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Therapeutic effect of *Azadirachta indica* (L.) flowers ethanolic extract (AIEE) against DMBA induced Breast cancer in Swiss Albino mice

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Abstract

Most often, aberrant cell proliferation and subsequent tumour formation are the causes of cancer development. Globally, millions of people are estimated to die from various types of cancer. Among various types of cancer, breast cancer is the most prevalent type in women throughout the world. It continues to have a high death rate even with different treatment approaches. Because of the complex natural etiology of breast cancer, its treatment is much challenging. Owing to substantial treatment hurdles, natural chemicals are being investigated as possible substitutes. The plant *Azadirachta indica* (L.); is extensively recognised since ancient times for various therapeutic uses in Ayurveda, Unani, and homeopathic medicine system. Scientifically, the phytoremedial compounds and anticancer properties of this plant have been widely studied in term of its preventive, protective, tumour-suppressive, immunomodulatory, and apoptotic actions against different cancers and their molecular processes. The present study focuses on therapeutic effect of *Azadirachta indica* (L.) flowers ethanolic extract (AIEE) against DMBA induced breast cancer in Swiss albino mice. Eighteen female mice weighing 30 ± 5 g were divided into two groups. Group-I: control (n=6) and Group-II: (n=12); were orally induced by DMBA (3 mg/ml dissolved in olive oil) at an interval of fourteen days until breast tumours (about 2.0 cm) were developed. Then after, Group-II was further divided into two sub-groups. Sub-Group-I; (n=6) as DMBA-Treated were sacrificed at same time and sub-group-II (n=6) were orally administered with AIEE (100mg/Kg body weight) for 5 weeks, and the last tumour volume were measured. The study results that, the mammary tumour volume significantly ($p<0.05$) reduced. Moreover, there was significant alteration ($p<0.0001$) observed at the level of hepatic and renal biomarkers. Conclusively, entire study depicts that AIEE administration possesses anti-proliferative activity by suppressing progression of breast tumour in mice model. AIEE administration also possesses significant ($p<0.0001$) improvement in the hepato-renal parameters. Therefore, *Azadirachta indica* flowers can be targeted as a novel and safe anti-cancer drug against breast cancer.

Keywords: DMBA, *Azadirachta indica* flowers, breast tumour, Swiss albino mice, haematological, biochemical and histopathology.

Introduction

Cancer is known as multifactorial genetic disorder that is caused by uncontrolled cellular division in addition with abnormal cellular proliferation in body tissues and organs (Kashyap and Dubey, 2022). The cancerous cells can either reside in fluids, as in case of leukaemia or they can invade nearby tissues and spread through the blood lymph system to form colonies in other parts of the body (Follain, et al., 2020). When these cells migrated to other regions in the body, the cancer is disseminated or metastasized. Often, various types of cancer are originated in the body that are named by their location, such as lung, colon, breast, skin, ovarian or prostate cancer (Saini, et al., 2020).

Globally, breast cancer is the most common cancer among women, with an annual-increases in new cases. Over the last three decades, the incidence and mortality of breast cancer have increased in both developing and developed countries (Lei, et al., 2020). Cancer statics reveals that from global record; one in every six death is cancer related where women mostly affected through breast cancer. Given the significant disease burden associated with breast cancer, it is essential to identify risk factors and preventive intervention that can reduce cancer incidence (Arnold, et al., 2022). Epidemiological research has demonstrated the importance of genetic susceptibility and family history, in addition to a range of hormonal and reproductive exposures that may increase breast cancer risk. Role of lifestyle, and dietary factors has also been explored but preventive strategies are limited. Reducing exposure to environmental toxins through dietary regulation and public health policies is one understudied potential preventive technique (Martin, et al., 2022; Turner, et al., 2020).

According to International Agency for Research on Cancer (IARC-2020) globally around 2.3 million newly female breast cancer were diagnosed (Lei, et al., 2020). The genetic factor, age, and the exposure of toxicant are some important factors that increases the rate of breast cancer, such as heavy metal toxicants, exposure to polycyclic aromatic hydrocarbons resulting from burning coal, factories, and automobile fuel (Ali, et al., 2024; Hussain, et al., 2020). Among carcinogenic polycyclic aromatic hydrocarbons is 7, 12-dimethylbenz (a) anthracene (DMBA), which is used to cause breast cancer in experimental mice (Gan, et al., 2019). The mice model is the most common model of breast cancer which develops in mice mimics' breast cancer that develops in humans. In the breasts, this carcinogen is activated into dMBA-3, 4-diol-1,2-epoxide (DMBA-DE disrupting and interrupting of tissue redox balance, which leads to oxidative stress (Hamza, et al., 2022; Akhouri, et al., 2020; Gan, et al., 2019). The outputs of reactive oxygen species (ROS) cause damage to DNA or a protein cell cycle and may lead to uncontrolled division and growth of cells. There is an increase in estrogen receptor-positive cancer incidences and a decrease in estrogen receptor-negative cancer incidences. The estrogen receptor-positive cancer is under the influence of estrogen, which helps to increase the proliferation of cancer cells (Huang, et al., 2021; Hua, et al., 2018).

