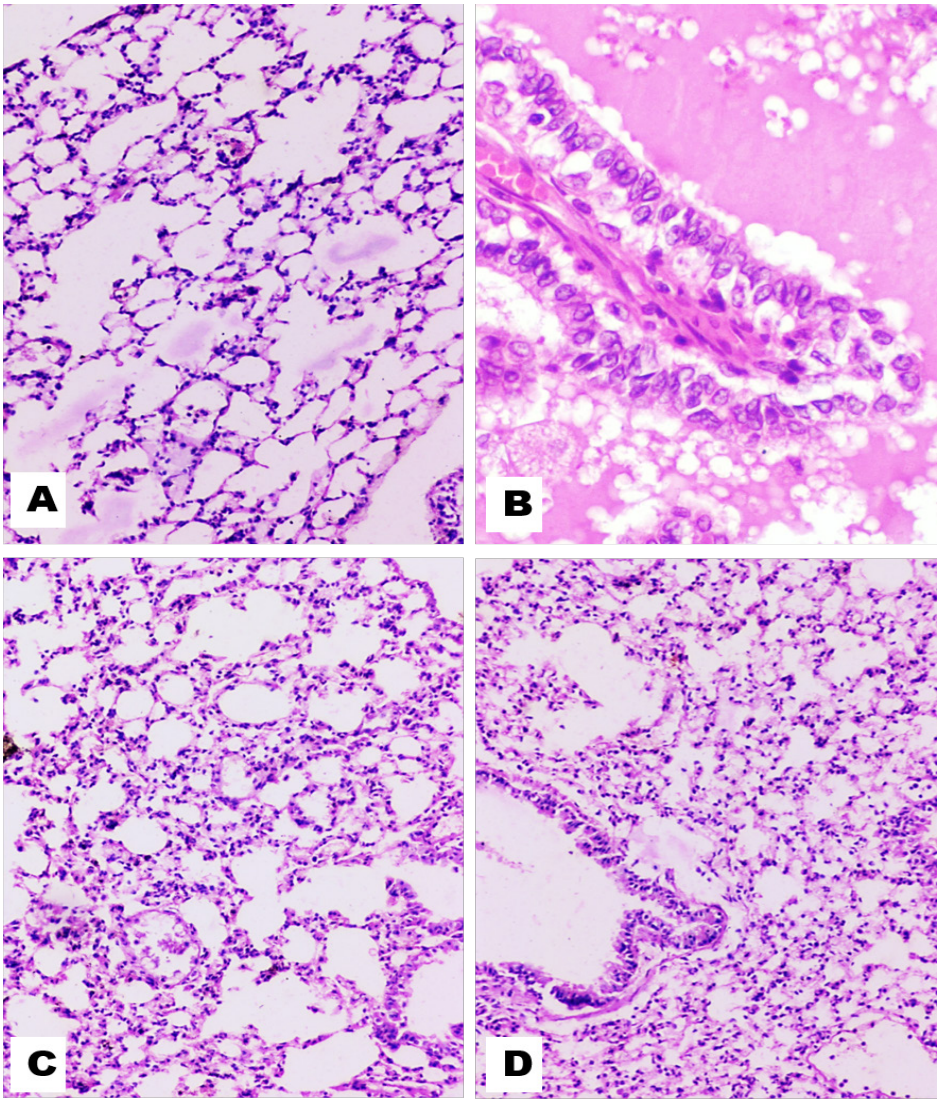

Figure 4 Showing microphotograph of lung tissue sections of (A) Control lung section showing normal architecture of alveolar sacs x 500 (B) Benzo[a]pyrene treated lung showing papillary tubulo- alveolar carcinoma x 500 (C) & (D) A.marmelos treated group showing significant normalization in the lung tissue with least residual disease x500.

to which humans are exposed to it frequently. In the present study, the lung tumor formed after the treatment with benzo[A]pyrene in the tissue was papillary tubulo-alveolar carcinoma, which is the prominent disease model formed in the present study. The benzo[A]pyrene exposure usually causes activates and increases the expression of the histone H3 lysine 9 methyltransferase. Moreover, it also suppresses the function of the tumor suppressor gene SOCS3 disrupting the functions of the caspase series and furthermore activating the Akt and Erkl/2 pathways leading to tumorigenesis in lungs. The similar model formation was made by various research workers (Wang et al., 2020; Martin & Fry 2018; Baltayiannis et al., 2008 Wang 2021; Gu et al., 2018; Chen et al., 2016) [37].