The treatment of breast cancer includes numerous drugs that protect the DNA, prevent the cell cycle and cell division, increases programmed death, and stop some of the pathways that cause abnormal growth of cells, such as expression of estrogen receptors (Rajan, et al., 2021; Mitra, et al., 2018). Phytochemicals as limonoids, azadirone, azadirachtin, flavonoids and some other derivatives present in *A.indica* (L.) has promising options for improving treatment efficiency and reducing adverse reactions in cancer patients (Moga, et al., 2018). Numerous studies have shown that, *A.indica* (Neem) possesses anti-inflammatory, antiarthritic, antipyretic, hypoglycaemic, antigastric, ulcer, antifungal, antibacterial, and antitumour activities (Sarkar, et al., 2021). The anti-neoplastic properties of *A.indica* (Neem) are gaining attention due to its cancer preventive, tumour-suppressive, anti-proliferative, apoptosis-inducing, anti-angiogenic, and immuno-modulatory effects via several molecular mechanism. The *A.indica* (Neem) or its derivatives have been shown to exert their antioxidant properties by decreasing TNF-α, increasing IFN-γ, and modulating antioxidant enzyme such as glutathione S-transferase (GST) and certain hepatic cytochrome P450-dependent mono-oxygenises. Its properties induce apoptosis cyclin B, cyclin D1, p53, and proliferation with chemotherapeutic drug like cyclophosphamide, cisplatin, 5-fluorouracil, or with radiotherapy. It potentiates their antitumour effect by activating pro-apoptotic signalling and negating survival signalling along with attenuating their side effects (Reddy, et al., 2022; Sarkar, et al., 2021, Singh, et al., 2017).

Notably, cisplatin is often used to treat solid cancers. It is the first agent in a series of anti-cancer drugs that contain platinum, nephrotoxicity, nausea, and vomiting, ototoxicity (hearing loss), electrolyte disruption, haemolytic anaemia, and other adverse effects may restrict the use of cisplatin (Brown, et al., 2019). Moreover, most cancer patients eventually experience cisplatin-resistant disease, which calls for combination therapy-a technique that combines various chemotherapeutic drug with chemo-preventive medications-to treat their illness (Coffetti, et al., 2023; Zheng, et al., 2017). Hence, at the above-mentioned facts present study focuses on therapeutic potential of *Azadirachta indica* (L.) flowers ethanolic extract (AIEE) against DMBA induced breast cancer in Swiss albino mice.

Materials and Methods

Chemicals and reagents

The chemical 7,12-dimethylbenz(a)anthracene (DMBA) were purchased from the licenced scientific chemical store Patna, Bihar, India. The chemical details were as follows: product code: 1009330344, Product number: D3254-1G, Lot number: PXLNG2901, and CAS number: 57-97-6. Here is the product code: 1009330344. None of the other substances were used in this study deviated from the standard of 99% purity.

Azadirachta indica (L.) Ethanolic Extract (AIEE) preparation:

Flowers of *Azadirachta indica* (Neem) were collected from the Patna Women's College, Patna campus and recognised by the famous Botanist of Patna University, Patna (Bihar), India. The flowers were washed, shrivelled from blotting paper, and lastly air dried at 37ºC. After that, flowers were ground and let them soak in 100% ethanol for 24 h. The mixture of ethanol- flowers extract was filtered to eliminate any remaining particles. The filtrate was then subjected to ethanol extraction in rota-vapour apparatus. After the completion of extract preparation dose was calculated by LD_{50} estimation and the final doses was titrated to 100mg/kg body weight.

Animals

Eighteen female mice weighing 30 ± 5 g were randomly selected which were provided by Mahavir Cancer Sansthan and Research Center's animal house, Patna (Bihar), India (CPCSEA Registration no. 1129/PO/ReBi/S/07/CPCSEA). The work of this experiment was approved by the Institutional Animal Ethics Committee (IAEC) with IAEC No. 2021/ID-06/10/21. All of the animal experimentation method was followed as per the guidelines of Committee for the Protection and Control of Experiments on Animal (CPCSEA), New Delhi, India. The mean of RT (room temperature) where animal was housed was maintained at 22 ± 2ºC with 12h light/dark cycle. Self- made notorious food and water were provided to the mice at *ad libidum*. All experimental mice were housed in conventional polypropylene case in small group (2 each) and were acclimatized under the laboratory housing conditions for 15 days prior to the beginning of the treatment.