In the present study, Beanzo[A]pyrene induced lung tumor model was developed and was confirmed histopathologically, as there was significant papillary tubulo- alveolar carcinoma formed in the lung. But, after the treatment with *A.marmelos* fruit pulp, there was significant reversal in the lung cells as the normal alveolar sacs were clearly observed. This result confirms that fruit pulp of *A.marmelos* contains anti-carcinogenic activities. The fruit pulp contains active ingredients such as marmelosin, lupeol, eugenol, citral, cineole and limonene which have probably anti-cancer effects [38]. Similar, *A. marmelos* extract effect have been reported as anticancerous effect in various models (Akhouri et al., 2020; Sain et al., 2014). Pynam and Dharmesh., 2018, have discovered its potent activity as antioxidant and anti-inflammatory in inhibition of TNF- alpha mediated tumor model. They have found marmelosin as the potent anti-tumor compound controlling the TNF-alpha mediated Akt signaling pathway. Agrawal et al., 2011, have reported the anticancerous effect against the chemically induced skin cancer in mice models [39].

Furthermore, in the present study there has been significant decrease ($p<0.05$) in the antioxidant effects in Benzo[A]pyrene treated group in comparison to the control group. There was

significant decrease ($p<0.05$) in the superoxide dismutase levels and catalase levels, while significant increase ($p<0.05$) in the lipid peroxidation levels. But after the treatment with the fruit pulp extract of *A.marmelos,* there was significant ($p<0.05$) normalization in the levels of superoxide dismutase, catalases and lipid peroxidation levels, The fruit pulp of *A.marmelos* contains flavonoids, vitamin B and C, thus shows the antioxidant, inflammatory, anti-ulcer activity. Various authors have correlated their study with the effect of pulp extract of *A.marmelos* (Agrawal et al 2011; Chaubey and Dubey 2020; Ahmad et al., 2021; Mujeeb et al., 2018; Venthodika et al., 2021) [40].

In the present study, to observe the effect of drug side effects, the vital organs such as liver and kidneys biochemical parameters were evaluated, which showed significant ($p<0.05$) increase in the levels of SGPT, SGOT, ALP bilirubin, urea and uric acid and creatinine levels but after the administration of the pulp extract of *A.marmelos*, there was significant ($p<0.05$) normalization in the levels in the liver and kidney biochemical parameters except SGPT, SGOT, bilirubin and creatinine levels. However, there was significant ($p<0.05$) decrease in the levels in comparison to the Benzo[A]pyrene treated group but still was higher than the normal ranges [42]. It could be probably due to the severe damage caused by the Benzo[A]pyrene and the anti-inflammatory activity in the liver and kidney organs might be slower as they are the metabolic organs. Furthermore, it is quite possible that if the dose of the pulp extract of *A.marmelos* could have increased for more duration, there could have been significant normalization in the biochemical parameters level. However, the significant decrease in the liver and kidney functions could be due to the presence of flavonoids which proves to be the major active constituent having the hepato-renal protective effect. The moderate regeneration is the vital effect of A.marmelos. Various other researchers have found the hepato-renal protective effect of *A.marmelos* (Sharma et al., 2022; Yang et al., 2020; Rajasekaran et al., 2009; Patel et al., 2012; Baliga et al., 2011; Dwivedi et al., 2017) [42].

Conclusion

From the entire study, it can be concluded that, Benzo[A]pyrene causes formation of lung tumors in Charles Foster rats, but there was significant reduction in the lung cancer disease due to the fruit pulp extract effect of the *A.marmelos.* Moreover, there was significant normalization in the free radicals levels-lipid peroxidation, superoxide dismutase and catalase activity. But, in the studied biochemical parameters of liver function tests and kidney function tests, there was significant normalization in the levels of ALP, urea and uric acid but moderate decrease in the levels of SGPT, SGOT, bilirubin and creatinine levels. The study finally concludes that *A.marmelos* possesses anticancerous, antioxidant and moderate hepato-renal protective activity. Furthermore duration of *A.marmelos* fruit pulp extract could have made more protection against liver and kidney organs. Hence, this drug has therapeutic anticancerous property against lung cancer.

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Competing Interests

The authors declare that they have no conflicts of interest.

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