Animal Grouping: The collected entire eighteen female mice weighing 30 ± 5 g were randomly selected and divided into two group. Group-I: control (n=6) and Group-II: (n=12); were orally induced by DMBA (3 mg/ml dissolved in olive oil) at an interval of fourteen days until breast tumours (about 0.5cm) were developed. Then after, Group-II were further dived into two sub-group. Sub-Group-I; (n=6) as DMBA-Treated were sacrificed at same time and sub-group-II (n=6) were orally administrated with flowers AIEE (100mg/Kg body weight) for 5 weeks, in last tumour volume were measured.

After the completion of entire treatment all group of mice were anaesthetized with ketamine and sacrificed during the diestrous phase of their estrous cycle. By using the orbital puncture technique blood was collected in EDTA coated and plain vacutainers. Serum was separated to analyse biochemical, inflammatory markers, and lipid peroxidation parameters.

Tumour induction

Mammary gland tumour ware induced in female Swiss Albino mice (25± 5 g). All these rodents which is used in this experiment were around 55 days old. As per the methodology of Liu, et al., (2017); single dose of 7,12-dimethylbenz(a)anthracene (DMBA) dissolved in olive oil at a concentration of 15mg/Kg body weight was delivered intragastrically. Except control group the rest mice were palpitated weekly in the beginning in the fourth week after DMBA injection to monitor tumour progression. Twenty weeks later, every single one of the twelve DMBA-Treated mice had developed tumours. The first tumour was exposed at 19th week.

Volume evaluation of mammary tumour

Volumes of the breast tumour was measured by using vernier calliper; where L and B are listed for perpendicular tumour diameters in centimetres (cm). The tumour's volume was calculated from V (cm^3) = (L B2)/2.

Haematological assay

The haematological examination as RBCs (red blood cells) count, WBCs (white blood cells) count, PLT (platelets) count, and HGB (haemoglobin) percentage were performed through the whole blood samples; analysed by using fully automated haematological analyser (BC 2800, Mindray China).

Biochemical assay

The biochemical analysis was performed through serum by standard Coral crest kit process on (UV-Vis) spectrophotometer (UV-10, Thermo Scientific, USA). For the liver functional test SGPT (serum glutamate pyruvate transaminase), SGOT (serum glutamate oxaloacetate transaminase) were measured by the method of Reitman and Frankel (1957), and ALP (alkaline phosphatase), were measured by Kind and King (1954) technique. For the Kidney functional test; urea level was analysed by Berthelot (1859) and, Fawcett & Scott (1960) method. Uric acid were measured by Fossati and Prencipe (1980), and the level of creatinine were measured by Bones and Tausky (1945) method.

Histopathological assay

Surgically, mice breast tissues were removed, sectioned, and preserved in 10% formalin for 24h. Thereafter, these breast tissues were dehydrated through graded ethanol concentration and embedded into paraffin. All paraffin embedded block was grossed at 5µm thickness through digital rotatory microtome (Microme HM, 340E, Thermo Scientific, USA), and stained with haematoxylin and Eosin (H&E) for further investigation of histopathological changes.

Statistical analysis

All the experimental outcomes are presented as mean ± standard deviation (SD) for six mice of individual groups. Total variations were represented in a set of data and were analysed through one way analysis of variance, (ANOVA) followed by Turkey's test with multiple comparisons. Value of $p < 0.05$ were considered as statically significant. Calculations were performed with the GraphPad Prism 5 (GraphPad Software, Inc., San Diego, USA).

Results

Morbidity and mortality

The six rats which were treated with DMBA all developed tumors in the region of their mammary teats 1, 3, 5, and 7. The remaining six rats from teats 1, 2, 3, and 5 were severely inhibited in their tumor development by the DMBA + *Azadirachta indica* group. Among all of the groups, there were no fatalities. Figure 1 is a graphical representation of the DMBA group as well as the DMBA + *Azadirachta indica* group **(Figure 1).**

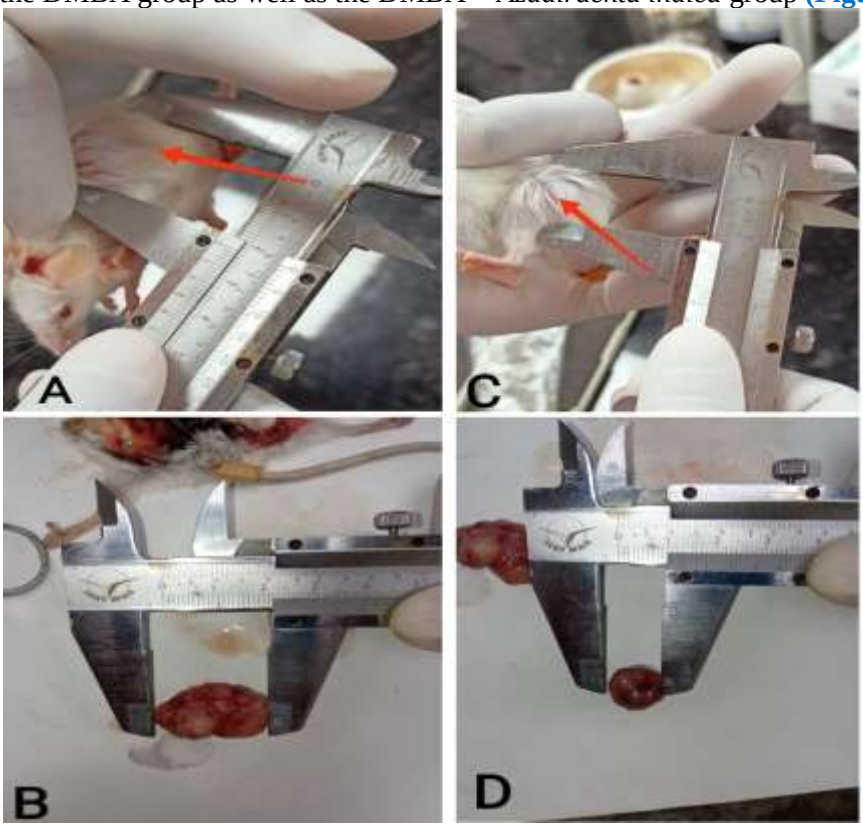

Figure 1: Photographs of rat mammary tumors, **1A** & **1B** of DMBA group (single dose of DMBA at 20 mg/mL in olive oil) and **1C** & **1D** of DMBA + *Azadirachta indica* group. *Azadirachta indica* was administered at the dose of 100 mg/kg body weight per day for 5 weeks after about 2.0 cm tumour development).

Evaluation of Tumour Volume: In the study, tumour size in the DMBA treated group increased significantly. The tumour volume was considerably decreased ($p<0.005$) in DMBA-induced rats when *Azadirachta indica* ethanolic flowers extract was given in the treatment, as shown in Figure 2, when compared to the treatment with DMBA alone. The ultimate outcome was a 48% reduction in tumour volume due to the *Azadirachta indica* flowers extract **(Figure 2)**.

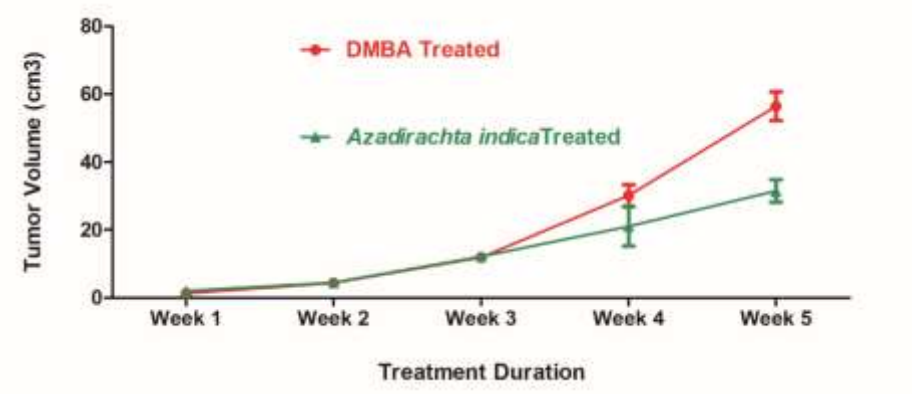

Figure 2: Effect of different treatments on tumour volume in the studied groups. DMBA group (single dose of DMBA at 20 mg/mL in olive oil), DMBA + *A.indica* group (*Azadirachta indica* at 100 mg/kg body weight per day for 5 weeks after about 2.0 cm tumour development). Values are expressed as mean ± SEM, n=6)

Effect on Haematological parameters

The haematological outcomes of DMBA-Treated mice showed significant ($p<0.0001$) reduction in the level of HGB, RBCs, WBCs, and PLT than the control group of mice, whereas the AIEE administration had significant ($p<0.0001$) restoration to normal. **[Table – 1]**

Table – 1: Haematological parameters of various mice group (n =6; values expressed as mean ± SD).

Parameters	**Control**	**DMBA Treated (2 Doses at the interval of 1 month)**	**AIEE administration at 100mg/kg body weight for 1 month**
RBCs Count ($\times 10^6 mm^3$)	8.23± 1.75	3.47 ± 1.93	6.90 ± 2.45
WBCs Count ($\times 10^3 mm^3$)	8.60 ± 6.36	3.25 ± 8.25	9.50 ± 7.34
PLT ($\times 10^5 mm^3$)	2.4 ± 1.53	1.7 ± 1.11	1.9 ± 0.45
HGB (g/dL)	14.2 ± 1.97	8.2 ± 2.56	11.7 ± 2.94

Effect on Biochemical parameters:

In comparison to control group, the DMBA-Treated group had significant ($p<0.0001$) elevation in the serum level of SGPT, SGOT, and ALP. The AIEE administration upon DMBA-treated group showed significant ($p<0.0001$) restoration in the serum level of SGPT, SGOT, and ALP in comparison to DMBA-treated group. **(Figure-3)**

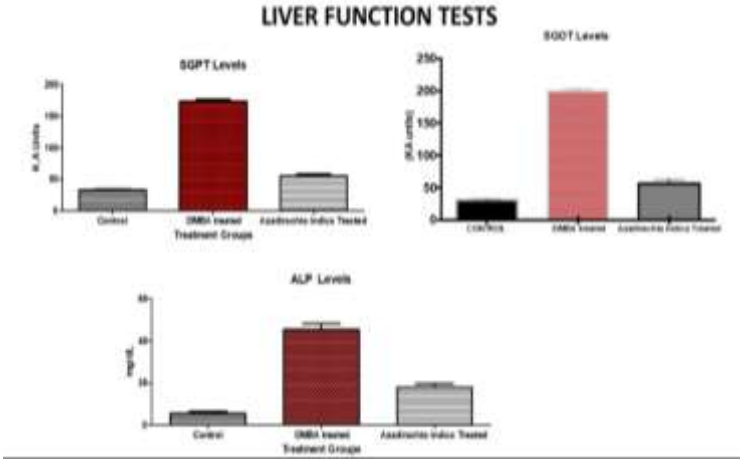

Figure -3: Comparative level of SGPT, SGOT, and ALP in various groups of mice (n=6), value is expressed as mean ± SD.

Compared to control, the DMBA-treated group had significant ($p<0.0001$) changes at the kidney functional biomarkers as urea, uric acid, and creatinine. The outcomes of AIEE administrated group upon DMBA-treated group of mice showed significant ($p<0.0001$) restoration in the serum level of urea, uric acid, and creatinine as compared to the DMBA-treated group of mice. **(Figure-4)**

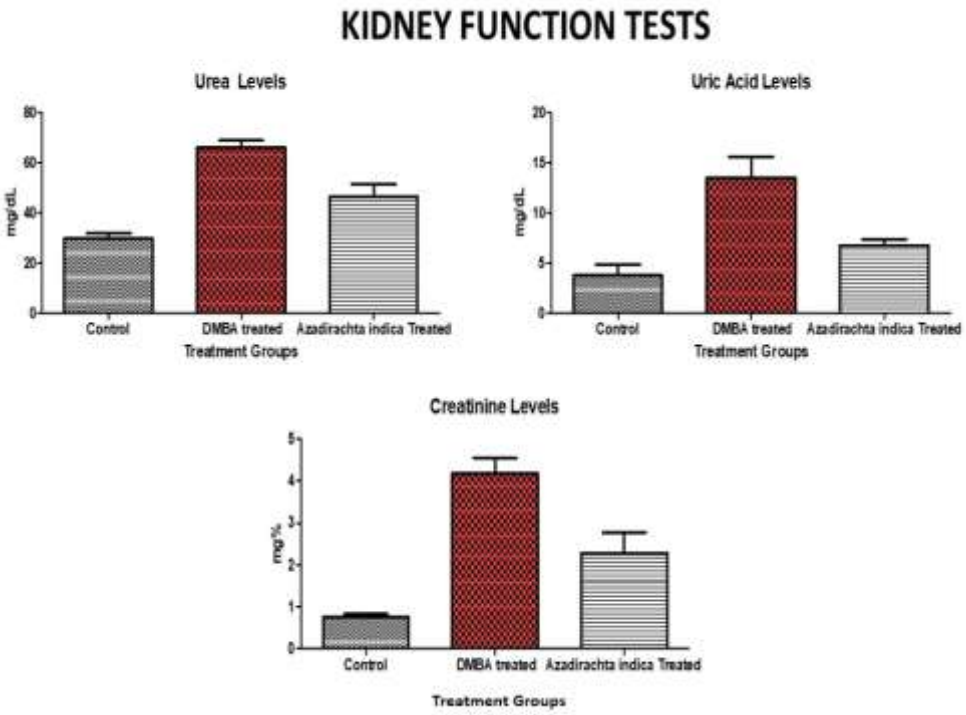

Figure -4: Comparative level of urea, uric acid and creatinine in various groups of mice (n=6), value is expressed as mean ± SD.

Changes in TNF alpha levels: The serum TNF alpha level was significantly higher in the DMBA-treated group compared to the control group ($p<0.05$). The serum TNF alpha level was lower in the DMBA + *A indica* group compared to the DMBA group ($p<0.05$) **(Figure-5).**

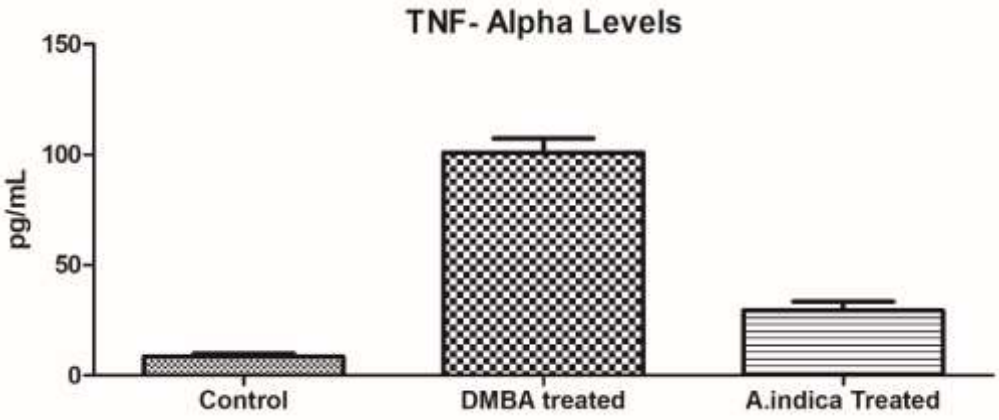

Figure -5: Comparative level of TNF alpha in various groups of mice. The data are presented as mean ± S.E, n = 6, significance at $p< 0.05$.

Changes in VEGF levels: The serum VEGF level was significantly higher in the DMBA-treated group compared to the control group ($p<0.05$). The serum VEGF level was lower in the DMBA + *A indica* group compared to the DMBA group ($p<0.05$) **(Figure-6).**

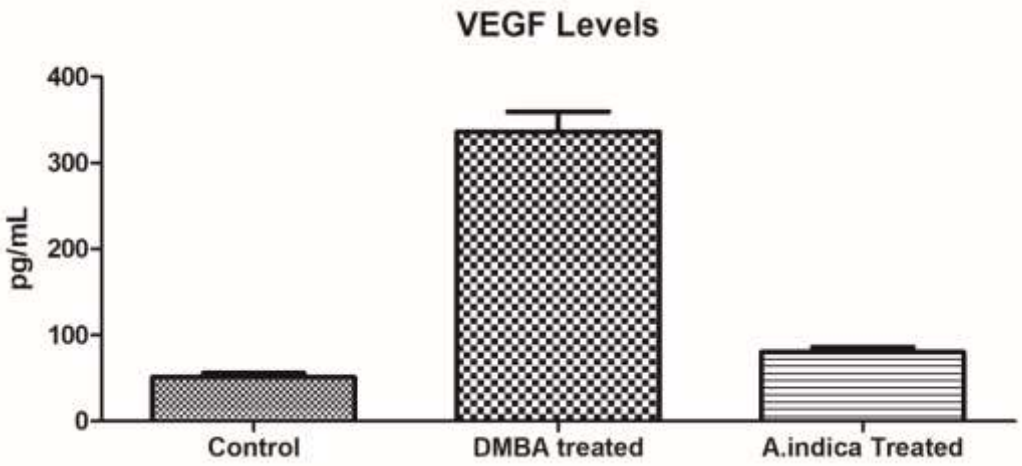

Figure -6: Comparative level of VEGF in various groups of mice. The data are presented as mean ± S.E, n = 6, significance at $p < 0.05$.

Histopathological changes

Histological section of the breast tissue of DMBA (3mg/ml) shows mammary tumours. The section shows presence of mucin in ductal lumen, discontinuous basement membrane with papillary outgrowth, dense and highly granulated cytoplasm and high degree of proliferation. **[Figure-4]**

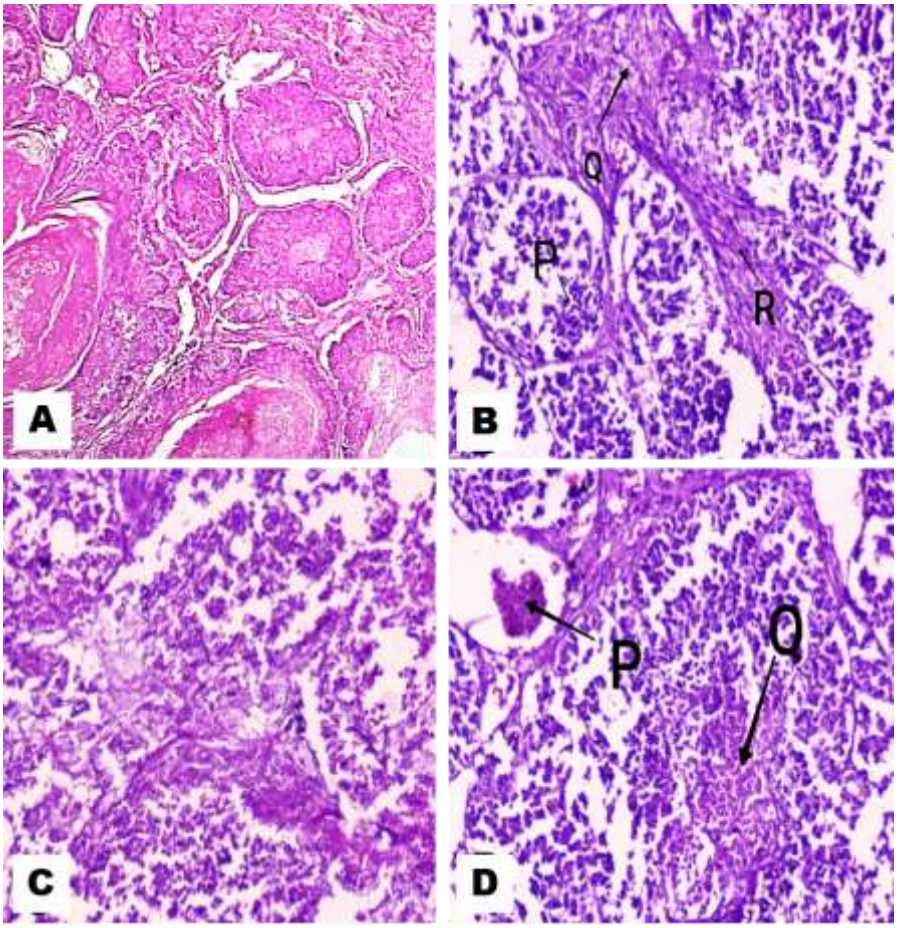

Figure -6: Histological section of tumour of Swiss Albino mice treated with DMBA and stained by Hematoxylin & Eosin **[A]** section of control mice breast having normal arrangement of tissues. **(HE-40x), [B] -** (P) in the ductal, (Q) discontinuous basement membrane & **(R)** presence of mucin (HE-40x), **[C]**- histological section of tumorous tissues. (HE-40x), **[D]**- (P &Q) Ductal normalisation after *A.indica* flower extract treatment (HE- 40x).

Discussion

The cytochrome p450 enzyme initiates the metabolic process that transforms DMBA into its potent carcinogen, DMBA-3,4-dihydrodiol-1,2-epoxide (DMBA-DE). Metabolic processes generate an abundance of reactive oxygen species (ROS), which may alter the tissue's redox balance. One result of lipid peroxidation (LPO) is malondialdehyde (MDA), which is enhanced by these reactive species. Generally, it is understood that elevated levels of malondialdehyde (MDA) are a sign of oxidative stress and antioxidant status in cancer individuals and animal models. In this study, the DMBA group had much greater serum MDA levels than the control group. Nonetheless, the DMBA+ *Azadirachta indica* group showed a significant reduction in serum MDA levels when contrasted with the DMBA group. Reducing malondialdehyde (MDA) levels is one way to measure the antioxidant capability of *Azadirachta indica* ethanolic flowers extract. The antioxidant properties of *Azadirachta indica* are due to its main phytochemical components. Since uncontrolled cell growth results from a redox imbalance, the antioxidant properties may have helped maintain the cells' redox state, which is often disturbed by carcinogen metabolism. An important pharmacological and therapeutic chemical, it contains the phytochemicals limonoids, azadirone, azadirachtin, flavonoids antioxidant, and glucosyl xanthone, all of which have significant antioxidant, anti-lipid peroxidation, immunomodulatory, cardiotonic, hypotensive, wound healing, antidegenerative, and antidiabetic activities (Tripathi et al., 2012; Mukherjee et al., 2014; Arumugam et al., 2016; Lee et al., 2017; Chaube et al., 2014; Sahu et al., 2017).

Translocation of nuclear factor-kB (NF-kB), a transcription factor involved in the survival and proliferation of neoplastic cells, into the nucleus is facilitated by tumor necrosis factor-alpha (TNF), a pro-inflammatory cytokine that is upregulated during the carcinogenic process induced by DMBA. One of the main factors that propels the progression of breast cancer is elevated TNF-levels. The present study revealed that serum TNF-levels were much greater in the DMBA group when contrasted with the control group. The DMBA+ *Azadirachta indica* group, however, had significantly lower serum TNF-levels than the DMBA group. A decrease in blood TNF-level is indicative of the anti-inflammatory properties of *Azadirachta indica* ethanolic flowers extract. It has also been shown that flavonoids and azadirachtin have important roles in regulating cell proliferation via their anti-proliferative actions on T cells.

Although the DMBA + *Azadirachta indica* group showed a tendency toward a reduced breast tumor volume compared to the DMBA group at the conclusion of the trial, the difference was statistically significant. Nevertheless, a tumor development inhibition of up to 48% was seen in the last week of treatment. It is quite likely that the medicinal plant group would have experienced a significant reduction in breast tumor volume had treatment been allowed to be prolonged (Arora et al., 2013; Ruslie and Darmadi 2020; Kandhare et al., 2017; Bharti et al., 2014; Elumalai et al., 2022; Kim et al., 2012; Pooladanda et al., 2019; Abdel-Gaber et al., 2023).

A large number of effective anticancer drugs are available today is encouraging, but many of these drugs come with significant side effects that may impact many different vital parts of the body, including the kidneys and liver. Therefore, it is of the utmost importance to assess how the *Azadirachta indica* flower extract affects the operation of vital organs like the kidneys and the liver. The detoxification process for xenobiotic compounds, such as DMBA, mostly occurs in the liver. Metabolic stress and liver damage were outcomes of the chemical carcinogen's metabolism. The DMBA group had significantly greater serum ALT, AST, and ALP levels than the other two groups. An increased serum hepatitis biomarker is a sign of liver damage. The DMBA + *Azadirachta indica* group had significantly lower blood total bilirubin, ALT, AST, and ALP levels than the DMBA group. The DMBA + *Azadirachta indica* group demonstrated reduced blood levels of liver biomarker tests, indicating that the ethanolic flowers extract of *Azadirachta indica* possesses hepatoprotective characteristics. There is much documentation of comparable research on other models (Koul et al., 2014; Haque et al., 2006; Bhanwra et al., 2000; Rahman et al., 2001; Rahman & Siddiqui 2004).

The kidney is an important organ because it filters out metabolic waste and makes substances the body needs. Because renal impairment might decrease the excretion and metabolism of chemotherapeutic medicines, it is possible that they have increased systemic toxicity. A number of renal indicators, including urea, creatinine, and uric acid, were shown to be considerably higher in the DMBA group. The elevation in kidney biomarkers demonstrates the nephrotoxic consequences of DMBA. Compared to the DMBA group alone, the DMBA + *Azadirachta indica* group showed a much larger reduction in serum renal indicators such as urea, creatinine, and uric acid. The fast restoration of serum kidney biomarker levels is another indication of the therapeutic effects of *Azadirachta indica* flower extract against DMBA-induced renal injury in mice. There is much documentation of comparable research on other models (Seriana et al., 2021; Abdel Moneim et al., 2014; Omóbòwálé et al., 2014; El-Beltagy et al., 2021; Hsieh et al., 2015; Srivastava et al., 2007).

Histopathological analysis of *Azadirachta indica* ethanolic flowers extract revealed anti-proliferative properties. The DMBA group showed signs of accelerated tumor growth in their mammary tissue sections, such as papillary projections, cystic dilatation, cellular sheet development, pleomorphism, and patches of embryonic mesenchymal cells, which are typical of tubular-papillary carcinoma of the breast. The DMBA + *Azadirachta indica* group shows a slower rate of tumor formation because most of the fibrous tissues are mature. The measurement of the final tumor volume in the two-treatment group provided further evidence of the antiproliferative properties. Akhouri et al. (2020[a,b]) has extensively documented investigations on several models that are similar.

Conclusion

These findings indicate to the antitumorigenic characteristics of *Azadirachta indica* ethanolic flowers extract, especially in its capacity to combat free radicals. The kidneys and liver have been protected from damage caused by the DMBA from the plant extract. So, it's safe to speculate that the plant extract may both prevent and cure breast cancer in mice that has been generated by DMBA. Research exploring the potential of an ethanolic *Azadirachta indica* flower extract as a chemotherapeutic agent for the treatment of breast cancer is also showing encouraging results. The molecular mechanism and action mode of *Azadirachta indica* flower extract should also be investigated via research. Nonetheless, the current investigation shows promising in regulating DMBA-induced breast cancer in mouse models.

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Declaration of conflicting interests

The authors declare that they have no conflict of interest.

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